

# **Cathosis: Cathepsins in particle-induced inflammatory cell death**

A Dissertation Presented

By

Gregory Michael Orlowski

Submitted to the Faculty of

the University of Massachusetts Graduate School of Biomedical Sciences,  
Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 1, 2015

Medical Scientist Training Program (MSTP)

**“Cathosis: Cathepsins in particle-induced inflammatory cell death”**

A Dissertation Presented  
By  
Gregory Michael Orlowski

The signatures of the Dissertation Defense Committee  
signify completion and approval as to style and content of the Dissertation.

Kenneth L. Rock, M.D., Thesis Advisor

Anne Marshak-Rothstein, Ph.D., Member of Committee

Douglas T. Golenbock, M.D., Member of Committee

Evelyn Kurt-Jones, Ph.D., Member of Committee

Neal Silverman, Ph.D., Member of Committee

Andrew D. Luster, M.D./Ph.D, External Committee Member

The signature of the Chair of the Committee signifies that  
the written dissertation meets the requirements of the Dissertation Committee.

Katherine A. Fitzgerald, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences  
signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D., Dean of the Graduate School of Biomedical Sciences

Medical Scientist Training Program (MSTP)  
May 1, 2015

© Copyright by  
Gregory Michael Orlowski  
2015

## **Dedication**

I dedicate this thesis to my family (Marie, Ireneusz, Lisa) for making me who I am, my wife (Shruti) for supporting and mentoring me as a person and as a scientist, and my animals (Buttercup, Libra and Leona) for their intellectual input.

## **Acknowledgements**

First and foremost, I would like to thank my wife, Shruti Sharma for her continuous support and mentorship as a senior scientist and my life partner. I thank my family, including my mom, dad and my sister (Lisa) for their continuous love and support. I thank my mother and father in-law in India, and my brother in-law (Anirudh) with whom I have collaborated, for their constant encouragement. And, I would like to thank all of my friends (you know who you are), without whom I would have no refuge from my tireless struggles throughout these last five years in the lab.

Next, I would like to thank my lab for everything they have done for me during the considerable amount of time we have worked together side-by-side. I thank Jeff Colbert for his invaluable mentorship, especially early on during my thesis, helping me with a variety of basic lab techniques and sharing his considerable knowledge on the subject of cathepsin biology. I thank Hiroshi Kataoka for spending long hours assisting me with mouse surgery, and Ke Yang for teaching me how to inject mice via the tail vein. I thank Lianjun “LJ” Shen for providing me with a constant source of social entertainment and intimate historical insight into every reagent, machine and experiment done in our lab over the past decade. I thank Dipti Karmarkar for being my confidant, bay mate, fellow gang member, and mentor early on in my work on IL-1. I thank Freidrich “Fred” Cruz and Jiann-Jyh “JJ” Lai for being there working beside me in the lab

on many many many evenings late into the night. I thank Eleanor Kincaid for her constant willingness to explain to me her complicated but well-informed views on just about anything. I also thank Diego Farfan for always sharing his cynically entertaining views on just about anything, including his years of personal struggle with a violent pit-bull on his route to work each day. Importantly, I thank Elena Merino-Rodriguez, LJ, Matt Janko, Zubin Patel, and especially Barry Kreigsman for being my friends inside and outside of the lab.

I would also like to thank Janice Bellisle for maintaining a tight ship and for accommodating the unconventional way I adapt my lab space with duct tape and magnets. I thank Joan Veinot for always being a source of friendly banter and helping me with so many things over the years (like putting together the copies of my thesis) and doing so much for the lab that I will never fully understand. I thank Sharlene Hubbard for keeping all my mice in check. And I thank Rosemarie Shooshan, not only for her management of all the glassware and pipette tips upon which I so unconditionally depend, but also for being my buddy.

I would also like to thank my collaborators, who have been acknowledged or recognized as authors in Chapters III and IV. I thank John McCullough as well for his guidance in my adventures into pharmacology and drug formulation. And I thank my committee for allowing me to take the extra year I needed to fully realize the potential of my thesis research. I especially thank my chair, Kate Fitzgerald, as well as Ann Marshak-Rothstein, for their extra support of both my wife and I in our research careers. Moreover, I would like to thank Douglas

Golenbock for being my first research advisor at UMMS and for his continuing support and interest in my work.

Finally, and certainly not least, I would like to thank my advisor and mentor, Kenneth Rock. Ken has always been an outstanding role model for me as a scientist. He is careful and contemplative, and he has taught me throughout my Ph.D. how to communicate better and think more clearly. He is one of the few people I know who stops and thinks after I ask him a question, rather than answering right away, so I have never taken his responses lightly. There are few people like Ken, with whom my instinct tells me first to be critical of my own position when I disagree with him about something. Indeed, in many instances, I have been an enlightened beneficiary of his wisdom. But also, Ken has given me free-range to troubleshoot and explore the many facets of my research in the lab, and this has truly made me an independent and self-confident scientist. Furthermore, by always valuing my input and keeping an open mind, Ken has motivated me to find wisdom in my science. After all this, I hope that I have given him something back, such that both Ken and I walk away from my thesis work forever enlightened by the ~1,800 days I spent in his lab.

## Preface

A year and a half ago, my committee told me that I was ready to graduate. I disagreed. Yes, I had probably put in enough effort, gathered enough data, and grown enough as a researcher to merit a Ph.D., but my goal was never to earn a Ph.D. My goal was to figure out what it really takes to be a good scientist. But, a year and a half ago, I had not yet connected point A to point B by answering my original research question: Are cathepsins B and L involved in NLRP3 activation? This question turned out to be much more complex than I originally anticipated; it was more complex than the field or even my committee understood at the time. But I knew how it looked. “Is this project really that complex, or is this student just confused and unfocused? Maybe it is best that he leave before this continues on forever.” I suppose this is how every committed scientist feels when faced with a challenging investigation that sometimes seems hopeless and nobody else seems to understand. This feeling really broke me down at times, even to the point of tears once or twice. However, deep down, I knew I was not deluded about the complexity of the problem before me. I knew that I was making progress that would break the story open sometime soon, and that persevering against these types of odds is a minimum requirement for survival in science. Thus, I met with each of my committee members to ask for just one more year of research, explaining to each of them why my luck has not been good and how my challenges have not been trivial.



I have no idea whether the types of challenges I faced during my thesis research were typical, but they were certainly numerous and unyielding. While my inhibitor experiments originally went according to expectations, my early *in vivo* acute peritonitis experiments produced results that were contrary to work previously published by our lab and collaborators. Was I just a bad scientist? While I cannot say for sure, I later found that the control mice used for previous *in vivo* experiments came from an outside vendor, rather than using control mice bred in-house as I had, suggesting that this may have affected previous findings **(see Appendix 7)**. In light of these findings, I had to readjust what I thought I knew. However, by this time, I had already begun to study an atherosclerosis model *in vivo*, including months and months of trying to crystallize cholesterol to the specifications I needed to study these particulates in parallel *in vitro*. At the conclusion of this two-year atherosclerosis study, my results were negative (agreeing with my findings in the peritonitis model) **(see Appendix 1)**. Moreover, while I had therapeutic success using inhibitors in my acute-dosing model, I spent months and months of amateur pharmaceutical formulation trying to setup a long-term dosing model with an experimental drug. Finally, after a consultation with a pharmaceutical company, I learned that my aptitude for drug formulation was not the problem, but the chemical nature of the drug itself was an inherent limitation **(see Appendix 12)**.

*In vitro*, I was able to generate double-knockout macrophages from non-viable mice, and studied them using the standard methodology of the field. At

first, my results were exciting, since I found a complete phenotype in immortal cells and a partial phenotype in primary cells. But something did not feel right about these experiments. Coming up with my own methods to control for cell death, cell number and intracellular cytokine levels, my original data turned out to comprise a panel of various artifacts reflecting different though still important biology. Among these new phenomena I was documenting, I made some interesting early findings that seemed to highlight the importance of cell death in my system. However, after presenting some of these findings at conferences, I was scooped by another group, sending me right back to square one. But, I felt that this group, which scooped my work, misinterpreted their data. Indeed, the more I learned about my field, the more I realized the prevalence of misinterpretation and the under appreciation of the biological complexity inherent to this system.

Then, a little more than a year and a half ago, I had a breakthrough. I obtained a reagent called BMV109 from a collaborator that allowed me to examine my system with more precision than almost anyone else in the field at the time. I developed two real-time assays to measure lysosomal and mitochondrial dynamics. I standardized a new approach to siRNA knockdown in primary macrophages using Endoport<sup>er</sup> in combination with RNAiMax to silence ~98% of the message for multiple genes at once with minimal toxicity. By diversifying the experimental approaches I was using and examining multiple

angles of inquiry, I had built my own literature base upon which I could consistently rely. All I needed was time.

The bulk of my dissertation comes from these breakthroughs during the last year and a half of my research. I could have taken it much further. But I have learned that answering one question just leads to more questions; there is inherent entropy to inquiry. For now, having answered my original research question, I reached my goal as a Ph.D. student. And, for whatever it is worth, I described what I believe to be an important biological concept, if not just a new term, which I call “cathosis” (cathepsin-mediated inflammatory necrosis).

## Abstract

Sterile particles underlie the pathogenesis of numerous inflammatory diseases. These diseases can often become chronic and debilitating. Moreover, they are common, and include silicosis (silica), asbestosis (asbestos), gout (monosodium urate), atherosclerosis (cholesterol crystals), and Alzheimer's disease (amyloid A $\beta$ ). Central to the pathology of these diseases is a repeating cycle of particle-induced cell death and inflammation. Macrophages are the key cellular mediators thought to drive this process, as they are especially sensitive to particle-induced cell death and they are also the dominant producers of the cytokine responsible for much of this inflammation, IL-1 $\beta$ . In response to cytokines or microbial cues, IL-1 $\beta$  is synthesized in an inactive form (pro-IL-1 $\beta$ ) and requires an additional signal to be secreted as an active cytokine. Although a multimolecular complex, called the NLRP3 inflammasome, controls the activation/secretion of IL-1 $\beta$  (and has been thought to also control cell death) in response to particles *in vitro*, the *in vivo* inflammatory response to particles occurs independently of inflammasomes. Therefore, I sought to better understand the mechanisms governing IL-1 $\beta$  production and cell death in response to particles, focusing specifically on the role of lysosomal cathepsin proteases. Inhibitor studies have suggested that one of these proteases, cathepsin B, plays a role in promoting inflammasome activation subsequent to particle-induced lysosomal damage, however genetic models of cathepsin B deficiency have argued otherwise. Through the use of

inhibitors, state-of-the-art biochemical tools, and multi-cathepsin-deficient genetic models, I found that multiple redundant cathepsins promote pro-IL-1 $\beta$  synthesis as well as particle-induced NLRP3 activation and cell death. Importantly, I also found that particle-induced cell death does not depend on inflammasomes, suggesting that this may be why inflammasomes do not contribute to particle-induced inflammation *in vivo*. Therefore, my observations suggest that cathepsins may be multifaceted therapeutic targets involved in the two key pathological aspects of particle-induced inflammatory disease, IL-1 $\beta$  production and cell death.

# Table of Contents

Dedication .....	iv
Acknowledgements .....	v
Preface .....	viii
Abstract .....	xii
Table of Contents .....	xiv
List of Figures .....	xviii
Common Abbreviations .....	xx
List of Publications.....	xxi
Chapter I: Introduction .....	2
<b>Overview</b> .....	2
<b>Particle-induced Sterile Inflammatory Disease</b> .....	3
<b>The Immune System: Protection from pathogens</b> .....	9
<b>Innate Sensing of Pathogens: Janeway’s Pattern Recognition Theory</b> ...	12
TLRs – Plasma Membrane and Endosomal Pathogen Recognition.....	14
CLRs – Plasma Membrane Recognition of Fungi and Dead Cells .....	17
NLRs – Cytosolic Recognition of Bacterial Patterns.....	18
RLRs – Cytosolic Recognition of Viral RNA .....	19
Cytosolic DNA Recognition .....	20
Inflammasomes .....	21
<b>Sterile Inflammation: Matzinger’s “Danger Model”</b> .....	27
DAMPs: Danger Signals Released from Necrotic Cells .....	28
IL-1: Master Regulator of Sterile Inflammation .....	32
<b>Mechanisms of Particle-Induced NLRP3 Activation</b> .....	36
Lysosomal Membrane Disruption (LMD) and Cathepsin B.....	39
Reactive Oxygen Species (ROS) .....	40
Potassium (K <sup>+</sup> ) Efflux.....	44
Cytosolic Calcium Influx .....	47
Mitochondrial DAMP Release.....	50
Importance of Priming for NLRP3 Activation .....	54
Non-canonical IL-1 $\beta$ Processing via RIP3 and Caspase-8 .....	58
<b>Cathepsins: Lysosomal Proteases in IL-1<math>\beta</math> Activation &amp; Cell Death</b> .....	59
What are cathepsins and how are they regulated? .....	59
Basic Pathways of Cell Death .....	67
Cathepsins and Lysosomal Cell Death.....	70
Lysosomal-Mitochondrial Crosstalk in Cell Death .....	72
<b>Summary, Research Question, Hypothesis</b> .....	75
Chapter II: Materials and Methods.....	83
<b>Reagents and Antibodies</b> .....	83
<b>Production and Measurement of Cytokines &amp; Cell Death</b> .....	84

<b>Animal and Cell Lines</b> .....	88
<b>Generation of Bone Marrow Chimeras</b> .....	88
<b>siRNA Knockdowns</b> .....	89
<b>Immunoblotting &amp; Live-cell Cathepsin Activity Labeling</b> .....	90
<b>Neutrophil and Monocyte Recruitment to Peritoneal Cavity</b> .....	90
<b>K777 Treatment of Mice by Injection or Alzet Pump Infusion</b> .....	91
<b>Real-time Measurement of LMD &amp; MMP</b> .....	91
Chapter III: Multiple Cathepsins Promote Pro-IL-1 $\beta$ Synthesis and NLRP3-Mediated IL-1 $\beta$ Activation .....	95
<b>Abstract</b> .....	96
<b>Introduction</b> .....	98
<b>Results</b> .....	103
Genetic and biochemical analysis of the impact of individual cathepsin deficiency on particle-induced IL-1 $\beta$ secretion.....	103
Analysis of small molecule cathepsin inhibitors.....	108
Analysis of compound cathepsin deficiencies .....	118
Analysis of endogenous cathepsin inhibitors.....	125
Analyzing the effect of small molecule cathepsin inhibitors on pro-IL-1 $\beta$ synthesis .....	132
Analyzing the effect of cathepsin inhibitors on Signal 2 of NLRP3 activation .....	139
<b>Discussion</b> .....	144
<b>Acknowledgements</b> .....	153
<b>Grant Support</b> .....	153
<b>Summary of Major Contributions in Chapter III</b> .....	154
Chapter IV: Lysosomal disruption induces cell death that depends on multiple cathepsins and requires intact mitochondria to promote NLRP3-mediated IL-1 $\beta$ secretion. ....	157
<b>Abstract</b> .....	158
<b>Introduction</b> .....	160
<b>Results</b> .....	165
Particle-induced cell death is inflammasome-independent.....	165
Particle-induced cell death and IL-1 $\beta$ secretion are co-dependent on cathepsins and the Bcl-2 family .....	169
Particle-induced cell death is dependent on multiple redundant cathepsins. ....	179
Bcl-2 inhibition induces inflammasome & RIP3-independent cell death and IL-1 $\beta$ secretion.....	185
Particle-induced IL-1 $\beta$ secretion is antagonized by mitochondrial disruptive cell death.....	193
Particle-induced IL-1 $\beta$ secretion and pro-IL-1 $\beta$ synthesis require an intact MMP. ....	201
Particle-induced sterile inflammation is suppressed by cathepsin inhibition <i>in vivo</i> . ....	206

<b>Discussion</b> .....	210
<b>Acknowledgments</b> .....	217
<b>Grant Support</b> .....	217
<b>Summary of Major Contributions in Chapter IV</b> .....	218
Chapter V: Discussion .....	220
<b>Cathepsins as Therapeutic Targets for the Treatment of Particle-induced Sterile Inflammatory Disease</b> .....	220
<b>K777 as a Therapeutic for Particle-induced Sterile Inflammatory Disease</b> .....	229
<b>Importance of Cell Death during NLRP3 Activation</b> .....	237
<b>Importance of Priming for NLRP3 Activation</b> .....	247
<b>Silica Vs. Nigericin-induced NLRP3 Activation</b> .....	257
<b>Lysosomal tropism, Lysosomal pH &amp; Cytosolic Acidification in IL-1<math>\beta</math> Activation</b> .....	262
<b>Cathepsin X in Nigericin-mediated NLRP3 Activation</b> .....	269
<b>An Evolutionary Perspective on LMD-Mediated NLRP3 Activation</b> .....	271
<b>Model of Particle-Induced NLRP3-mediated IL-1<math>\beta</math> Secretion and Cell Death</b> .....	277
Appendices.....	280
<b>Appendix 1:</b> Cathepsins B, L or B&L in bone-marrow-derived cells are not essential for atherogenesis. ....	280
<b>Appendix 2:</b> 8-OH-dG does not inhibit NLRP3 activation, mitochondrial RNA induces some IL-1 $\beta$ secretion, and silica-induced IL-1 $\beta$ secretion can be enhanced by binding to dAdT. ....	281
<b>Appendix 3:</b> Novel Protocol: Timing the harvest of fetal livers for hematopoietic stem cell collection by predicting date of conception based on weight curve of pregnant females. ....	282
<b>Appendix 4:</b> Novel Protocol: Optimal siRNA knockdown is achieved in PMs with minimal toxicity by combining Endoportor and RNAiMax.....	283
<b>Appendix 5:</b> Novel Protocol: Acridine Orange and TMRM 96-well format for the real-time monitoring of lysosomal and mitochondrial disruption.....	286
<b>Appendix 6:</b> Twenty-five different cathepsin inhibitors all exhibit similar efficacy for inhibiting silica and nigericin-induced IL-1 $\beta$ secretion and cell death in LPS-primed macrophages.....	287
<b>Appendix 7:</b> Compilation of additional in vivo acute peritonitis experiments. ....	289
<b>Appendix 8:</b> K777 selectively suppresses IL-1 $\beta$ secretion and cell death induced by particulate NLRP3 stimuli without affecting general phagocytic machinery. ....	291
<b>Appendix 9:</b> A cathepsin D inhibitor (Pepstatin A-penetratin), staurosporine, and chemotherapeutics (doxorubicin and etoposide) induce inflammasome-independent IL-1 $\beta$ secretion with variable sensitivity to suppression with K777. ....	292



<b>Appendix 10:</b> Cathepsin inhibitors suppress pro-IL-1 $\beta$ synthesis regardless of priming mechanism and silica/nigericin-induced cell death depends on MyD88 and/or TRIF.....	293
<b>Appendix 11:</b> ROS inhibitors selectively attenuate particle-induced IL-1 $\beta$ secretion, but not cell death. ....	295
<b>Appendix 12:</b> Drug Formulation: K777 requires low pH to remain soluble at high concentrations, but the ELP formulation permits absorption of K777 as a monodispersed suspension that causes local inflammation. ....	297
<b>Appendix 13:</b> Lysotropic drugs (amphiphilic weak bases) and lysosome/cytosolic acidification inhibitors selectively suppress particle-induced IL-1 $\beta$ secretion and cell death.....	299
<b>Appendix 14:</b> Lower concentrations of KCl selectively suppress IL-1 $\beta$ secretion induced by Silica and LLOMe (& NLRP3-dependent stimuli), but higher concentrations of KCl suppress IL-1 $\beta$ non-selectively.....	300
<b>Appendix 15:</b> P2X7R, IL-1R1, or TNF-R1&II do not contribute to IL-1 $\beta$ synthesis or particle-induced IL-1 $\beta$ activation and cell death in LPS-primed macrophages. ....	301
<b>Appendix 16:</b> ATP and Nigericin rapidly disrupt lysosomal pH gradients and intracellular cathepsin activity. ....	303
<b>Appendix 17:</b> Alkaline medium suppresses IL-1 $\beta$ synthesis, as well as particle-induced IL-1 $\beta$ secretion, while acidic medium potentiates inflammasome-mediated IL-1 $\beta$ secretion.....	305
<b>Appendix 18:</b> Regularly spaced cysteine motifs on the LRR of NLRP3 and NLRP3 cleavage sites.....	306
<b>Appendix 19:</b> NLRP3-mediated IL-1 $\beta$ secretion is completely dependent on cathepsins B&L in immortalized bone-marrow-derived macrophages (IMMPs). ....	307
<b>Appendix 20:</b> Nigericin-induced NLRP3 activation requires strong binding of integrins to a substrate and extracellular cathepsin activity. ....	308
<b>Appendix 21:</b> Attenuation of robust LMD-mediated cell death enhances IL-1 $\beta$ secretion, while early mitochondrial disruption suppresses IL-1 $\beta$ secretion..	309
Bibliography.....	310

## List of Figures

**Figure 1.1** Particle-induced sterile inflammatory pathology results from a repeating cycle of particle-ingestion, lytic cell death, and the release of inflammatory mediators.

**Figure 1.2:** General Model of Inflammasome Activation as Exemplified by the NLRP3 Inflammasome.

**Figure 1.3:** A Brief List of the Various Activators of the 4 Best-Studied Inflammasomes.

**Figure 1.4:** Hypothetical model for the role of cathepsins in particle-induced sterile inflammatory pathology.

**Figure 2.1:** Standard Assay for Understanding Inflammasomes.

**Figure 3.1:** Sterile particle-induced IL-1 $\beta$  secretion does not require cathepsins B, L, C, S or X, but nigericin is partially dependent on cathepsin X.

**Figure 3.2:** Both Ca074Me and K777 inhibit multiple cathepsins at concentrations needed to block IL-1 $\beta$  secretion.

**Figure 3.3:** Cathepsin inhibitors suppress particle-induced IL-1 $\beta$  secretion independently of individual cathepsins.

**Figure 3.4:** Compound cathepsin-deficiency causes a minor reduction in particle-induced IL-1 $\beta$  secretion.

**Figure 3.5:** Endogenous cathepsin inhibition by Cystatins C & B regulates particle-induced IL-1 $\beta$  secretion and LPS-induced pro-IL-1 $\beta$  synthesis.

**Figure 3.6:** Small-molecule cathepsin inhibitors suppress pro-IL-1 $\beta$  synthesis.

**Figure 3.7:** Cathepsin inhibitors also suppress NLRP3 activation independently of effects on pro-IL-1 $\beta$  synthesis.

**Figure 4.1:** Particle-induced cell death is inflammasome-independent in LPS-primed macrophages.

**Figure 4.2:** Particle-induced cell death and IL-1 $\beta$  secretion are co-dependent on cathepsins and the Bcl-2 family during NLRP3 activation.

**Figure 4.3:** Particle-induced cell death is initiated by multiple redundant cathepsins, and does not require cathepsin B, during NLRP3 activation

**Figure 4.4:** Bcl-2 inhibition induces inflammasome and RIP3-independent cell death and IL-1 $\beta$  secretion, but cathepsin inhibition still suppresses IL-1 $\beta$  secretion.

**Figure 4.5:** Particle-induced cell death and IL-1 $\beta$  secretion do not require mitochondrial disruption, which actually antagonizes IL-1 $\beta$  secretion.

**Figure 4.6:** Particle-induced IL-1 $\beta$  secretion and pro-IL-1 $\beta$  synthesis, and not cell death, requires an intact MMP.

**Figure 4.7:** Silica-induced acute peritonitis is suppressed by systemic treatment with a cathepsin inhibitor, K777, independently of cathepsin C.

**Figure 5.1:** Cathepsins as Therapeutic Targets.

**Figure 5.2:** A lysosomally dominant crosstalk involving mitochondria is optimal for particulate-mediated IL-1 $\beta$  secretion.

**Figure 5.3:** Model of Particle-Induced NLRP3-mediated IL-1 $\beta$  Secretion and Cell Death

## Common Abbreviations

absent in melanoma 2 (AIM2)  
acridine orange (AO)  
antigen presenting cell (APC)  
antimycin A (AntA)  
carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP)  
cholesterol crystals (CC)  
c-type lectin receptor (CLR)  
cycloheximide (CHX)  
danger-associated molecular pattern (DAMP)  
dendritic cell (DC)  
electron transport chain (ETC)  
interleukin-1 (IL-1)  
interleukin-6 (IL-6)  
Leu-Leu-OMe (LLOMe)  
leucine-rich repeat (LRR)  
lysosomal membrane disruption (LMD)  
mitochondrial membrane potential (MMP)  
mitochondrial outer membrane permeabilization (MOMP)  
monosodium urate crystals (MSU)  
NOD-like receptor containing a pyrin domain 3 (NLRP3)  
nuclear factor kappa B (NF- $\kappa$ B)  
oligomycin A (OMA)  
pathogen-associated molecular pattern (PAMP)  
pattern recognition receptor (PRR)  
peritoneal macrophage (PM)  
staurosporine (STS)  
poly(deoxyadenylic-deoxythymidylic) acid (dAdT)  
potassium (K<sup>+</sup>)  
quantitative PCR (qPCR)  
reactive oxygen species (ROS)  
RIG-I-like receptor (RLR)  
tetramethyl rhodamine methyl ester (TMRM)  
tumor necrosis factor alpha (TNF- $\alpha$ )  
toll-like receptor (TLR)  
voltage-dependent anion channel (VDAC)  
wild-type (WT)

## List of Publications

**G.M. Orlowski**, S. Sharma, J. D. Colbert, M. Bogyo, S. A. Robertson, F. K. Chan and K. L. Rock. (2015). Lysosomal disruption induces cell death that depends on multiple cathepsins and requires intact mitochondria to promote NLRP3-mediated IL-1 $\beta$  secretion. (*IN PREPARATION*).

**G.M. Orlowski**, J. D. Colbert, S. Sharma, M. Bogyo, S. A. Robertson, and K. L. Rock. (2015). Multiple cathepsins promote pro-IL-1 $\beta$  synthesis and NLRP3-mediated IL-1 $\beta$  activation. *Journal of Immunology* (*IN REVIEW*).

A. Sharma, **G.M. Orlowski**, Y. Zhu, S.Y. Kim, A. Hubel, B. Stadler. (2015). Inducing cells to self-disperse magnetic nanowires via integrin-mediated responses. *Nanotechnology*.

S. Sharma, A. Campbell, J. Chan-Kim, S.A. Schattgen, **G.M. Orlowski**, R. Nayar, A.H. Huyler, K. Nundel, C. Mohan, L.J. Berg, M.J. Shlomchik, A.M. Rothstein, K.A. Fitzgerald. (2015) Suppression of autoimmunity by the innate immune adaptor STING. *PNAS*.

K. Moriwaki, J. Bertin, P.J. Gough, **G.M. Orlowski**, F.K.M. Chan. (2015). Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. *Nature: Cell Death & Disease*.

H. Kono, **G.M. Orlowski**, Z. Patel, K.L. Rock. (2012). The IL-1-dependent sterile inflammatory response has a substantial caspase 1-independent component that requires cathepsin C. *Journal of Immunology*.

C. Liu, H. Miller, **G. Orlowski**, H. Hang, A. Upadhvava, W. Song. (2012). Actin reorganization is required for the formation of polarized B cell receptor signalosomes in response to both soluble and membrane-associated antigens. *Journal of Immunology*.

M.D. Siegelin, T. Dohi, C.M. Raskett, **G.M. Orlowski**, C.M. Powers, C.A. Gilbert, A.H. Ross, J. Plescia, and D.C. Altieri. (2011). Exploiting the mitochondrial unfolded protein response for cancer therapy in mice and human cells. *JCI*.

S. Sharma, **G. Orlowski** and W. Song. (2009). Btk regulates B cell receptor-mediated antigen processing and presentation by controlling actin cytoskeleton dynamics in B cells. *Journal of Immunology*.

# **Chapter I: Introduction**

# Chapter I: Introduction

## Overview

Pathology comes from the Greek roots “pathos” (suffering) and “logia” (the study of), and is therefore defined as “the study of suffering”(1). More practically, pathology is a branch of biological and medical sciences concerned with understanding the process of how something that was going right (physiology), ends up going wrong (pathology). Therefore, in order to understand any particular type of pathology, one needs to first understand the complementary physiology. Since the scope of this thesis is concerned with understanding a mechanism of pathology, whereby the immune system goes wrong, a brief background on what the immune system normally does right will be described as well.

The goal of this thesis is to examine the pathological basis of the immune response to disease-causing sterile particles and to identify tractable therapeutic targets with the potential to prevent or alleviate human suffering. It is certain that pathology during particle-induced inflammatory disease is initiated by the particles themselves, and therefore, the focus of this thesis will be on the cellular mechanisms governing the initial response of the immune system to sterile particles. But first, I will begin with an overview of particle-induced sterile inflammatory disease as it relates to human suffering.

### **Particle-induced Sterile Inflammatory Disease**

Exogenous and endogenous sterile particles are known to drive the pathogenesis of debilitating and often incurable inflammatory diseases(2). The exogenous particles responsible for such diseases include silica and asbestos, which cause two of the most common fibrotic restrictive lung disorders in world today, silicosis and asbestosis. It should be noted that asbestos is itself classified as a silicate(3). Lung disorders of this type are referred to as pneumoconioses, and inhalation of these particles is commonly associated with blue-collar jobs, such as construction, textile work, masons (especially those who work with sandstone), and miners(4). These diseases usually manifest over a period of 10 years(3, 4). Typical presentations for any pneumoconiosis include progressive decline in respiratory function, reported by patients as breathlessness, and fibrotic changes detected by X-ray as pleural plaques (asbestosis) or dense pulmonary nodules (silicosis)(3, 4). In the case of asbestosis, prognosis in mild cases is usually good, with little progression following cessation of exposure and effective treatment with bronchodilators and supplemental oxygen(3, 4). However, more severe cases lead to steady decline in pulmonary function that may require a pulmonary transplant if the disease has not already progressed to lung cancer (mesothelioma), as it often does(3, 4). Silicosis, however, is relentless and progressive, even following cessation of exposure(3, 4).



Progressive massive fibrotic and coalescent nodules may be present in the lungs even before symptoms occur(3, 4). Like asbestosis, silicosis often progresses to cancer, the risk of which is higher in smokers(3, 4). Additionally, silicosis increases the risk of pulmonary infections, like tuberculosis(3, 4). Finally, silicosis is also often associated with the development of autoimmune disorders, like lupus, rheumatic arthritis, and scleroderma(3, 4). Unfortunately, the only effective treatment for silicosis is a lung transplant(3, 4). There are numerous other disease-associated particles that will not be discussed further, but they include the following: titanium dioxide (5, 6), coal dust(7), and cigarette smoke(8), to name a few. Currently, the most effective public health measures have been regulations that reduce exposures to these noxious environmental particles.

Compared to exogenous particles, preventing exposure to endogenous inflammatory particles is not as simple. These particles form inside the body upon supersaturation of their precursor solutes within body fluids or their overproduction in pathological states. Common diseases like atherosclerosis, gout, and pseudogout are known to be driven by crystalline particulates, such as cholesterol crystals (CC)(9-11), monosodium urate (MSU)(12), and calcium pyrophosphate (CPP)(13), respectively(12). There are numerous other crystals associated with diseases in the body: Charcot-Leyden crystals (allergic asthma and parasitic diseases)(14), malarial hemozoin (malaria)(15), and calcium/uric acid renal stones, gallstones and calculi (gall bladder and renal disease)(1), to name a few. Furthermore, there are numerous particulate protein complexes also

associated with common and/or debilitating human diseases: islet amyloid polypeptide (type II diabetes)(16), amyloid A-beta (Alzheimer's)(17), alpha-synuclein and tau (Parkinson's), serum amyloid A (SAA) (systemic amyloidosis)(18-20), beta-2 microglobulin (dialysis-related amyloidosis), transthyretin (familial amyloidosis), immunoglobulin light chain (light chain amyloidosis), to name a few(21).

Given the amount of human suffering associated with this vast array of exogenous and endogenous particles, there is considerable impetus for understanding how they cause disease. Therefore, I focus here on understanding the pathogenesis of these diseases by examining two key events that occur concomitantly during the initial immune response to sterile particles: lytic cell death and the generation of inflammatory mediators(2, 7). Almost every particulate mentioned above has been shown to directly induce lytic cell death and inflammation. This phenomenon raises important questions: What evolutionarily basis is there for cells to respond to particles this way? How does this response cause disease? Here, my investigation is based on the following two assumptions:

(1) Lytic cell death and the inflammatory recruitment of immune cells is an appropriate and protective host response that can lead to sterilizing immunity when host cells are burdened with intracellular pathogens(22-24).

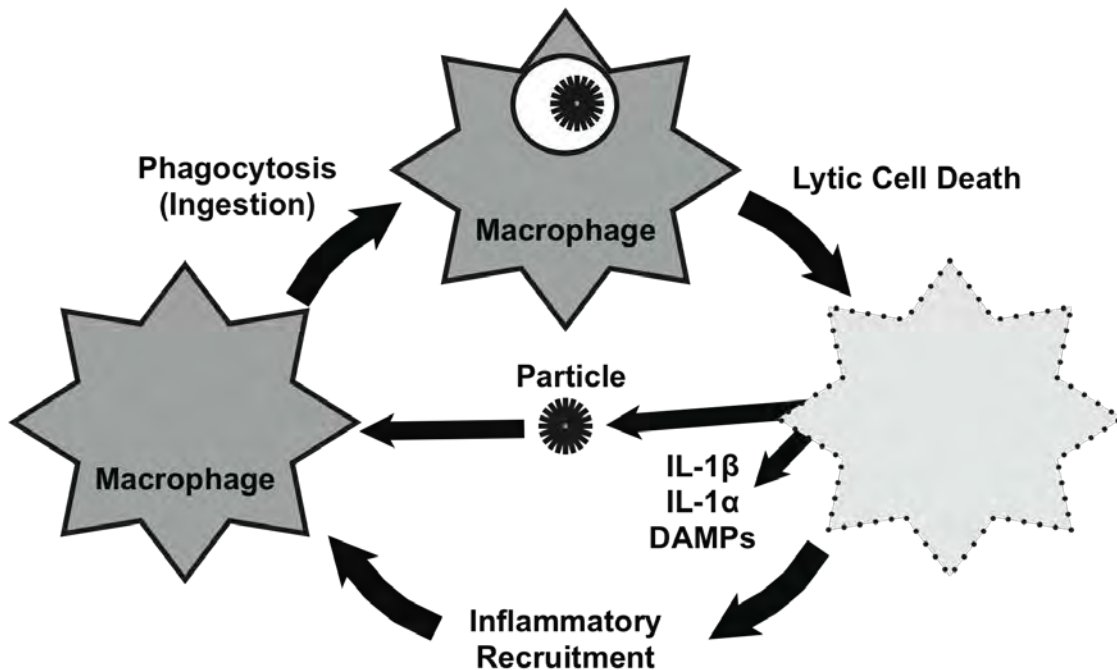
(2) When induced by sterile particles, such a response has almost entirely pathologic consequences(25, 26).

Unlike a bacterium or a virus, sterile particles are not themselves dangerous pathogens and many of them cannot be destroyed or broken down by immune defense mechanisms(9). There is simply nothing there that needs to be killed. As noted above, chronic exposure to these particles can lead to severe and debilitating pathology over time, characterized by functional decline of the affected organs brought on by fibrotic change. This is evident in the non-compliant lungs of patients with silicosis or asbestosis and in arteries that have been thickened and narrowed by atherosclerosis(27, 28). Therefore, engaging normal immune defense mechanisms to these particles does not improve their clearance, but instead, a continual stimulation of the immune response leads to chronic inflammation. Specifically, it seems that particle-induced diseases occur as a result of an ongoing cycle of cell death and inflammation(7, 29). Particles are engulfed (phagocytosed) by white blood cells of the innate immune system called macrophages (named as such according to their main function; literally translated, “big eaters”). Next, these macrophages die, releasing intracellular debris, inflammatory mediators and any engulfed particles back into the environment. These inflammatory mediators recruit more innate immune cells to this site, where they engulf these particles, and the cycle begins once again. Trying to kill/clear something that cannot be killed or cleared, they destroy the surrounding healthy tissue and replace it with fibroses (thick plaques of collagen) to isolate (via walls of fibroses) the undefeatable threat. Each time this cycle

occurs, another step is taken toward disease and away from normal tissue structure, homeostasis and health.

Although a common understanding of the pathological mechanisms that occur downstream of this cycle is still incomplete, I can be certain that particles are absolutely required to initiate particle-induced inflammatory disease. Therefore, my focus has been on identifying potential therapeutic targets involved in the two primary events incited by particles, cell death and inflammation. I expect that suppressing these inciting events might prevent the downstream pathology. Moreover, I sought to better understand these events by examining the primary phagocytic sentinels of the innate immune system, present in every tissue in the body, mononuclear phagocytes (macrophages).

**Chapter I, Figure 1: Particle-induced sterile inflammatory pathology results from a repeating cycle of particle-ingestion, lytic cell death, and the release of inflammatory mediators.** When macrophages encounter inflammatory particles, they phagocytose (or ingest) these particles, which lead to lytic cell death and the release of the particles & inflammatory mediators, like IL-1 $\beta$ , IL-1 $\alpha$ , and danger-associated molecular patterns (DAMPs). These mediators recruit more innate immune cells, like macrophages and neutrophils, which can once again phagocytose the released particles and begin the cycle again.



## **The Immune System: Protection from pathogens**

The immune system evolved to protect the body from pathogenic microorganisms, including bacteria, fungi, viruses, and parasites(30). Defense against these pathogens is generally broken down into two distinct branches of immunity: Innate Immunity and Adaptive Immunity(30).

The innate immune system provides constant surveillance and protection from pathogens that breach the primary epithelial barriers in the skin and mucosa(30). This response is rapidly initiated by sentinel cells normally present in all tissues of the body called mononuclear phagocytes, referred to herein for the sake of simplicity as macrophages (in tissues) or monocytes (in the circulation)(30). The vast majority of pathogens are controlled at this stage, where they are phagocytosed and digested(30). Minor exacerbations of this response, when epithelial barriers are disrupted, often lead to a more robust "induced response"(30). During the induced response, macrophages secrete proteins that signal changes in the activity of other cells or direct their movement, called cytokines and chemokines, respectively(30). Among other things discussed below, this recruits secondary innate effectors not normally present in tissues called neutrophils, which ingest and enzymatically/chemically degrade localized pathogens(30). This induced response produces all the hallmarks of inflammation(30). Heat and redness result from vasodilation, which increases the activity and delivery of immune cells and humoral (in the blood) factors to the

area(30). Pain and swelling result from vascular permeability, which focuses the attention of the organism to the problem and facilitates the penetration of immune cells and humoral factors into the tissue(30). When severe, but still localized, inflammation can also lead to loss of the surrounding tissue and/or organ dysfunction(30). And, when systemic, inflammation can lead to either chronic organ damage over long periods of time or acute and catastrophic organ system collapse and death(30). Therefore, regulation of inflammation is critical for an effective yet self-limiting response to pathogens(30).

Though many pathogenic challenges are resolved quickly and effectively by the innate response, as we are under constant challenge from external pathogens, sometimes the innate response is overwhelmed(30). In such cases, the adaptive immune response is called into action(30). Unlike the innate response, the adaptive response is slow to develop, but it has the capacity to generate highly specific, antigen-based (“antigen” is term that originally served as an abbreviation for “antibody generator”) responses to pathogens that leave the organism with lifelong immunological memory(30, 31). This memory allows the adaptive immune system to respond to a second challenge by the same pathogen rapidly and robustly(30). This is the principle relied upon for vaccination(30). While these qualities make the adaptive immune system superior to the innate immune system in some ways, the innate response is critical for the induction of the adaptive response(30).

The induced innate response queues up the adaptive immune system by activating specialized antigen-presenting cells (APCs), most notably dendritic cells (DCs), but also including macrophages and B cells (discussed below)<sup>1</sup>(30). Factors (cytokines and chemokines) produced by innate immune cells enhance the expression of co-stimulatory molecules on APCs, allowing them to take the information they have acquired through their innate ability to sense pathogens and transmit it to adaptive effector cells, which they activate(30). This information comes in the form of a molecular signature of the pathogen that is displayed by the APC to these adaptive immune cells expressing matching cognate receptors(30). Therefore, it is from these APCs, which ingest and process pathogen-derived antigens, that the appropriate cells of the adaptive immune system receive instructions about when/where to proliferate and what pathogen to destroy(30). Moreover, the panel of cytokines and chemokines secreted by innate immune cells differs based on the nature of the inciting pathogen, and in this way they shape the adaptive response as needed for a particular pathogenic challenge(30).

Adaptive effector cells, which are comprised of lymphocytes, like thymus-derived T cells or bone-marrow-derived B cells, depend on the information conveyed by innate cells to execute many of their functions(30). While cytotoxic T cells engage and kill infected cells directly, they require chemotactic signals

---

<sup>1</sup> B cells are the only strictly adaptive immune cells that are also APCs. 32.Rock, K. L., B. Benacerraf, and A. K. Abbas. 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med* 160:1102-1113.



from local innate cells to direct them to the area of infection(30). Moreover, similar information from innately cued APCs is required for the activation and efficacy of the “helper” T-cell ( $T_h$ ) responses, which administrate the entire adaptive immune response(30).  $T_h$  responses, broadly characterized as Type I helper T cells (“cell-mediated immune responses”) act primarily by augmenting the ability of macrophages to kill ingested pathogens, and type II helper T cells (“humoral immune responses”) primarily activate B cells to produce copious quantities of antibodies that aid in the opsonization/neutralization/destruction of extracellular pathogens/toxins for ingestion and digestion by macrophages<sup>2</sup>(30). Therefore, the innate immune system is vital for the engagement, direction, and implementation of adaptive immunity, and inappropriate or excessive activation of the innate immune system has the potential to initiate and/or exacerbate autoimmune disease(30). For the purpose of this thesis, my focus will be on the role of macrophages in the innate immune response, since these sentinel cells are at the interface between physiologic responses to pathogens/cellular debris and pathologic responses to sterile particles.

### **Innate Sensing of Pathogens: Janeway’s Pattern Recognition Theory**

---

<sup>2</sup> Antibodies are also important biological tools in laboratory settings. 33. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J Immunol* 122:2491-2497.

Chemokines and cytokines form the basis of the innate inflammatory response, and the cytokines IL-1, TNF- $\alpha$  and IL-6 are master regulators of this process(34). These cytokines initiate all the hallmarks of inflammation, discussed above. Another important class of inflammatory cytokines(34), Type I and II IFNs, direct critical facets of antiviral immune responses, cell-mediated immunity, and antigen presentation by APCs(34). Importantly, preventing pathological initiation of innate and adaptive responses requires that these inflammatory cytokines are produced only in response to pathogens (or cell injury, discussed below in the section on sterile inflammation). Therefore, macrophages, as well as nonprofessional immune cells that participate in innate immune responses (ex- epithelial cells), have evolved a set of receptors that recognize foreign pathogens whilst maintaining tolerance and measured responsiveness to self-derived stimuli(34).

In 1989, Charles A. Janeway Jr. proposed that, “effector mechanisms [are] activated by nonclonally distributed receptors whose specificity developed over evolutionary time to recognize patterns found on the surfaces of large groups of microorganisms”(35). Indeed, this has proven to be correct. These germline-encoded receptors are called PRRs (pattern/pathogen-recognition receptors), and they respond to PAMPs (pathogen-associated molecular patterns) by initiating the transcription of pro-inflammatory genes, like those encoding the cytokines just mentioned above(34). Unlike antigen receptors of the adaptive immune system, which are highly adaptable and specific receptors encoded on B cells and T cells, PRRs are immutable and have broad specificity for conserved

PAMPs(34). Moreover, PRRs allow innate immune cells to immediately recognize and respond to these PAMPs, which are evolutionarily conserved structures common to a wide variety of pathogens(34). The PRRs encoded by macrophages generally fall into four main categories: the cell surface or endosomal TLRs (Toll-like receptors), the cell surface CLRs (C-type lectin receptors), and the cytosolic NLRs (NOD-like receptors), and RLRs (RIG-I-like receptors)(34). There is also a group of cytosolic DNA sensors sometimes referred to as the ALRs (AIM2-like receptors)(34). Since, RLRs and CLRs fall outside the scope of this thesis, they will be mentioned only briefly. Importantly, a particular group of these receptors can form complexes called inflammasomes, which are activated by various pathogenic and injurious stimuli(34). One of these inflammasomes, NLRP3, governs the inflammatory response to lytic cell death and sterile particles(34). Therefore, NLRP3 will be discussed in great detail later on.

## **TLRs – Plasma Membrane and Endosomal Pathogen Recognition**

TLRs are named after the receptor Toll, originally discovered for its role in directing the development of the dorsal-ventral axis in the body plan of *Drosophila melanogaster* (fruit fly)(36). Toll was later shown to be important for innate immune signaling in the fruit fly(37). Signaling through Toll by the protein

Spatzle, which is activated upon exposure to gram-positive bacteria, fungi and parasites, leads to nuclear translocation of a transcription factor homologous to mammalian NF- $\kappa$ B (nuclear factor kappa-B), called Relish(38, 39)<sup>3</sup>. In fruit flies, this leads to the synthesis and secretion of antimicrobial peptides. Mammalian TLRs have an analogous signaling pathway, culminating with the nuclear translocation of NF- $\kappa$ B. Among other things, mammalian NF- $\kappa$ B drives the transcription of a diverse set of pro-inflammatory mediators, like TNF- $\alpha$  (tumor necrosis factor alpha), IL-6 (interleukin six), pro-IL-1 $\alpha$  and pro-IL-1 $\beta$  (pro-interleukin-1 alpha and beta). IL-6 and TNF- $\alpha$  induction and secretion is fastidiously regulated transcriptionally and post-transcriptionally by several mechanisms(41-44). Importantly, unlike TNF- $\alpha$  and several other cytokines, the IL-1 $\beta$  transcript is translated into a precursor, pro-IL-1 $\beta$ , which is inactive and not automatically secreted following synthesis(34). A second signal is required for the cleavage-based activation of the 37 kDa precursor pro-IL-1 $\beta$  into the 17 kDa mature and bioactive IL-1 $\beta$ , which leads to its secretion by an unknown mechanism(34). This extra level of post-translational regulation over active IL-1 $\beta$  secretion (there are several additional regulatory mechanisms that will not be discussed here) is a testament to the fact that inappropriate IL-1 $\beta$  secretion has pathologic potential. Therefore, in the coming sections, much of this thesis will focus on the process of IL-1 $\beta$  activation/secretion.

---

<sup>3</sup> Activation of this pathway requires the kinase TAK1. 40. Silverman, N., R. Zhou, R. L. Erlich, M. Hunter, E. Bernstein, D. Schneider, and T. Maniatis. 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. *J Biol Chem* 278:48928-48934..

All TLRs are membrane bound, containing an intracellular Toll/IL-1R homology (TIR) domain for signaling, and an extracellular/luminal LRR (leucine-rich repeat) domain(34). The LRR domain is the ligand-sensing component of TLRs and it determines their specificity(34). The plasma-membrane-bound TLRs include TLRs 1,2,4,5 & 6, which sense a variety of lipid and sugar-modified pathogen-associated structural and biochemical motifs(34). The endosomal TLRs include TLRs 3,7,8 & 9, which sense various forms of microbial (and in some cases, host) nucleic acids introduced by invading microbes(34). TLR-mediated signaling generally proceeds through either of two adaptors: MyD88 (myeloid differentiation primary response gene 88) or TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ )<sup>4</sup>(34). While TLRs 1,2,4,5,6,7,8 & 9 signal through MyD88 leading to NF- $\kappa$ B translocation, TLR3 does not(34). Instead, TLR3 signals solely through the adaptor TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ )(34). Unlike, MyD88, TRIF signaling causes slow/weak NF- $\kappa$ B translocation and primarily leads to activation of the transcription factor IRF3 (interferon regulatory factor 3), which induces the production of the antiviral cytokine IFN- $\beta$  (interferon-beta)(34). However, in specialized DCs (plasmacytoid DCs), TLRs 7,8 & 9 also activate the transcription factor IRF7 through MyD88 leading to the production of the functional counterpart

---

<sup>4</sup> MyD88 requires the bridging adaptors TIRAP ((TIR) domain-containing adaptor protein) and Mal (MyD88-adaptor-like)<sup>45</sup>. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, D. McMurray, D. E. Smith, J. E. Sims, T. A. Bird, and L. A. O'Neill. 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413:78-83..

to IFN-  $\beta$ , called IFN- $\alpha$ , which are together referred to as Type I IFNs(30). Type I IFNs are critical antiviral immune regulators, but they are outside the scope of this thesis and will not be discussed here in detail.

Among the TLRs, the best studied is TLR4, which senses a major structural component in the cell wall of gram-negative bacteria called LPS (lipopolysaccharide)(34). Interestingly, sensing of LPS by TLR4 occurs in cooperation with the soluble molecules Lbp (LPS-binding protein) and myeloid differentiation factor 2 (MD-2), as well as the membrane-bound co-receptor CD14, which is critical for binding free LPS(46). Moreover, TLR4 is the only TLR that signals through both the MyD88 and TRIF pathway(34). In fact, MyD88-dependent signaling by TLR4 has recently been shown to occur at the plasma membrane, while TRIF-dependent signaling involves CD14-mediated internalization of TLR4 and proceeds from the endosome to induce Type I IFNs and a second wave of NF- $\kappa$ B signaling(47, 48).

## **CLRs – Plasma Membrane Recognition of Fungi and Dead Cells**

CLRs are plasma-membrane-bound receptors that bind to carbohydrate moieties most often associated with fungal pathogens(34). However, CLRs can also sense some bacterial and viral pathogens via surface expressed carbohydrates as well(34). Two CLRs important for initiating host defense against *Candida albicans* are dectin-1 and 2, which recognize beta-glucans in the fungal cell wall

and initiate inflammatory cytokine production via Syk tyrosine kinase(34). In addition to other transcription factors, this pathway also can lead to NF- $\kappa$ B activation, although the exact signaling mechanisms are not well described(34).

Importantly, several CLRs are involved in the sterile immune response to cellular debris and are also important mediators of inflammation and vaccine responses(34). Lytic cell death exposes intracellular components to the innate immune system that are not normally present in healthy tissues(34). Because these components are not normally seen by innate immune cells, this debris is a sign of ongoing infection or cell injury requiring the activation of immune defenses or clean-up and repair(34). This concept will be discussed later in detail in the section on sterile inflammation. Two well-studied CLRs worth mentioning are Mincle (Clec4e) and DNGR-1 (Clec9a)(34). Mincle is expressed on macrophages and neutrophils, and it senses spliceosome-associated protein 130 (SAP130) released during necrosis(34, 49). Also, DNGR-1 is expressed on DCs and senses filamentous actin released from necrotic cells(50, 51). Importantly, the DNGR-1 response is critical for the cross-presentation of dead-cell associated antigens often incorporated into vaccine designs aimed at inducing strong cell-mediated immunity(52-54). Therefore, CLRs are important for sensing and responding to both pathogens and tissue injury(34).

## **NLRs – Cytosolic Recognition of Bacterial Patterns**

There are three main families of cytosolic PRRs, the NLRs and the nucleic acid-sensing PRRs (RLRs and DNA sensors/ALRs described below)(34). NLRs are named after the cytosolic receptors NOD1 and NOD2 (nucleotide-binding oligomerization domain-containing proteins 1 and 2)(34). Like plasma membrane-bound TLRs, NOD1 & 2 primarily sense structural components of bacteria via LRR domains(34). Upon activation, NODs oligomerize via their NOD domains, and recruit effectors through their signaling domains called CARDs (caspase-activation and recruitment domains), which leads to NF- $\kappa$ B activation similarly to membrane-bound TLRs(34). Importantly, the NLR family includes several cytosolic proteins capable of forming a multimolecular complex known as the inflammasome, which drives the proteolytic cleavage/activation and secretion of IL-1 $\beta$  (discussed below)(34).

## **RLRs – Cytosolic Recognition of Viral RNA**

RLRs (RIG-I-like receptors) are cytosolic and include RIG-I, MDA5 (melanoma differentiation-associated gene 5), and LGP2(34). Though they are also primarily cytosolic, RLRs have different ligand sensing domains than NLRs and generally respond to cytosolic nucleic acids (mostly RNA), thereby inducing Type I IFN production(34). Thus, their sensing repertoire and gene induction profiles are more similar to endosomal TLRs(34). And like endosomal TLRs, they are critical for mounting antiviral responses(34). RLRs share the same downstream



transcription factors as these TLRs, but are critically dependent on the mitochondrial bound adaptor IPS-1 (IFN- $\beta$ -promoter stimulator 1, aka MAVS)(34). Moreover, together with cytosolic DNA-sensing receptors (discussed below), they also depend on TBK1 (tank-binding kinase 1) and the ER-bound adaptor STING to activate IRF3 & 7-dependent transcription(34).

## **Cytosolic DNA Recognition**

Cytosolic DNA, usually introduced by DNA viruses or intracellular bacteria, also activates strong innate responses through multiple receptors and is typified by two classes of cytosolic DNA-sensors(55). The first class includes, cGAS (cyclic guanylate adenylate synthase), IFI16 (interferon inducible protein 16), DDX41 (Dead-box protein 41), and DAI (DNA-dependent activator of IRF), and are mainly characterized by their participation in the induction of Type I IFN responses(55). As many of these receptors are upregulated by Type I IFNs, have overlapping tissue expression, and can stimulate similar downstream responses, they may play redundant roles in innate immunity(55).

However, the second class include a non-redundant and critical aspect of cytosolic DNA sensing; the induction of IL-1 $\beta$  responses through the DNA-sensing inflammasome receptor AIM2 (absent-in-melanoma 2)(56). AIM2, like several of the NLRs, forms an IL-1 $\beta$ -activating inflammasome in response to

cytosolic dsDNA (a synthetic ligand often used to stimulate this response is poly(dA-dT), referred to herein as dAdT (inflammasomes are discussed below)).

Importantly, nucleic acid-based (especially DNA-based) vaccines are often used to provoke protective immune responses involving aspects of both classes of DNA-sensors and downstream signaling(57, 58). However, the simultaneous induction of these responses can also have disastrous consequences under circumstances where DNA accumulates in the cytosol, as it does in several pathological conditions. Cytosolic nucleic acid sensors, like AIM2, RIG-I and the battery of known Type I IFN inducing DNA sensors can sense undigested nucleic acid complexes, which accumulate as a result of DNase or RNase deficiency, leading to IFN-driven autoimmune diseases that cause robust inflammatory pathology(59-61). Importantly, antibody immune complexes carrying nucleic acids (or other TLR ligands) in autoimmune disease are also inflammatory particulates, and thus, they relate back to the theme of this thesis.

## **Inflammasomes**

The term “inflammasome” was originally coined by the late Jürg Tschopp in 2002(62). Inflammasomes are cytosolic multimolecular IL-1 $\beta$ -activating complexes that assemble in response to various cellular stresses and pathogenic signals(63). As mentioned earlier, inflammasome activation leads to the activation/cleavage of pro-IL-1 $\beta$  and subsequent secretion of active mature IL-

1 $\beta$ (64, 65). However, basally, pro-IL-1 $\beta$  expression is too low to permit a productive response(63). Therefore, IL-1 $\beta$  activation by inflammasomes is usually described as a two-step signaling process(62, 66). Signal 1 involves the “priming” of pro-IL-1 $\beta$  synthesis through TLRs, NLRs, various cytokine receptors, and any other receptor that activates NF- $\kappa$ B(67, 68). Signal 2 leads to inflammasome “activation” and the secretion of mature IL-1 $\beta$ (63). As noted earlier, the additional post-translational regulation of IL-1 $\beta$  activation by inflammasomes represents a safeguard to the potent and potentially harmful effects of its overproduction. Evidence of this fact can be seen in patients with gain-of-function mutations in inflammasome components, which cause autoinflammatory diseases(69, 70). This thesis will focus on one of these inflammasomes (NLRP3), which is activated inappropriately by disease-causing sterile particles.

Each inflammasome is named after its protein sensor(63). The four most highly studied inflammasomes include NLRP1 and NLRP3 (NOD-like receptor containing a pyrin domain 1 and 3), NLRC4 (NOD-like receptor containing a CARD domain 4), and AIM2(56, 62, 71). In general, activation of these proteins leads to the recruitment and oligomerization of the adaptor ASC (apoptosis-associated speck-like protein) and the effector protease, caspase-1(63). The prototypic inflammasome, NLRP3, is made up of the NLRP3 protein with an LRR sensing domain (similar to TLRs), a nucleotide-binding domain (NBD) also called the NACHT (NAIP, C2TA, HET-E, and TP1) ATPase domain to mediate ATP-

dependent oligomerization, and a Pyrin domain (PYD)(63). Upon activation, the PYD of NLRP3 interacts with the PYD of ASC(63). ASC also has a caspase-activation and recruitment domain (CARD), which then allows it to recruit and interact with the CARD domain of the 45 kDa pro-caspase-1, leading to its autocatalytic activation into the 10 and 20 kDa subunits of mature caspase-1(63). Though the process just described is typical for the assembly of any inflammasome upon activation, each inflammasome has unique structural features and activating stimuli.

AIM2 is distinct from the other inflammasomes, which are NLRs containing LRR domains(63). Instead, AIM2 has a HIN200 domain that directly senses AT-rich dsDNA(63). Like NLRP1 and NLRP3, AIM2 has a PYD(63). However, NLRP1 also has a CARD, while NLRC4 (and mouse NLRP1b) has a CARD instead of a PYD(63). Therefore, NLRP1 and NLRC4 are both capable of directly activating caspase-1 via their CARDS without ASC, but ASC greatly enhances activation(63). Interestingly, NLRC4 is further distinguished by the fact that it relies on NAIP (neuronal apoptosis inhibitory proteins) proteins in order to sense bacterial ligands, namely flagellin(63). NAIPs bind these ligands, oligomerize, and these ligand-NAIP complexes are sensed by the LRR of NLRC4(63). Especially noteworthy is the fact that NLRP1b senses anthrax lethal toxin through the proteolytic cleavage of its LRR(63). It has been shown that expression of NLRP1 or NLRP3 without LRR domains makes them constitutively active, so it can be inferred that the LRR domains autoinhibit inflammasome

activation and their cleavage could lead to activation(63). When bound or modified by the appropriate stimulus, the LRRs may also change conformation leading to or facilitating inflammasome activation(63). However, in the case of NLRP3, the exact mechanism responsible for its activation remains unresolved despite over a decade of intense investigation(63). Since the NLRP3 inflammasome controls the IL-1 response to both necrotic cells and sterile particles, I will focus on the mechanisms proposed to govern NLRP3 activation later in this chapter.

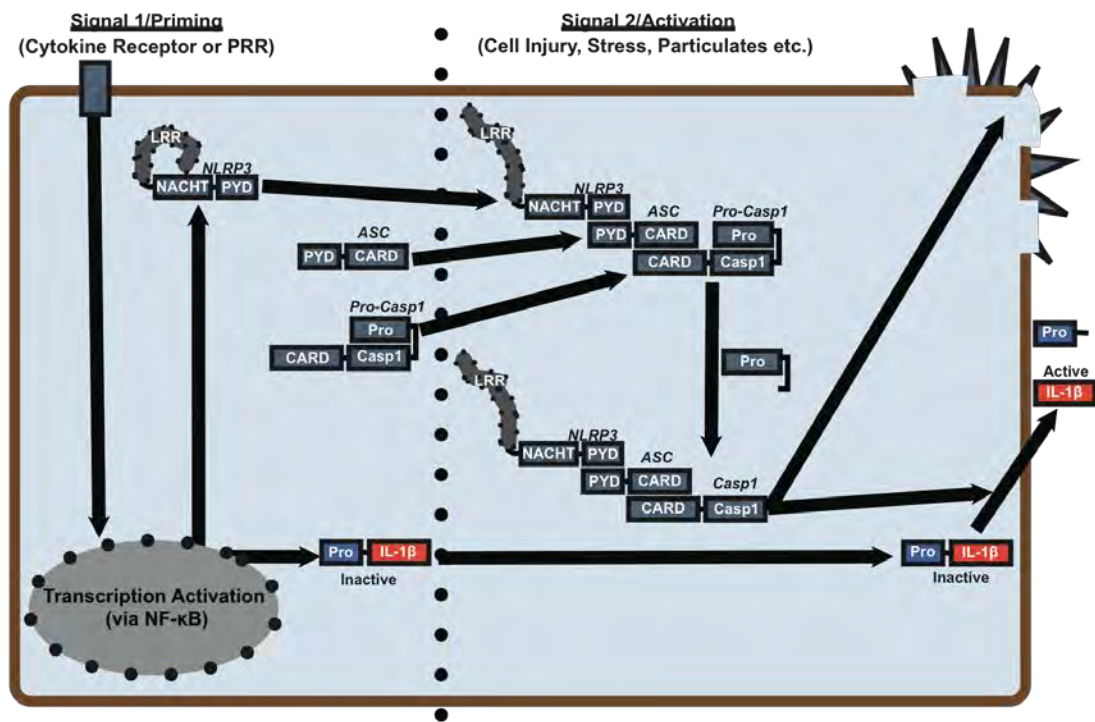
A few additional notes about inflammasomes are worth mentioning here. Inflammasomes also activate/secrete another pro-inflammatory cytokine, IL-18(63). Moreover, caspase-11 also contributes to inflammasome activation by non-canonical NLRP3-activating stimuli, like cholera toxin and a variety of intracellular gram-negative bacteria(72-75). These topics are outside the scope of this thesis and will not be discussed in further detail.

Finally, and importantly, inflammasome activation not only leads to IL-1 $\beta$  activation and secretion, but it also causes a lytic form of cell death called pyroptosis, which depends on either caspase-1 or caspase-11 (discussed later)(24). Originally, it was suggested that caspase-1 activation and IL-1 $\beta$  secretion precede pyroptotic cell death(76). However, these studies were conducted in bulk cellular assays, so they lacked the precision of single-cell analytic techniques. Indeed, more recently, it was shown using single-cell FRET (fluorescence resonance energy transfer) techniques that caspase-1 activation

and secretion of IL-1 $\beta$  occur simultaneously with pyroptotic cell death(77). Moreover, this study showed that inflammasome activation is an all-or-none event (if one molecule of caspase-1 is activated, all molecules of caspase-1 in that cell are also activated), and no cells that secreted IL-1 $\beta$  survived. Intriguingly, two back-to-back studies have recently shown that inflammasome activation releases inflammasome complexes into the extracellular environment during pyroptosis, which can act as particulate stimuli that propagate NLRP3 activation in other cells(78, 79). Thus, lytic cell death and IL-1 $\beta$  activation/secretion are two phenomenon intimately tied to one another during inflammasome activation, which necessarily releases a particulate inflammatory complex into the surrounding environment.

**Chapter I, Figure 2: General Model of Inflammasome Activation as Exemplified by the NLRP3 Inflammasome.**

The figure shows the two signals or steps that occur in order to activate an inflammasome (in this case, the NLRP3 inflammasome). Signal 1 or “Priming” is initiated by binding of ligands to cytokine receptors or PRRs, which causes NF- $\kappa$ B-mediated transcription and translation of pro-IL-1 $\beta$  (and in the case of the NLRP3 inflammasome, NLRP3 is also synthesized during priming). Upon Signal 2 or “Activation” of inflammasomes, the adaptor-binding domain (PYD) of inflammasome main sensor protein is exposed, allowing it to bind and recruit the adaptor ASC via homotypic (PYD-PYD) domain interactions. ASC can then recruit pro-caspase-1 via homotypic (CARD-CARD) domain interactions. This leads to the autocatalytic activation of pro-caspase-1 to the active caspase-1. Caspase-1 can then cleave and activate pro-IL-1 $\beta$  into the mature IL-1 $\beta$  cytokine, which is then immediately secreted. At the same time, caspase-1 induces a type of lytic cell death called pyroptosis (depicted on the upper right as membrane disruption).



### **Sterile Inflammation: Matzinger's "Danger Model"**

The immune system is generally considered for its role in protecting the body from pathogens(30). However, inflammation does not occur only in the presence of microbial stimuli(30). For example, neutrophils and monocytes were shown as early as the 1960s to infiltrate sites of cellular necrotic injury in the absence of any microbial stimuli(80). Realizing that the immune system also rejects sterile organ transplants, Polly Matzinger came up with a model arguing that the immune system is not simply discriminating between human tissues and microbial pathogens. Instead, she proposed in her "Danger Model" that, "the immune system is far more concerned with danger and potential destruction than the distinction between self and non-self"(81). In other words, she believed that the immune system's apparent self-non-self discrimination may be an epiphenomenon of a higher order process meant to recognize danger regardless of the distinction between self and non-self. Indeed, inducing cell injury in the absence of microbial stimuli can act as an adjuvant to prime the DC-based adaptive response employed by vaccines(82, 83). Moreover, the innate immune system is responsible for clearing cellular debris released from damaged tissues and promoting the healing process(84). This inflammatory response, which is initiated in the absence of microbial stimuli, is now referred to as "sterile inflammation." Since innate immune sentinels, like macrophages, are not sensing



microbes in this situation, it has been inferred that what is being sensed are self-derived “danger signals” released by stressed, damaged or dying cells(30). Thus, these danger signals are referred to as DAMPs (danger-associated molecular patterns)(30).

It should be noted that the sterile inflammatory response is operationally defined as occurring in the absence of microbial stimuli, but microbes are also able to stress, damage or kill host cells(30). Therefore, components of the sterile inflammatory response are also often highly active during microbe-associated inflammation(30). Moreover, many of the microbe-sensing receptors appear to cross-react with DAMPs, suggesting that sterile inflammatory and microbe-induced inflammatory responses are not mutually exclusive(30). In fact, microbial recognition can prime or enhance otherwise sterile inflammatory responses(30). Also, in various pathological states, sterile inflammation may even be triggered inappropriately by non-microbial stimuli, like particles, via similar mechanisms that are actually meant to sense and defend against invading microbes(30).

### **DAMPs: Danger Signals Released from Necrotic Cells**

Many DAMPs have been identified, but only a few will be covered here. It should be noted that DAMPs are classically thought to be released from cells dying by necrosis, which is an unregulated form of inflammatory lytic cell death. DAMPs are generally not released from cells dying by apoptosis, which is a non-

inflammatory programmed cell death (necrosis vs. apoptosis will be discussed in more detail later). For example, HMGB1 (high mobility group box 1) is a canonical DAMP released from necrotic cells that induces acute inflammatory responses through the receptor RAGE (receptor for advanced glycation end-products) and TLRs during sterile liver injury(85). HMGB1 is not released from apoptotic cells, since apoptosis induces stronger binding of HMGB1 to nuclear chromatin(85). Interestingly, HMGB1 does not always act by itself to induce pro-inflammatory cytokines, as first proposed(86). Instead, it often binds cytokines and DNA, carrying them to cytokine receptors and TLR9(87-89). HMGB1 also binds LPS to signal through TLR4, IL-1 $\beta$  to signal through IL-1R1, and nucleosomes to signal through TLR2(90). In fact, the delivery of TLR ligands via various carriers is a common phenomenon. Indeed, this is also the case for hemozoin, a crystalline particulate generated by malaria after it digests heme in red blood cells. Originally, it was proposed that hemozoin directly activates TLR9(91). However, it was later shown that hemozoin actually binds and delivers DNA to TLR9(92). Therefore, DAMPs like HMGB1 are released by necrotic cells in a mixture of interacting molecules that promote inflammation through a number of complex and variable mechanisms.

HMGB1 is just one among several known DAMPs participating in inflammatory responses. Another DAMP, IL-33, can be released from necrotic fibroblasts in the peritoneum and it induces an inflammatory response that depends on the ST2 receptor on mast cells(93, 94). In contrast to IL-33, HMGB1

is not essential for this particular response. HMGB1-deficient necrotic fibroblasts induce a similar level of inflammation as WT fibroblasts(85, 95). Thus, multiple DAMPs can play redundant roles in inflammatory responses and the dominant DAMP can depend highly on cell-specific and stimulus-specific conditions(96, 97).

ATP is yet another potent DAMP released from necrotic cells. ATP binds the P2X7 receptor (P2X7R), which recruits a pore-forming protein, pannexin-1, to induce K<sup>+</sup> efflux(98). This somehow activates the NLRP3 inflammasome, leading to IL-1 $\beta$  secretion and intravascular neutrophil recruitment(99-101). Conversely, ATP is released in smaller quantities from apoptotic cells, which recruit monocytes into the area for clearance by signaling through P2Y2 receptors, instead of pro-inflammatory signaling through P2X7R(102). Together, HMGB1 and ATP represent two soluble DAMPs that can promote NF- $\kappa$ B-mediated cytokine production and/or directly induce IL-1 $\beta$  activation. But necrotic death can also lead to the formation of particulate DAMPs, such as that formed upon the precipitation of uric acid.

Uric acid was arguably the first DAMP identified(103). It was shown that uric acid activates DCs and primes *in vivo* CD8<sup>+</sup> T cell responses to dead cell-associated antigens. As a byproduct of purine metabolism by xanthine oxidase, uric acid can reach high concentrations in cells and can precipitate as pro-inflammatory crystals upon necrotic cell death, a consequence of dietary excess or of decreased clearance/increased metabolism of purine nucleotides(96, 104).

These crystals can accumulate in the joints, causing a chronic and painful inflammatory condition, called gout. In fact, the crystalline form of uric acid, MSU, is the active form. For example, it has been shown that human eosinophils respond to MSU to induce chemokine and cytokine secretion, but they do not respond to soluble uric acid(105). Indeed, not only does MSU drive inflammatory pathology in gouty arthritis, as discussed earlier, but it is also a proven activator of the NLRP3 inflammasome(12). *In vivo*, MSU has since been shown to be an important inflammatory mediator of fibrotic lung injury that depends on NLRP3 and it also drives NF- $\kappa$ B-mediated inflammatory responses in renal disease(106). Therefore, as a particulate created by cell death that causes inflammation and cell death, MSU is the embodiment of particle-associated pathology.

Stepping back, I introduced particles as agents of pathology that cause necrosis and inflammation. Moreover, a plethora of inflammatory DAMPs can become complexed with one other or with inflammatory cytokines after they are released from cells. These DAMPs are then sensed by a variety of different pro-inflammatory receptors during necrosis. Thus, one might expect that therapeutically blocking all of these responses could be difficult or even impossible. Maybe, preventing the initial cell death event would be a more feasible strategy. This may prove to be the case indeed. However, it is important to note that *in vivo*, necrotic cells and sterile particles actually generate an acute inflammatory response that is almost entirely dependent on a single receptor, IL-1R1, which is a proven interventional target for certain inflammatory diseases.

## **IL-1: Master Regulator of Sterile Inflammation**

IL-1 was the first interleukin identified. Interleukin comes from the root “inter” (between) and the suffix “-leukin” (referring to leukocytes; aka white blood cells) and signifies the role of interleukins as cytokines that transmit information between white blood cells(70). Originally, IL-1 was described as an endogenous pyrogen because it triggers fever during systemic inflammatory responses that resemble the effects of LPS (called at the time endotoxin - a toxin that is intrinsic to the bacteria’s makeup – and also referred to as the exogenous pyrogen) during sepsis with gram-negative bacteria(70). IL-1 influences cellular activity at very low concentrations by many different cells and tissues, leading to fever induction by the hypothalamus, proliferation and survival of bone-marrow-derived cells, T-cell activation and differentiation, chemokine and adhesion molecule upregulation for recruitment of leukocytes by endothelial cells, and acute-phase protein production in the liver for mediating systemic inflammatory responses(70). One of the earliest observations made about IL-1 was that it was important for protection against microbial pathogens(70, 107). Here, I am concerned with the role of IL-1 in sterile inflammation resulting from lytic cell death and sterile particulates.

In the past decade, models of acute inflammatory peritonitis have shown that the master receptor of the sterile inflammatory response to necrotic cells is

IL-1R1(95). In this model, it has been shown that the recruitment of neutrophils and macrophages into the peritoneal cavity of mice is almost entirely absent in mice lacking IL-1R1. Specifically, IL-1R1 signaling upregulates adhesion molecules and chemokine secretion from endothelial cells that enable neutrophils to extravasate into the area of the original insult down a gradient of chemokines signaling through CXCR2, like CXCL1(101, 108). While various DAMPs have been shown to activate TLRs, *in vivo* responses to necrotic cells were minimally reduced in TLR2 & TLR4 double-deficient mice, but not affected at all in mice lacking only single TLRs. Conversely, this response was dramatically reduced in MyD88-deficient mice, but not mice lacking TIRAP/Mal or TRIF(95). The dependence of this response on MyD88 providing a critical clue that led to the identification of the IL-1R as the master receptor for this response. As mentioned above, IL-1 comprises two distinct cytokines, IL-1 $\alpha$  and IL-1 $\beta$ , both of which activate IL-1R1. Like TLRs, IL-1R1 signals through a cytoplasmic TIR domain that requires the adaptor MyD88. Indeed, IL-1R1 on radioresistant cells, and not bone-marrow-derived cells, was shown to be absolutely necessary for *in vivo* acute inflammatory responses to dead or dying cells(95). Follow-up studies focused on identifying the cellular sources of IL-1 driving this response. One such study found that CD11b<sup>+</sup> cells (macrophages), and not CD11c<sup>+</sup> cells (DCs), were critical sources of IL-1(109). Macrophages were the dominant source of IL-1 $\alpha$ , while bone-marrow-derived myeloid cells (which include macrophages/monocytes, neutrophils, and some types of DCs) and

radioresistant cells were together the dominant sources of IL-1 $\beta$ . Similarly, in liver injury models, monocyte-derived Kupffer cells were shown to be the NLRP3-dependent source of IL-1 $\beta$  in response to necrotic death(70, 100, 110).

In order to understand their unique roles in mediating inflammatory responses via IL-1R1, IL-1 $\alpha$  and IL-1 $\beta$  activation/secretion must be differentiated. As mentioned earlier, both IL-1 $\alpha$  and IL-1 $\beta$  are synthesized with C-terminal pro-peptides. Pro-IL-1 $\beta$  must be cleaved into mature IL-1 $\beta$  to be secreted as a biologically active cytokine capable of mediating local and systemic inflammatory responses(111, 112). Conversely, pro-IL-1 $\alpha$  is constitutively active and usually expressed as a membrane-associated cytokine mediating local inflammatory responses(113). Moreover, IL-1 $\alpha$  is present mainly in epithelial and endothelial cells throughout the body, while IL-1 $\beta$  is made primarily by monocytes/macrophages and DCs(70). Importantly, the activation/secretion of IL-1 $\beta$  in these cells is controlled by inflammasome-mediated caspase-1 activation, which also controls the secretion of IL-1 $\alpha$  by driving a lytic form of cell death called pyroptosis(114, 115). Interestingly, it has also been shown that certain stimuli require pyroptosis to release HMGB1(85, 116, 117). Indeed, like HMGB1, IL-1 $\alpha$  is itself a canonical DAMP. Cells such as DCs or vascular smooth muscle have been shown to release IL-1 $\alpha$  upon necrotic death, whereas apoptosis sequesters IL-1 $\alpha$  in the nucleus(108, 118). In fact, IL-1 $\alpha$  has a nuclear localization sequence and is thought to have transcription-related functions(70). Moreover, during necrosis, it has been shown that pro-IL-1 $\alpha$  is cleaved by a

calcium-dependent protease, calpain, converting it into its optimally active form(119). Another study found that IL-1RII, a decoy receptor that neutralizes IL-1 $\alpha$  or IL-1 $\beta$ , can bind and prevent IL-1 $\alpha$  from signaling following release from certain types of necrotic cells, like vascular smooth muscle cells(120). Conversely, macrophages dying by pyroptosis were shown to utilize caspase-1 to degrade IL-1RII, thereby liberating IL-1 $\alpha$ . In cells that do not express IL-1RII, necrosis was shown sufficient to release active IL-1 $\alpha$ . Therefore, cells dying by necrosis or pyroptosis will release pro-IL-1 $\beta$ , pro-IL-1 $\alpha$  or IL-1 $\alpha$ , HMGB1 and other DAMPs. While it is presumed that only cells dying by pyroptosis will secrete active mature IL-1 $\beta$ , it is also possible that cells that have already activated IL-1 $\beta$  intracellularly could release it via necrosis as well(70).

It is clear that IL-1R1 is critical for these *in vivo* responses, but the unique ways in which IL-1 $\alpha$  and IL-1 $\beta$  influence these responses are still under active exploration. Interestingly, both IL-1 $\alpha$  and IL-1 $\beta$  are critical for the *in vivo* inflammatory response to necrotic cells and sterile particles, as shown in mice lacking either gene alone(109, 121). Although the reason for this is not entirely clear, since both IL-1 $\alpha$  and IL-1 $\beta$  signal via the same receptor, a recent study suggested that IL-1 $\alpha$  is directly bound by IL-1 $\beta$  and requires IL-1 $\beta$  for its own secretion(122). Therefore, preventing the activation and secretion of IL-1 $\beta$  should prevent the IL-1R1-driven response triggered by both of these cytokines. However, neutralizing antibodies targeting IL-1 $\alpha$ , and not IL-1 $\beta$ , nearly ablated the inflammatory response to dead or dying cells *in vivo*, suggesting IL-1 $\alpha$  is the



dominant mediator(95). As a word of caution on this conclusion, it was proposed that the neutralization of IL-1 $\beta$  may have been incomplete in this study. However, a subsequent study found again that macrophages deficient in IL-1 $\beta$ , and not macrophages deficient in IL-1 $\alpha$ , were able to reconstitute these responses in IL-1R1-deficient mice(109). So the question remains: Why do IL-1 $\alpha$ -deficient or IL-1 $\beta$ -deficient mice have phenotypes equivalent to IL-1 $\alpha,\beta$  double-deficient or IL-1R1-deficient mice?

Future studies will be necessary to clarify the relative contribution of IL-1 $\alpha$  and IL-1 $\beta$  to the IL-1-dependent acute inflammatory response to dead cells and sterile particles. Nonetheless, it is worth emphasizing once again that IL-1-dependent responses can be driven either by necrosis, leading to the release of pro-IL-1 $\beta$ , IL-1 $\alpha$ , HMGB1 and other DAMPs, or by inflammasome-dependent pyroptosis, leading to the release of active IL-1 $\beta$ , IL-1 $\alpha$ , HMGB1 and other DAMPs. As mentioned earlier, the NLRP3 inflammasome is the only known mediator of IL-1 $\beta$  activation and pyroptosis in response to sterile particles and necrotic cells. Therefore, I will now describe the theorized mechanisms of NLRP3 activation as they relate to sterile particles.

### **Mechanisms of Particle-Induced NLRP3 Activation**

NLRP3 is activated by the most physically and chemically diverse set of stimuli of any known inflammasome or PRR (**see Fig. 3 below**). Moreover, NLRP3 is

known to govern the IL-1-response to sterile inflammatory insults, including necrotic cells and particulates(123, 124). Direct sensing of such a vast range of dissimilar ligands is not likely. Instead, it has been proposed that there is some type of non-specific stress that activates NLRP3 under all these conditions(124, 125). K<sup>+</sup> efflux(15, 126-136), ROS(127-130, 135, 137-140), and cathepsins(17, 126, 128, 136, 140-153) have each been proposed as necessary for nearly all NLRP3 stimuli. These, and other stress-related cell biological mechanisms implicated in the process of NLRP3 activation, will be discussed in detail below. However, I will begin here with some specific requirements that have been shown unique to particulate stimuli.

**Chapter I, Figure 3: A Brief List of the Various Activators of the 4 Best-Studied Inflammasomes.** The figure shows the main activating stimuli for the NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes. The list shown here is not exhaustive, but is meant as a reference tool for this thesis. Moreover, it should be apparent how many stimuli can activate NLRP3 compared to other inflammasomes. The diversity of these stimuli suggest that NLRP3 is activated by cellular stress induced by these stimuli, rather than by directly recognizing these stimuli through binding.

### **Brief List of Inflammasome Activators: NLRP3 Stands Out**

**NLRP1** – Muramyl Dipeptide (Bacterial Cell Wall), Lethal Toxin/LT (*Bacillus anthracis*)

**NLRP3** –

Particulates/Crystals (CC, Silica, MSU, CPP, Alum, Hemozoin, TiO<sub>2</sub>, Asbestos)

1,3 beta glucans (Fungi - *Aspergillus*, *Candida*)

Amyloid (SAA, A-Beta, IAPP)

Lysosomal Membrane Disruption (FFA, H<sub>2</sub>O<sub>2</sub>, Lysosomotropic detergents)

Ionophores (ATP, Nigericin, Maitotoxin)

Gram(-) & Intracellular Bacteria (*Chlamydia*, *Neisseria*, *Listeria*, *Klebsiella*, *Legionella*, *E. coli*, *Citrobacter*, *Haemophilus*, *Vibrio*, *Enterobacter*, *Shigella*, *Proteus*)

Gram(+) (GBS, *Pneumococcus*, *Staph*)

Pore/Toxin (*Staph* (PL), *Strep* (SLO), *Listeria* (LLO), *Vibrio* (hemolysin), Anthrax (LeTx), *Neisseria* (LOS), *Aeromonas* (AL), Ricin)

Viruses (*Adenovirus*, Influenza, Reovirus) Nucleic Acids (Bacterial/Viral/Mitochondrial RNA, DNA)

Other: Antivirals, Hyaluronan, (Glucose Elevation in Beta cells), (UVB, HSR Irritants in Keratinocytes), Oxidized Heme

**NLRC4** - Flagellin (~TLR5), Intracellular Bacteria (*Salmonella*, *Shigella*, *Legionella*, *Pseudomonas*)

**AIM2** – dsDNA [host, viral, bacteria] (ex-**Poly(dA-dT)**) & Vaccinia

## **Lysosomal Membrane Disruption (LMD) and Cathepsin B**

Particulate stimuli require phagocytosis and lysosomal acidification to activate NLRP3(12, 144), while lysosome-disrupting detergents require only lysosome acidification(154). Originally, the NLRP3-activating mechanism proposed for sterile particles and lysosome-disrupting detergents was that lysosomal membrane disruption (LMD) releases the cysteine protease, cathepsin B, into the cytosol, somehow leading to NLRP3 activation(10, 11, 144). This subject will be explored in more depth in chapters III and IV, so I will not go into great detail here. However, it should be noted that the involvement of cathepsin B in this pathway is controversial given contradictory results obtained by studies using a supposedly cathepsin B-specific inhibitor, Ca074Me, and genetic models of cathepsin B deficiency(10, 15, 155-163). In brief, Ca074Me blocks NLRP3 activation, while cathepsin B-deficiency does not. Also, although several studies have provided direct evidence supporting the idea that particulate stimuli specifically activate NLRP3 (and not other inflammasomes) by inducing LMD and the release of cathepsins into the cytosol(17, 124, 136, 144, 145, 148, 150), LMD and the release of cathepsins has also been observed during NLRP1 activation(162). Indeed, Ca074Me has been shown to block NLRP1b-mediated IL-1 $\beta$  secretion induced by anthrax lethal toxin(162). Moreover, Ca074Me blocks IL-1 $\beta$  secretion by both particulate and non-particulate stimuli, including ATP, nigericin, antiviral compounds, A-beta amyloid, silica, alum, cholesterol crystals,

titanium dioxide, bacteria, toxins, fungi, viruses, dextran sodium sulfate, DNA and RNA(17, 126, 128, 136, 140-153). Therefore, cathepsins may play a role in NLRP3 activation by any type of stimulus, and they could possibly have a role in mediating the responses of other inflammasomes, so it is worth going into some detail here about the other mechanisms proposed for NLRP3 activation and how these may relate to activation with sterile particles.

## **Reactive Oxygen Species (ROS)**

One of the most well supported mechanisms proposed for NLRP3 activation involves ROS(127-130, 135, 137-140). Originally, it was found that ROS inhibitors blocked NLRP3 activation and that shRNA knockdown of a cellular ROS inhibitor, thioredoxin, enhanced IL-1 $\beta$  activation by sterile particles, like MSU and asbestos(130, 133). This is consistent with the fact that the inflammatory character of sterile particles is correlated with their ability to induce ROS in host cells(164-166). One of these studies investigating the relationship between ROS and sterile particles found that the sequence of events during particle-induced NLRP3 activation proceeds in the following order: phagocytosis, ROS production, LMD, NLRP3 activation(166). The connections between these events will be discussed in more detail later. For now, I will consider the fact that the interaction between large (“Frustrated Phagocytosis”) or indigestible particles and phagocytic cells initiates a sustained respiratory burst of ROS(167). This

respiratory burst is usually used by macrophages to kill and digest pathogens, and it is generated by the phagosomal enzyme complex NADPH oxidase, which generates superoxide radicals. Indeed, it was shown that shRNA knockdown of the p22phox subunit of this complex diminished NLRP3 activation(130). However, ROS production occurs with non-particulate stimuli as well, like ATP and the antiviral TLR7 ligand, R837, suggesting that ROS are important for NLRP3 activation in general(12, 138). On the other hand, it is well known that TLR activation causes robust ROS production, but this appears to be insufficient for NLRP3 activation(168). Also challenging the ROS hypothesis, it was later shown that cells from patients with chronic granulomatous disease, lacking functional NADPH oxidase complexes, can respond to particles and actually have elevated IL-1 $\beta$  activation(169). Furthermore, elevated ROS by macrophages deficient in superoxide dismutase-1 (SOD1), an endogenous ROS inhibitor like thioredoxin, causes covalent oxidative modification and inhibition of caspase-1(170). Moreover, mice lacking SOD1 are actually protected from caspase-1/11-dependent endotoxic shock and have depressed innate immune responses in the lungs(170, 171). Therefore, it seems that elevated superoxide production can suppress NLRP3 activation. However, it has been suggested that there may be another source of ROS, besides NADPH oxidase, that is important for NLRP3 activation(172).

A recent landmark study demonstrated that the critical source of ROS for NLRP3 activation is the mitochondria(173). This study found that low

concentrations of rotenone (inhibitor of complex I in the ETC (electron transport chain)) or antimycin A (AntA; inhibitor of complex II in the ETC) induce mitochondrial ROS production and NLRP3-dependent IL-1 $\beta$  activation, whereas higher concentrations lead only to cell death. Moreover, they showed that inhibition of mitophagy, which is a process used to digest old or damaged mitochondria that are prone to spontaneous ROS production, promotes NLRP3 activation. On a side note, this was also shown later by a group that knocked out key proteins involved in autophagy, which is a more general mechanism for digesting old or damaged intracellular components(174). Additionally, it was shown that upon stimulation with MSU or nigericin (a potassium efflux-inducing toxin and robust NLRP3 activator), NLRP3 localizes near the outer mitochondrial membrane and the endoplasmic reticulum together with the protein TXNIP (thioredoxin-interacting protein)(173). Interestingly, prior to this study, it was shown that knockdown of TXNIP reduces NLRP3 activation by a number of different stimuli(175). They showed further that ROS liberates TXNIP from the antioxidant protein thioredoxin, prior to its binding to NLRP3. Moreover, using a yeast two-hybrid overexpression system and HEK293 cells, they found that TXNIP directly binds to the LRR domain of NLRP3. Finally, in a mouse model of diabetes, when they stimulated pancreatic beta-cells with high levels of glucose, the resultant IL-1 $\beta$  secretion was dependent on TXNIP. Based on these findings, these studies proposed a comprehensive model in which stress-induced mitochondrial ROS activates NLRP3 by causing TXNIP to dissociate from

thioredoxin, thereby allowing TXNIP to bind and activate NLRP3(137, 176-178). This dynamic was shown to occur following stimulation with ATP, silica and MSU. Although these results could not be reproduced by one follow-up study(16), another study found that amino-functionalized nanoparticles accumulate in lysosomes, triggering LMD and cathepsin B-dependent mitochondrial ROS production with concomitant release of TXNIP from thioredoxin(179). This made TXNIP available to bind and activate NLRP3. Moreover, they showed *in silico* that TXNIP binding to NLRP3 caused a conformational change that moved the LRR away from the PYD, allowing NLRP3 to bind ASC. Finally, in the landmark study mentioned above, they examined the role of VDAC1 (voltage-dependent anion channel 1), which is located on the outer mitochondrial membrane and is required for mitochondrial ROS production. They found that VDAC1 was essential for NLRP3 activation by particulate and non-particulate stimuli(173). It is worth noting here that overexpression of the anti-apoptotic protein Bcl-2, which regulates VDAC1, reduces mitochondrial calcium levels and ROS production(180). Moreover, Bcl-2 overexpressing macrophages from transgenic mice showed reduced IL-1 $\beta$  secretion in response to MSU, alum and nigericin(173). Therefore, this connection between Bcl-2 and ROS is important to remember, as it relates to concepts discussed in several sections below. Finally, the most direct evidence that ROS can activate NLRP3 was reported by a study that stimulated LPS-primed macrophages with H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide),



showing that it was sufficient to induce NLRP3 activation(175). However, this particular finding has not yet been corroborated (**see Appendix 11e**)(172).

Despite the evidence provided, whether ROS provide a final common pathway for NLRP3 activation remains in question. ROS studies may be confounded given the fact that Signal 1 stimuli, like TLR activators, also evoke ROS, but they are insufficient for NLRP3 activation(123, 124, 181-183). Moreover, a subsequent study showed that ROS inhibitors suppress Signal 1/priming and not NLRP3 activation(184). This study demonstrated that due to NLRP3's inherent dependence on priming - basal levels of NLRP3 are too low to activate the inflammasome without priming – it is easy to misinterpret variables that affect only NLRP3 as being specific to NLRP3 activation and not simply an affect on priming(185). Nonetheless, this does not rule out the possibility that both priming and ROS are more important for NLRP3 activation and less important for the activation of other inflammasomes. Thus, the role of ROS in NLRP3-mediated IL-1 $\beta$  secretion remains to be further elucidated(172).

### **Potassium (K<sup>+</sup>) Efflux**

K<sup>+</sup> efflux has been considered in many reports to be a common requirement for all NLRP3 stimuli(15, 126-136). In cell-based systems, the role of K<sup>+</sup> efflux is exemplified by a K<sup>+</sup> ionophore, nigericin, and ATP-mediated activation of K<sup>+</sup> efflux via the P2X7 receptor (P2X7-R)(186). This mechanism was corroborated

by an early study where Martinon et. al. noticed that low K<sup>+</sup> triggered NLRP3 assembly in a cell-free system(62). Moreover, it was shown that high levels of extracellular K<sup>+</sup> (KCl) or a K<sup>+</sup> channel blocker (glyburide) inhibit NLRP3 activation(133, 187). Finally, a recent study suggested that K<sup>+</sup> efflux is indeed an absolute requirement for NLRP3 activation(154). They showed that even though nigericin, ATP and another K<sup>+</sup> ionophore, gramicidin, all activate NLRP3, gramicidin does so without affecting mitochondrial respiration. Instead, depression of mitochondrial respiration was detected 45 min after IL-1 $\beta$  activation was observed, suggesting that this effect is downstream of NLRP3 activation. Moreover, this study showed that inducing ROS production with mitochondrial electron transport chain (ETC) inhibitors does not activate NLRP3, and that ROS inhibitors do not suppress NLRP3 activation or K<sup>+</sup> efflux induced by gramicidin. Likewise, gramicidin did not increase ROS production, although they did not specifically measure mitochondrial ROS. They also claim that ROS inhibitors do not affect priming, since gramicidin-induced IL-1 $\beta$  secretion was not reduced when ROS inhibitors were given prior to LPS priming. However, although K<sup>+</sup> efflux has also been implicated in LPS-signaling in macrophages and a variety of other cell types(188-192), this study did not examine whether pro-IL-1 $\beta$  levels (a.k.a. priming pathways) were dependent on K<sup>+</sup> efflux(172).

Interestingly, this study also found that particulate NLRP3 activators (silica, alum, CPPD) and a lysosomal detergent (LLOMe) all cause K<sup>+</sup> efflux, and they assumed this was not a result of cell death because they examined this in

NLRP3-deficient macrophages that are unable to undergo pyroptosis(154). It should be noted here that they made the assumption that cell death induced by particles and LLOMe requires NLRP3. Later, I will provide data that this assumption is incorrect. They also demonstrate that LPS priming enhances K<sup>+</sup> efflux caused by these particles and LLOMe, but not by nigericin or ATP, proposing that priming does this by enhancing phagocytosis(193, 194). Additionally, they found that the Ca074Me or inhibition of lysosomal acidification prevents K<sup>+</sup> efflux in response to particulates and LLOMe, but not nigericin or ATP. Therefore, this evidence suggested that particulates induce K<sup>+</sup> efflux, which is enhanced by priming and requires cathepsins and acidified lysosomes. Finally, this study found that incubating cells in K<sup>+</sup>-free medium spontaneously induced IL-1 $\beta$  secretion in LPS primed macrophages, supporting their conclusion that K<sup>+</sup> efflux is both necessary and sufficient for NLRP3 activation. However, how K<sup>+</sup> efflux does this remains unclear. One possible contribution that should be noted is that K<sup>+</sup> efflux causes osmotic changes in lysosomal compartments and cytosolic acidification, both of which may contribute to NLRP3 activation(126, 195-201). On the other hand, lysosomal disruption can also acidify the cytosol, which would cause K<sup>+</sup> influx and H<sup>+</sup> efflux at the cell membrane(202). Therefore, K<sup>+</sup> efflux and LMD may both be independent mechanisms for acidifying the cytosol, which could be important for NLRP3 activation. However, cytosolic pH effects have never been examined in this context(172).

## Cytosolic Calcium Influx

Another ion, calcium, has been implicated in NLRP3 activation. Two studies have shown that G-protein coupled receptors on the plasma membrane, CASR (calcium-sensing receptor)(203) and GPRC6A(204), respond to elevated extracellular calcium by inducing extracellular calcium influx, which activates ER-mediated cytosolic calcium release via PLC (phospholipase C), IP3 (inositol-3-phosphate), and the IP3-receptor on the ER membrane. In each study, activation of these receptors led to NLRP3 activation. Moreover, TRP (transient receptor potential) cation channels, like TRPV2 and TRPM7, have been shown to induce calcium fluxes in response to various cell stresses(205). It was found that IL-1 $\beta$  secretion in response to activation of these channels occurs during hypotonic stress and induces phosphorylation of TAK1 (transforming growth factor kinase 1). Indeed, knockdown of TAK1 suppressed IL-1 $\beta$  secretion. Another study showed that the ROS-responsive TRPM2 channel also plays a role in NLRP3 activation by facilitating calcium influx(206). Multiple other studies have since found a role for calcium as well(205-209). Importantly, calcium influx induced by high extracellular calcium levels or ATP is inhibited by high levels of extracellular KCl(154, 207). However, one would expect any downstream effects of ATP-induced K<sup>+</sup> efflux to be blocked by high levels of extracellular KCl anyway. Moreover, two other studies found, to the contrary, that elevated extracellular KCl does not affect calcium influx during NLRP3 activation by other stimuli(208, 210).

Additionally, a subsequent study showed that the concentration of extracellular calcium used in the study on the CASR actually precipitates into particulates in the growth medium that was used(154). When tested in other mediums that do not cause precipitation of calcium bicarbonate crystals, no NLRP3 activation was observed. Perhaps, high concentrations of localized calcium release from intracellular stores during cell death in bicarbonate buffered mediums could induce particulate formation as an incidental byproduct inducing subsequent NLRP3 activation. Whether this detracts from the findings of the multiple studies implicating calcium in NLRP3 activation is unclear. But this again identifies precipitated calcium crystals, which form in necrotic tissues, as endogenous sterile particles that potentially activate NLRP3(1).

The comprehensive calcium-dependent model posited for NLRP3 activation is as follows. Calcium and cAMP (cyclic AMP) are in balance prior to stimulation, with cAMP directly inhibiting NLRP3 activation(203). Indeed, they found that cAMP can directly bind to the NBD of NLRP3, and that it is unable to bind the NBD for NLRP3 proteins carrying mutations associated with NLRP3 hyperactivity in human patients. Upon stimulation with particulate and non-particulate NLRP3 stimuli, which have been shown dependent on CASR using siRNA, it was suggested that the induction of calcium influx inhibits adenylate cyclase. Inhibition of adenylate cyclase, which normally converts ATP into cAMP, causes cAMP levels to fall thereby disinhibiting/activating NLRP3. Moreover, they proposed that either a rise in cytosolic calcium or a drop in cAMP is sufficient to

activate NLRP3. However, a follow-up study examining the effects of activating or inhibiting adenylate cyclase during extracellular-calcium-induced NLRP3 activation found this had no effect on IL-1 $\beta$  secretion(204). Nonetheless, two additional studies examining responses in epithelial cells found that calcium influx induced mitochondrial dysfunction and ROS production, either by causing ER stress or inducing uptake of calcium directly by the mitochondria(207, 208). Similar to the study cited above, which found that mitochondrial ROS induces the localization of NLRP3 with the mitochondria, these studies found that calcium influx caused NLRP3 to localize with the mitochondria and that this process could be inhibited by calcium chelation. Therefore, it may be that calcium influx generates ROS or that ROS generates calcium influx and that one or both of these events contribute to NLRP3's localization with mitochondria, but this has not been examined closely. Importantly, the original study on the CASR also showed that it was not important for responses to the AIM2 activator dAdT or the NLRC4 activator flagellin, suggesting that calcium-mediated pathways (at least those requiring CASR) are specific to NLRP3(203). Indeed, this group meticulously tracked pro-IL-1 $\beta$  levels, which were unchanged in all experiments, suggesting that the effects observed also did not play a general role in transcriptional priming. However, just like ROS production and K<sup>+</sup> efflux, calcium efflux has been shown to result from LPS signaling in macrophages and dendritic cells by several studies showing that calcium influx is critical for NF- $\kappa$ B and IRF3-mediated transcription(211-216). Thus, it is unclear why Ca<sup>2+</sup>, or calcium

mobilizing channels, were shown to have no role in Signal 1 or pro-IL-1 $\beta$  synthesis in the above studies(172).

## **Mitochondrial DAMP Release**

Together, the mechanisms described above require further investigation before any conclusive statements can be made about the definitive pathway for NLRP3 activation. However, they all seem to indicate that mitochondria are important. It should be noted that mitochondria are thought to derive from an evolutionarily favored endosymbiotic relationship whereby a pre-eukaryotic cell engulfed an ancient prokaryote. Instead of this prokaryote harming the pre-eukaryote, or the pre-eukaryote killing it in turn, the fitness of both cells increased; the prokaryote was protected inside the pre-eukaryote, and in return, the prokaryote generated a copious supply of ATP for its host. Thus, modern eukaryotes harbor mitochondria derived from these ancient prokaryotes, suggesting that mitochondrial components may be recognized as DAMPs by innate immune cells when released into or out of their host cells. Indeed, there are a number of known mitochondrial DAMPs, such as mitochondrial DNA (mtDNA) and N-formyl peptides, which are released into the circulation following tissue injury(217). Given this fact, it is tempting to predict that NLRP3 may sense one of these DAMPs following mitochondrial damage induced by various stimuli(172).

Two recent studies have proposed that disruption of mitochondria releases mtDNA into the cytosol, which can directly activate NLRP3. One of these studies showed that defects in autophagy lead to the leakage of mtDNA out of old/damaged mitochondria and spontaneous NLRP3 activation in LPS-primed macrophages(174). Moreover, they showed that LPS and ATP induced NLRP3 activation by causing the release of mtDNA into the cytosol. However, their data suggested that NLRP3 is necessary for inducing mitochondrial ROS and mtDNA release. It is conceptually challenging to understand how the release of mtDNA or ROS requires NLRP3 activation while at the same time NLRP3 activation requires the release of mtDNA or ROS. If this is true, it may indicate that these events represent a positive feedback loop. However, a follow-up study by Shimada et. al. showed that ATP, nigericin and an apoptosis inducer, staurosporine (STS), induced mitochondrial dysfunction, the release of oxidized mtDNA into the cytosol, and NLRP3-dependent IL-1 $\beta$  activation(218). Moreover, they showed that oxidized mtDNA directly binds NLRP3. Indeed, NLRP3 activation was prevented by inhibiting mtDNA synthesis and the binding of mtDNA to NLRP3 was blocked with an oxidized nucleotide analog (8-OH-dG). However, this finding has not yet been corroborated (**see Appendix 2a**). Again, it should be noted here that they also found that overexpression or knockdown of a protein that prevents mitochondrial disruption, Bcl-2, inversely regulates NLRP3 activation. Pointing out that both K<sup>+</sup> efflux and LMD can lead to mitochondrial ROS production and mitochondrial disruption, Shimada et. al. proposed that the



release of oxidized mtDNA represents a unifying terminal mechanism for NLRP3 activation (219). However, whether or not the release of oxidized mtDNA into the cytosol occurs or is necessary during NLRP3 activation by sterile particles, remain open questions. Moreover, pro-IL-1 $\beta$  levels were not closely examined in these reports and so the role of these pathways in mediating Signal 1 remains to be examined(172).

Another study also found that a component of the inner mitochondrial membrane, called cardiolipin, might directly activate NLRP3. Iyer et. al. showed that an oxazolidinone antibiotic, linezolid, activated NLRP3 independently of ROS production by inducing the release of mitochondrial cardiolipin, which directly bound to NLRP3(220). Like ATP and silica, they blocked linezolid-induced NLRP3 activation using high extracellular KCl or an inhibitor of mitochondrial membrane pore formation, cyclosporine A (CsA). Unlike ATP and silica, linezolid responses were not prevented by ROS inhibitors or by the ROS-generating ETC uncouplers, rotenone and AntA. This suggested that neither ROS nor the ETC were necessary for linezolid-induced NLRP3 activation. Moreover, they show that ATP, silica and linezolid all induce mitochondrial dysfunction and that silica and linezolid induce the physical association of NLRP3 with the mitochondria. Additionally, they found that cardiolipin binds the NLRP3 LRR domain (although technically the domain they define as the LRR disagrees with public databases). Using a “broken cell” system, where they disrupted cell membranes and mixed in cardiolipin, cardiolipin was sufficient to induce caspase-1 activation. Finally, they

found that inhibition of cardiolipin synthase suppressed IL-1 $\beta$  secretion induced by ATP, silica and linezolid, but not the NLRC4 activator *Francisella tularensis*. Although these results are intriguing, several weaknesses emerge from this study. Firstly, while they show caspase-1 activation in response to cardiolipin in the broken cell system, they did not confirm whether this activation depended on NLRP3. Secondly, almost all of these experiments were done in a macrophage cell line (J774.1) and not replicated in primary macrophages. Therefore, it remains to be seen whether these results are generalizable to primary cells. Thirdly, the above study did not closely examine whether “priming” or the appropriate synthesis of pro-IL-1 $\beta$  was affected in these experiments, which would invariably contribute to changes in IL-1 $\beta$  secretion. It is also worth noting here that during a process of cell death driven by “death receptors” like TNF-R1, called extrinsic apoptosis (discussed below), activation of caspase-8 requires binding to cardiolipin on the outer mitochondrial membrane(221). This process is carried out by phospholipid scramblase 3 (PLS3), which transfers cardiolipin from the inner to the outer mitochondrial membrane as a result of ROS production during apoptosis(222, 223). Therefore, if the above model is true, it remains to be shown why NLRP3 is not generally activated during apoptosis as a result of cardiolipin transfer to the outer mitochondrial membrane(172).

The two models above suggest that mitochondrial dysfunction leads to the release of mitochondrial components that directly activate NLRP3(174, 218, 220). Moreover, mild mitochondrial dysfunction with low concentrations of ETC

uncouplers seems to generate ROS and activate NLRP3(173, 218), while higher concentrations of ETC uncouplers induce severe mitochondrial dysfunction and actually prevent NLRP3 activation(173, 218, 224). Therefore, it has been posited that a partial/temporary disruption of the ETC-dependent mitochondrial membrane potential (MMP) can promote NLRP3 activation, while severe disruption leads to death and prevents NLRP3 activation(172). One study proposed that the reason for this is that two mitochondrial outer membrane proteins, mitofusin 1 and 2 (Mfn 1 and 2), directly bind NLRP3 upon activation with several RNA viruses(224). Moreover, they show that this association depends on an intact MMP and that Mfn2 is required for NLRP3 activation and IL-1 $\beta$  secretion. However, this study also does not examine whether this association depends on ROS and/or K<sup>+</sup> efflux or whether any of their experiments affected pro-IL-1 $\beta$  synthesis and Signal 1(172).

## **Importance of Priming for NLRP3 Activation**

In many of the various studies examining the mechanisms of NLRP3 activation, little attention has been given to the role of Signal 1. As mentioned earlier, priming can be achieved by the activation of NF- $\kappa$ B transcription driven by receptors like TLRs, NLRs, IL-1R1, and TNF-R1&2(67, 68). One outcome of priming is pro-IL-1 $\beta$  synthesis, which is necessary because pro-IL-1 $\beta$  is not normally synthesized at resting state. To this extent, priming is required for all

inflammasomes to activate and secrete mature IL-1 $\beta$ . While they are unable to activate and secrete IL-1 $\beta$  without priming, other inflammasome proteins, including ASC, pro-caspase-1 and pro-IL-18, can support AIM2 or NLRC4 inflammasome complex formation and IL-18 secretion independently of priming as result of adequate basal synthesis(67, 68, 225). Like pro-IL-1 $\beta$ , and unlike other inflammasomes, NLRP3 synthesis is dramatically enhanced by priming and basal levels of NLRP3 are thought to be insufficient for NLRP3 inflammasome assembly(67). Therefore, priming is thought to be especially important or even an absolute requirement for NLRP3 activation(172).

In contrast to previous expectations, a more recent study showed that basal NLRP3 protein levels are actually adequate for inflammasome activation, but that non-transcriptional priming events are required for NLRP3 activation(226). In this study, they showed that NLRP3 activation occurred after stimulation with ATP following only 10 minutes of prior priming, which is too short a time for new protein synthesis. Moreover, they showed that when NLRP3 was artificially expressed in NLRP3-deficient macrophages, priming substantially increased the kinetics and magnitude of NLRP3 activation. Indeed, it has been suggested that MyD88 (along with the kinases IRAK1 & 4) is responsible for NF- $\kappa$ B-dependent transcriptional priming of NLRP3 and pro-IL-1 $\beta$ , while TRIF (and IRAK1) is responsible for non-transcriptional priming(67, 172, 227, 228).

Investigation into non-transcriptional priming of NLRP3 activation has begun only recently. So far, it has been shown that NLRP3's LRR is basally

ubiquitinated prior to priming, and that it becomes deubiquitinated upon activation of BRCC3 deubiquitinase (shown dependent on mitochondrial ROS production, which occurs during priming and is augmented in response to NLRP3 activating stimuli)(226, 229). Indeed, several studies have shown that knockdown or inhibition of BRCC3 with small molecules prevents NLRP3 activation without affecting synthesis of NLRP3 or pro-IL-1 $\beta$ (226, 229, 230). Moreover, deubiquitination is also a requirement for activation of ASC(230). Another non-transcriptional regulatory mechanism of NLRP3 occurs through its stabilization by Hsp90 (heat shock protein 90), which binds to the LRR and NACHT domains of NLRP3 to prevent its spontaneous autoactivation following its synthesis(231). Hsp90 performs this task in association with its co-chaperone SGT1 (a protein known to associate with ubiquitin ligases). This study suggested that Hsp90 and SGT1 dissociate from NLRP3 upon priming. Moreover, they show that Hsp90 stabilizes both NLRP3 and pro-IL-1 $\beta$  as they are synthesized, while SGT1 only plays a role in regulating NLRP3(172).

I expect that more mechanisms involved in non-transcriptional priming of NLRP3 activation will be described in the near future. For now, it is important to realize that the mechanisms I just described for priming imply that the two processes of Signal 1 and Signal 2 for NLRP3-mediated IL-1 $\beta$  secretion are continuous with one another, rather than two discrete and independent events. Indeed, this may relate directly to particle-induced NLRP3 activation, since other studies have suggested that priming specifically enhances K<sup>+</sup> efflux induced by

this class of activators, as described earlier(193, 194). Therefore, additional stimulus-specific priming events are also likely to be uncovered in the near future(172).

There are a few final notes worth mentioning here. First, exogenous priming is not necessary *in vivo*, as inflammasome activation and IL-1 $\beta$  secretion occur without it upon stimulation, for unknown reasons(232). No dominant trigger for priming has been identified *in vivo*. Since IL-1-dependent responses require MyD88, and multiple cytokine and DAMP receptors signal through MyD88, it is reasonable to suggest that many different stimuli could provide this signal as a result of stimulus-induced cellular stress or cell death. Second, priming alone can activate IL-1 $\beta$  in circulating monocytes, even though this does not occur in tissue macrophages(233). The reason for this is that caspase-1 is constitutively active in these cells(234). This is the principle reason that LPS or TNF- $\alpha$  can both induce IL-1 $\beta$  & IL-18-dependent septic shock(235). Moreover, in macrophages from patients with hyperactivating mutations in NLRP3, it has been shown that several constraints on inflammasome and caspase-1 activation, like K<sup>+</sup> efflux, are no longer required(236). Third, stimulation of bone-marrow-derived DCs with LPS alone can activate IL-1 $\beta$  via the RIP3 and caspase-8-dependent pathways, described below(237). Therefore, in these two latter situations, priming is the one and only critical signal driving IL-1 $\beta$  activation(172).

## Non-canonical IL-1 $\beta$ Processing via RIP3 and Caspase-8

There are several recently documented non-canonical, caspase-1-independent, intracellular platforms for IL-1 $\beta$  processing that depend on either RIP3, caspase-8 or both. As will be explained in more detail later, caspase-8 is an initiator caspase that mediates the downstream signaling of TNF-receptor family members. Caspase-8 has been shown important for IL-1 $\beta$  activation and synthesis in response to various stimuli. One seminal study showed that stimulation of TLR4 or TLR3 can activate IL-1 $\beta$  independently of NLRP3 and caspase-1, but depended instead on TRIF and caspase-8(238). Conversely, a follow-up study was unable to repeat these findings, showing that activation of TLR3 by poly(I:C) generates Type I IFN production that inhibits Signal 1/priming(239). However, since then, other studies have found similar caspase-8-dependent IL-1 $\beta$  activation is induced by Fas-ligand (activates the receptor Fas of the TNF-R family)(240), Dectin-1 receptor activation by fungi ( $\beta$ -glucans of the fungal cell wall) and mycobacteria(241, 242), pro-apoptotic chemotherapeutics or ER stress(243-245), and bacterial infection of macrophages (*Yersinia*, *Salmonella*, *Escherichia coli*, *Citrobacter rodentium*)(246-248). Importantly, it was subsequently discovered that these pathways depend on a downstream TNF family receptor kinase called RIP3 (receptor interacting protein kinase 3)(237).

RIP3 is now well-known for mediating a process of inflammatory cell death driven by either RIP1 (receptor interacting protein kinase 1)-dependent TNF

receptor family activation or by TRIF-dependent TLR3/TLR4 activation(249). This process, called necroptosis, occurs under conditions where TRIF is upregulated via TLR4 or Type I IFNs or when the synthesis of a proteins like cFLIP or IAPs(inhibitor of apoptosis proteins)/TAK1 are too low to allow or prevent apoptotic signaling, respectively (discussed below)(249-252). For instance, it has been shown the treatment of LPS-primed macrophages with Smac mimetic (Smac is discussed below), which inhibits IAPs, causes RIP3 and caspase-8-dependent IL-1 $\beta$  activation and cell death(253). This same study also found that IL-1 $\beta$  activation via this pathway is also partly NLRP3-dependent. Unlike in necroptosis, RIP3's involvement in caspase-8-mediated IL-1 $\beta$  activation is independent of its kinase function, depending instead on its ubiquitinase activity(237). Also, RIP3 and caspase-8 normally regulate each other, leading to embryonic lethal phenotypes in capase-8—deficient mice due to hyperactive RIP3-mediated necroptosis and vice versa(254-256). However, under the conditions described above, RIP3 and caspase-8 work together in a complex to mediate IL-1 $\beta$  activation.

### **Cathepsins: Lysosomal Proteases in IL-1 $\beta$ Activation & Cell Death**

**What are cathepsins and how are they regulated?**



Lysosomes were discovered in 1955 by Christian de Duve, an achievement that earned him the Nobel Prize in physiology and medicine in 1974(257-259). He observed that these intracellular granules contained numerous hydrolytic enzymes that are active at acidic pH, and therefore called them “lysosomes”(258, 259). Lysosomes represent one of two main protein degradation centers inside cells, the other being the cytosolic proteasome(259). However, lysosomes also contain a variety of hydrolases that include proteases, amylases, lipases and nucleases(259). The proteases are divided into three groups: aspartic, serine and cysteine proteases(259). Cathepsins are cysteine proteases, with the exception of the aspartic proteases cathepsin D and E, and the serine proteases cathepsins A and G(259). They are designated as being part of the cysteine protease clan CA, which are highly evolutionarily conserved among species, and can be further classified in the subgroup of the C1 family of papain-like cysteine proteases(259). Proteases in this family exist in plants, like papain in papaya, parasites, helminthes, insects, viruses, and of course, mammalian lysosomes(259).

The name cathepsin comes from the Greek word “kathepsin,” which means “to digest”(259). Indeed, much like the stomach of mammals, which contains acidic fluid facilitating optimal digestion by its enzymes, lysosomes contain cathepsins in a low pH environment (pH ~3.5-5) in which they are optimally active and perform a similar function(260). However, cathepsins have other roles beyond intracellular (and extracellular) digestion, which will be

discussed shortly. In humans, there are eleven cysteine cathepsins, including cathepsins B, L, C, S, and X, which will be examined in this thesis(259). However, there are also cathepsins K, H, F, O, V, and W(259). Additionally, in mice, there are a total of nineteen cathepsins, eight of which are not expressed in humans and are expressed mostly in the placenta(261).

Cathepsins are synthesized as prepro zymogens(259). A preprocathepsin carries an N-terminal signal peptide that is cleaved off upon transport into the ER during N-linked glycosylation with a mannose-6-phosphate that targets the immature procathepsin to the endosomes and lysosomes(259). The pro-peptide folds over into the active site, blocking enzyme activity(259). Upon acidification in endosomes, or lysosomes, procathepsins generally undergo autocatalytic activation into the mature cathepsin, which can be in the form of single or disulfide-linked double-chain enzymes(259). However, according to some reports, cathepsins C and X require cathepsins L or S for their activation(259, 262). Additionally, glycosaminoglycans (GAGs) are particularly influential in accelerating the autocatalytic activation of cathepsins, as has been shown for cathepsins B and S(259, 263, 264).

Generally, the cellular compartmentalization and acidic pH dependence of cathepsins prevents them from degrading important intracellular components instead of ingested proteins(259). However, cathepsins do remain active for a short period of time in neutral environments before their activity is lost as a result of irreversible unfolding, and they can also be stabilized by binding to their

substrates(259, 265-268). Moreover, disruption of lysosomes, as occurs during some types of cell death (discussed below) can lead to cytosolic acidification(259, 269). Indeed, cathepsins exhibit activity in a range of various non-lysosomal environments, including the nucleus, cytoplasm, and plasma membrane(259). Moreover, they can also be secreted into the extracellular environment(259). In the nucleus, cathepsins L and F have been shown to associate with and proteolytically process histones, like histone H3(259, 270). Cathepsin S is unique in that it maintains almost normal enzymatic activity at neutral and slightly basic pH, which suggests that it is capable of functioning efficiently if released into the cytosol(259). Indeed, several cathepsins have been shown to target certain cytosolic substrates directly relevant to their roles in initiating cell death, as will be discussed shortly(259). Moreover, it has been shown that at the cell membrane, and in the extracellular environment, glycosaminoglycans (GAGs) within the matrix can facilitate the activity of certain cathepsins whilst inhibiting others(259). Therefore, not only do GAGs play an important role in facilitating cathepsin activation, but they also regulate the ability of cathepsins, such as K and L, to degrade extracellular tissue matrix components like collagen and elastin(259). Likewise, cathepsin X is predominantly located in the extracellular matrix on the plasma membrane, where it plays a part in activating integrins and modifying T-cell activity and/or migration(259, 271-274).

Tissue expression of cathepsins is similarly variable(259). Cathepsins B, L, C, X, H, F, and O are expressed in virtually all tissues(259). However, cathepsin K is primarily expressed in epithelial cells, synovium and osteoclasts, and plays an important role in bone resorption(259). Cathepsin V, which is closely related to human and mouse cathepsin L, is most highly expressed in the thymus where it plays a role in antigen presentation(275, 276), and it also resides in the testes. Cathepsin W is primarily expressed in CD8 T cells and NK (natural killer) cells and presumably participates in cell-mediated toxicity of infected cells. Cathepsin S is expressed predominantly in APCs, especially DCs and macrophages, and has a role in antigen presentation(276). Finally, and critically, cathepsin C is required for the activation of several neutrophil serine proteases(277). Importantly, these neutrophil proteases have been shown capable of activating pro-IL-1 $\beta$  in cell-free systems(121, 278-281). Therefore, specific functions of cathepsins are determined to some extent by the tissues/cells in which they are expressed.

As might be expected of digestive enzymes, charged with breaking down almost any substrate that can be engulfed and encountered in lysosomes, each cathepsin can potentially cleave a wide array of different substrates with few exceptions. Their only restriction seems to be a general preference for cleaving proteins after basic and hydrophobic residues(282). Most cathepsins are endopeptidases, cleaving at positions within proteins between the C (carboxy) and N (amino)-terminus. However, cathepsin X is strictly a

carboxymonopeptidase, cleaving only single amino acids at the C-terminus, and cathepsin C is strictly an aminodipeptidase, cleaving two amino acids at the N-terminus. Moreover, in addition to acting as endopeptidases, cathepsin B is also a carboxydipeptidase and cathepsin H is also an aminopeptidase.

The regulation of cathepsin activity is complex. Once active, the ionic environment, pH, redox conditions, GAGs and surrounding molecular chemistries strongly influence cathepsin activity and specificity, making it difficult to generalize their function in cell-free assays to cellular or *in vivo* environments(259, 283, 284). A clear example of this is that cathepsins B, L, H and K have been shown to cleave a protein called kininogen in cell-free systems, however, only cleavage by cathepsin K was found to occur at the same cleavage site in the natural substrate from *in vivo* samples(259, 285, 286). Moreover, cathepsins are under constant regulation from several families of endogenous cathepsin inhibitors(259).

Endogenous cathepsin inhibitors are specific inhibitors of the C1 clan of cysteine cathepsins, and can be classified first into emergency inhibitors and regulatory inhibitors(259). The emergency inhibitors are not normally inhibiting cathepsins, and only do so when cathepsins are displaced from physiological locations during cell death and lysosome disruption or when they secreted by invading pathogens(259). The regulatory inhibitors, such as cathepsin pro-peptides, prevent cathepsin activity only prior to activation or before encountering a substrate(259). The main class of emergency inhibitors is the cystatins, which

can be further sub-classified into Type 1 Cystatins (Stefins), Type 2 Cystatins (Cystatins) and Type 3 Cystatins (Kininogens)(259). Type 1 Cystatins are generally cytosolic, but can also be found in body fluids, and in humans they include stefins A and B (a.k.a. cystatins A and B)(259). Type 2 Cystatins are found in a wider variety of locations than Type 1, but they are generally secreted(259). This group includes the most dominant cystatin, cystatin C, as well as cystatin D, E/M, F, and salivary cystatins. There are other less well-characterized groups, including the Type 3 Cystatins, Thyropins and the less cathepsin-selective Serpins. For the purpose of this thesis, cystatins B and C are the most important to understand. Cystatin B inhibits cathepsins L and H almost equally, and cathepsin B about 150-fold less well. Cystatin C inhibits cathepsins L very strongly, and cathepsins H and B equally to about the same degree as cystatin B. Other cathepsins are inhibited by the cystatins as well, but these hierarchies are less well studied.

In summary, it is critical to recognize that the non-specific nature of cathepsins for various substrates, and their complex multi-faceted regulation, has made it notoriously difficult to assign specific functions to specific cathepsins. It is only in the few cases listed above, for cathepsins L, V, K, C and S, that non-redundant activity or specific localization has allowed specific functional assignment with some degree of confidence. Cell permeant inhibitors tend to be non-specific, as do fluorogenic cellular substrates. This was shown clearly in one study showing that a supposedly specific cathepsin B inhibitor, Ca074Me,

inhibited nigericin-induced cell death, while knockdown of cathepsin B did not(147). Moreover, two other studies have found that lysosome disruption correlates with increased cleavage of a supposedly cathepsin B-specific fluorogenic substrate, Magic Red R-R, but this cleavage was inhibited by 100  $\mu$ M CA074Me and not 10  $\mu$ M (a concentration of CA074Me already shown to completely block cathepsin B activity), suggesting that other cathepsins can also cleave this substrate(142, 157, 162). There are numerous examples of this in the literature, several of which will be described later in chapters III and IV(259). However, newer and better tools for cathepsin research are being developed all the time.

The recent development of activity-based probes (ABPs) has made it possible to determine the relative activity of specific cathepsins under various conditions in living cells and whole animals with a high degree of confidence(287). Combining these probes with quenched fluorescent substrates, which fluoresce only when the probe is bound to active cathepsins, has made the non-invasive assessment of intracellular, *in vivo*, and inhibitor activity easier and more accurate than ever before(288-293). Upon labeling of active cathepsins in live cells, their activity and localization can be visualized with fluorescent imaging tools. Additionally, the probe-bound cathepsins can be extracted from these tissues, then separated and specifically identified by molecular weight. In this case, not only can one determine the exact identity of the cathepsins, but also their level of activity can be correlated directly with the intensity of the signal

generated by fluorescent probe. Indeed, the use of these tools will likely prompt researchers to revisit earlier conclusions made with less precise technology.

## **Basic Pathways of Cell Death**

Understanding how cathepsin-mediated cell death (discussed below) stands in relationship to known pathways for cell death requires some basic background. Multiple pathways have been described for cell death(294). However, cell death can generally be broken down, with a few exceptions, into necrosis and apoptosis(294). Pyroptosis, described earlier as being caspase-1/11-dependent cell death that occurs during inflammasome activation has features of both necrosis and apoptosis; membranolysis being the important necrosis-like characteristic worth considering for this thesis(24). Necrosis is generally considered pro-inflammatory, non-programmed cell death that results in cellular swelling and the loss of membrane and organelle integrity (a.k.a. lytic cell death)(294). Generally, necrosis is caused by sudden or extensive cell damage following the chemical or physical disruption of cells(294). However, there are pathways described for programmed necrosis, like necroptosis, which depends on RIP1, RIP3 and caspase-8, as discussed above(295). Another necrotic or apoptotic pathway involves cathepsins and LMD, which will be discussed in more detail below(294). In contrast to necrosis, apoptosis is generally non-inflammatory programmed cell death that results in nuclear condensation, DNA



fragmentation and membrane blebbing without loss of membrane integrity(294). Furthermore, apoptosis can be subdivided into two pathways, intrinsic apoptosis and extrinsic apoptosis(294).

Intrinsic apoptosis can be initiated by LMD, DNA-damage, growth factor withdrawal, and other various cellular perturbations that lead to mitochondrial outer membrane permeabilization (MOMP)(294). In general, MOMP is regulated by the Bcl-2 family, which is made up of anti-apoptotic (ex- Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic proteins (Bid, Bax and Bak)(294). Anti-apoptotic and pro-apoptotic Bcl-2 family members are in a constant balance, neutralizing one another under basal conditions(296). When the balance between anti- and pro-apoptotic proteins is tipped in favor of apoptosis, the key terminal effectors required for MOMP, Bax and Bak, form a pore in the mitochondrial outer membrane. This dissipates the proton gradient generated across this membrane required for the production of ATP by oxidative phosphorylation, which consequently is also required for the production of ROS. MOMP also causes the release of pro-death factors from inside in the mitochondria, which include cytochrome C and Smac. Importantly, in order for cytochrome C to be released from sequestration, an inner mitochondrial membrane lipid, cardiolipin, must undergo ROS-mediated peroxidation, which is initiated by activated Bid, cytosolic phospholipases or neutral sphingomyelinases that disrupt the mitochondrial respiratory chain(297-300). Upon release from mitochondria in the cytosol, cytochrome C binds and activates apoptotic protease-activating factor 1 (Apaf-1),

which forms a multimolecular cytosolic complex closely resembling inflammasomes. This complex activates the initiator caspase-9, which then activates the executioner caspases 3,6 & 7. Caspases 3,6, & 7 begin a cascade of downstream enzymatic hydrolysis throughout the cell leading to all the final morphologic features of apoptotic death. Smac, on the other hand, inhibits the X-chromosome-linked inhibitor of apoptosis protein (IAP) called XIAP, as well as the cellular IAPs, cIAP1 and cIAP2, which normally inhibit caspases 3,7 & 9 to prevent apoptosis.

Similarly, the terminal steps in the extrinsic pathway of apoptosis also activate the executioner caspases 3,6 & 7. However, this pathway is initiated by death-receptor signaling through TNF receptor superfamily members (TNF-R1, TNF-RII, Fas, TRAIL-R) on the plasma membrane, which are activated by stimuli outside of the cell(294). Under conditions where XIAP, cIAP1 and cIAP2 are unable to perform their usual function of promoting NF- $\kappa$ B-mediated transcription of pro-survival factors, like when protein translation is blocked, this leads to the formation of a death-inducing signaling complex (DISC)(294). The DISC activates caspase-8 (or caspase-10 in some cases)(294). Caspase-8 can directly activate caspases 3,6, & 7 in some cell types, but in others it leads to cleavage of Bid, which activates Bax and Bak, leading to MOMP and the terminal pathway described above(294). It is worth noting here that Bid can be cleaved by a number of proteases, including caspase-8, caspase-3, granzyme B, calpain, cysteine cathepsins, and cathepsin D(294, 301). Therefore, it is thought that Bid

senses cell injury or stress that may result in any one of multiple pathways for intracellular protease activation and cell death(294).

## **Cathepsins and Lysosomal Cell Death**

Lysosomal cell death was first recognized and described by Christian de Duve(257, 302). Although this pathway of cell death was acknowledged by the field early on(257, 303), it was soon dismissed as just another caspase-dependent form of apoptotic death since caspase inhibitors, now known to inhibit cathepsins as well, suppressed this response(257, 304). However, genetic models of cathepsin deficiency, where LMD has been confirmed to be causative for cell death, have shown otherwise(257). Cathepsin B-deficiency has been shown to protect cells from lysosomal cell death in response to growth factor withdrawal and etoposide toxicity in monocytes(257, 305), TNF- $\alpha$ /Death Receptor Activation/TNF plus protein synthesis inhibitors known to cause necrosis in either fibroblasts or hepatocytes/hepatocellular carcinoma cells(257, 306-310), parvovirus in brain tumors(257, 311), and LLOMe in breast cancer cells(257, 312). Similar results were obtained for growth factor withdrawal and TNF plus protein synthesis inhibitors in cathepsin L-deficient cells(257, 305, 306). Also, cathepsin D-deficiency (an aspartic protease) has also been protective in fibroblasts treated with adriamycin, etoposide or TNF- $\alpha$ (313, 314), spontaneous death of neutrophils(315), STS in T cells(257, 316), interferon-gamma and death

receptor activation HeLa cells(257, 317), and HIV-1/Nef killing of human CD4 T cells(257, 318).

Most of the pathways described for LMD-mediated cell death are described as apoptosis(257). However, it has been shown that while mild LMD leads to apoptosis, more severe LMD leads to necrosis(319, 320). Regardless of the ultimate cell death phenotype, LMD has been shown to cause caspase-independent cell death in response to a variety of different stimuli(197, 321-323), and in situations where inhibition of activated caspases fails to suppress cell death(311, 324). Therefore, LMD appears to initiate an independent pathway of programmed cell death. Although only a few intracellular death-related substrates have been described so far, cathepsins are considered by experts in the field to be “death-executing proteases” in their own right(257, 259).

Many different stimuli cause LMD, but LMD is not always the cause of cell death when it occurs(257). In fact, any type of cell death will mostly likely cause LMD at some point(257, 325). Moreover, LMD is easy to overlook since leakage of lysosomal contents can occur without causing readily observable changes in lysosomal ultrastructure(257, 326). Therefore, determining whether LMD is the cause or result of cell death requires either direct induction of LMD with lysosomotropic detergents, genetic evidence of dependence on cathepsins, or observation of LMD plus inhibition of cell death with cathepsin inhibitors in cases where other cell death pathways are ruled out(257). Lysosomotropic detergents accumulate in lysosomes (or other acidic compartments) because they are

weakly basic amphiphilic molecules that can diffuse across membranes at neutral pH, but they become protonated and no longer able to pass through membranes at acidic pH(257, 327). This is called, “Ion Trapping.” One of the most commonly used lysosomotropic detergents is LLOMe, which requires cathepsin C-mediated cleavage in lysosomes in order to induce LMD(328, 329). Importantly, other stimuli known to induce LMD are viruses, pore forming toxins (like nigericin) from bacteria, fungi, spiders and snakes, ROS, various lipids and their metabolites, ultraviolet light (UVA/B) and DNA-damage-mediated activation of p53(257). There are many other inducers of LMD, but an exhaustive list is outside the scope of this thesis. However, it is worth noting that a major intracellular inhibitor of LMD is Hsp70(324). This may have special relevance to inflammation. Hsp70 can be degraded by calpain (described earlier as the protease that cleaves pro-IL-1 $\alpha$ ), which has been shown to contribute to LMD by a variety of studies in neurons(199, 257, 330-333). Next, I will focus on one more mechanism for promoting LMD as it relates to the consequences of crosstalk between lysosomes and mitochondria in cell death.

## **Lysosomal-Mitochondrial Crosstalk in Cell Death**

One of the first cytosolic substrates established for cathepsins was a pro-apoptotic Bcl-2 family protein, Bid(334). This cleavage activates Bid, now called truncated Bid (t-Bid), and can be achieved by multiple redundant cathepsins

(including cathepsins B, K, L and H), as shown in both cell-free and cellular systems(335, 336). Indeed, cathepsins also have been shown to cleave and inactivate anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-xL, Mcl-1 and XIAP(200, 336). In fact, it has been shown that microinjection of cathepsins B or D directly into cells can trigger MOMP(337, 338). Indeed, many different LMD-inducing stimuli have been shown to induce MOMP via cathepsins, such as viruses(339) and amino-functionalized nanoparticles(179). Therefore, there seems to be a linear relationship between LMD, cathepsins, the Bcl-2 family and MOMP(257).

Interestingly, in cell-free conditions, cathepsin B has been shown capable of directly cleaving caspase-11 at neutral pH(142), and caspase-1 at acidic pH(308, 310, 340-343). Moreover, in a human monocyte cell line (THP-1 cells) cathepsin B was shown to directly cleave caspase-1. However, unlike its normal autocatalytic cleavage pattern (into 10 and 20 kDa subunits), this generated 37 and 40 kDa fragments making the significance of this cleavage in cellular systems unclear(142). Thus, cathepsins have multiple known cytosolic targets that can lead to MOMP and cell death via the pathways for intrinsic apoptosis or possibly pyroptosis, as described above(257).

Interestingly, the Bcl-2 family has also been suggested to regulate LMD. It has been shown that Bax can induce pore formation in isolated lysosomes and that it is responsible for inducing LMD in *in vitro* and *in vivo* models of Parkinson's disease(344, 345). Also, it has been shown that a pro-apoptotic Bcl-

2 protein, Bim, can recruit Bid to lysosomal membranes and enhance LMD following stimulation of the death receptor TRAIL-R in hepatocytes(346, 347). However, there are several studies showing that Bcl-2 overexpression or Bax-Bak double-deficiency do not prevent LMD under various circumstances, suggesting that the Bcl-2 family may be sufficient, but not necessary, to cause LMD(322, 323, 348-350). Nonetheless, activation of MOMP by cathepsins via the Bcl-2 family also causes a burst of ROS from the mitochondria, and ROS are important mediators of LMD.

Experts in the field of lysosomal cell death agree that ROS are among the most important inducers of LMD(78, 197-199, 219, 269, 294, 351-355). As mentioned earlier, phagocytosis of LMD-inducing particles generates ROS, and the inflammatory character of sterile particles is directly related to their ability to induce ROS production(164-167). Moreover, ROS-dependent LMD has been observed in neutrophils, neurons, and cardiac muscle cells(198, 315, 352, 356). The reason that lysosomes are especially susceptible to LMD caused by ROS is that various iron-containing molecules accumulate in the lysosomes of macrophages and other cells, either via phagocytosis of red blood cells or autophagy of old mitochondria. This “ferruginous material” can be oxidized by membrane permeant intracellular ROS, like H<sub>2</sub>O<sub>2</sub>, leading to conversion of the reduced ferrous form of iron into the oxidized ferric form (Fenton Reaction: H<sub>2</sub>O<sub>2</sub> + Fe<sup>2+</sup> → Fe<sup>3+</sup> + OH· + OH<sup>-</sup>), which results in the production of highly reactive hydroxyl radicals(294, 352). Indeed, H<sub>2</sub>O<sub>2</sub> is used in common models for LMD

and cell death(78, 175, 219). Hydroxyl radicals cause membrane lipid peroxidation leading to disruption of these phospholipid barriers. Interestingly, iron supplementation is contraindicated for patients suffering from inflammation or infection, and this makes sense given the current discussion(1). Finally, various endogenous lysosome-disrupting molecules in cells are activated by ROS, including PLA2 (phospholipase A2), N-aspartyl chlorin e6, and siramesine(323, 357, 358).

But where does all this ROS come from? Although NADPH oxidase can generate ROS, as mentioned earlier, the vast majority of ROS production in cells is generated by the mitochondria through the ETC pathway for oxidative phosphorylation(359). The release of lysosomal enzymes into the cytosol has been shown to cause mitochondrial dysfunction and a burst of mitochondrial ROS production(360). Thus, in a feed-forward loop, LMD can potentially enhance LMD via lysosomal-mitochondrial crosstalk. In summary, whether or not the Bcl-2 family directly or indirectly induces LMD via mitochondrial ROS, there are important interactions between lysosomes and mitochondria that are worth considering when evaluating LMD and cell death.

### **Summary, Research Question, Hypothesis**

Exogenous and endogenous particle-induced sterile inflammatory diseases cause widespread human suffering and most have no cures. Disease-causing



sterile particles initiate cycles of cell death and inflammation that are symptomatic of inappropriate innate immune macrophage activation meant for the destruction of intracellular pathogens. The type of lytic cell death caused by these particles is known to be inflammatory as a result of DAMP release and the activation of multimolecular inflammasome complexes, which further exacerbate a form of enhanced inflammatory cell death called pyroptosis. Despite the complexity of the inflammatory response generated by these particles, it seems to depend almost entirely on the IL-1R. The IL-1R can be activated by IL-1 $\alpha$  or IL-1 $\beta$ . Although the processing and secretion of these two cytokines is different, these events are inextricably linked to lytic cell death. Therefore, it is highly probable that blocking either or both lytic cell death and IL-1 $\alpha,\beta$  production/secretion induced by sterile particles has potential therapeutic benefits in the treatment of particle-induced sterile inflammatory disease.

My main research question is as follows. **What role do cathepsins play, if any, in particle-induced sterile inflammation?** Moreover, there is another more peripheral question I have been asking during my investigation into the role(s) of cathepsins in particle-induced sterile inflammation that has direct clinical relevance. **Are cathepsins tractable therapeutic targets mediating IL-1 $\alpha,\beta$  production/secretion and lytic cell death induced by sterile particles in macrophages?** A key word in this question is “tractable.” Besides vaccines, the vast majority of successful therapeutics target enzymes (ex- proteases). Other successful targets are receptors and ion channels. While the field of biologics,

which deals with mostly blocking-proteins, has been promising, there are significant drawbacks to this approach. Patients suffering from genetic mutations that cause hyperactive or inappropriate inflammasome activity, Hereditary Periodic Fever (HPF) syndromes, respond dramatically to IL-1-neutralizing biologics(361-363). Moreover, the use of these biologic for treatment of various inflammatory disease has demonstrated that IL-1 is a valid therapeutic target(364), and such treatments have even been shown to attenuate atherosclerosis in mice(69). However, the IL-1-blocking biologics tested so far have been complicated by expensive production and storage requirements, short half-lives, injection-site reactions, development of neutralizing antibodies and infectious adverse effects that are exacerbated as the IL-1-blocking agent becomes more effective(364). On the other hand, small-molecule drugs that target enzymes, receptors or ion channels have significant advantages. Firstly, the cost of synthesis and stability of small molecules makes long-term preventative studies possible. Furthermore, membrane penetrating small molecules would allow intracellular targeting of particle-specific NLRP3 activating pathways. Greater specificity translates into less-generalized immunosuppression. Importantly, compared to IL-1-blocking, specific inhibition of NLRP3 would actually allow broader coverage over the effectors mediating its pathology by reducing both IL-1 $\beta$  and IL-18 activation. If pro-IL-1 $\beta$  and pro-IL-1 $\alpha$  synthesis and particle-induced cell death can be inhibited as well, this would be

even better. Therefore, intracellular targets that can be accessed by small-molecule drugs are what I mean here by “tractable.”

Another key term in my second research question is “therapeutic.” That is to say, what tractable drug targets will give the effects I want; reduction of particle-induced cell death and IL-1 secretion? Firstly, ROS have been implicated by all the different mechanisms of NLRP3 activation, including those relating to sterile particles. However, decades of clinical trials testing antioxidants have failed to demonstrate significant therapeutic efficacy in a number of inflammatory conditions, mostly because of the inherent difficulty in targeting a specific type of ROS at the right time and in the right location(365). Therefore, these targets are currently not tractable. Potassium efflux and calcium influx both seem integral to NLRP3 activation, but neither has been directly implicated as being necessary for lytic cell death by sterile particles. Moreover, systemic KCl treatment is known to directly induce sudden cardiac death, and therefore would not be therapeutic. Likewise, inhibitors of mitochondrial ROS that block the ETC are also deadly poisons. Given all of the various IL-1 $\beta$ -activating and cell death mechanisms discussed above, there are two likely candidates as tractable therapeutic drug targets: caspase-1/11 or cathepsins.

My hypothesis is as follows: **Particle-induced IL-1 $\beta$  secretion and cell death are both dependent on cathepsins, and therefore, cathepsin inhibitors and genetic models of cathepsin deficiency should suppress these responses *in vitro* and exhibit an anti-inflammatory effect *in vivo* that**

**surpasses the effect of caspase-1/11 deficiency.** Therefore, I believe cathepsins may be the tractable therapeutic drug targets I am seeking. Chapters III and IV will introduce the rationale for this hypothesis, and describe the controversies that investigating this hypothesis will invariably address. Moreover, these chapters will describe my interrogation of this hypothesis in great detail.

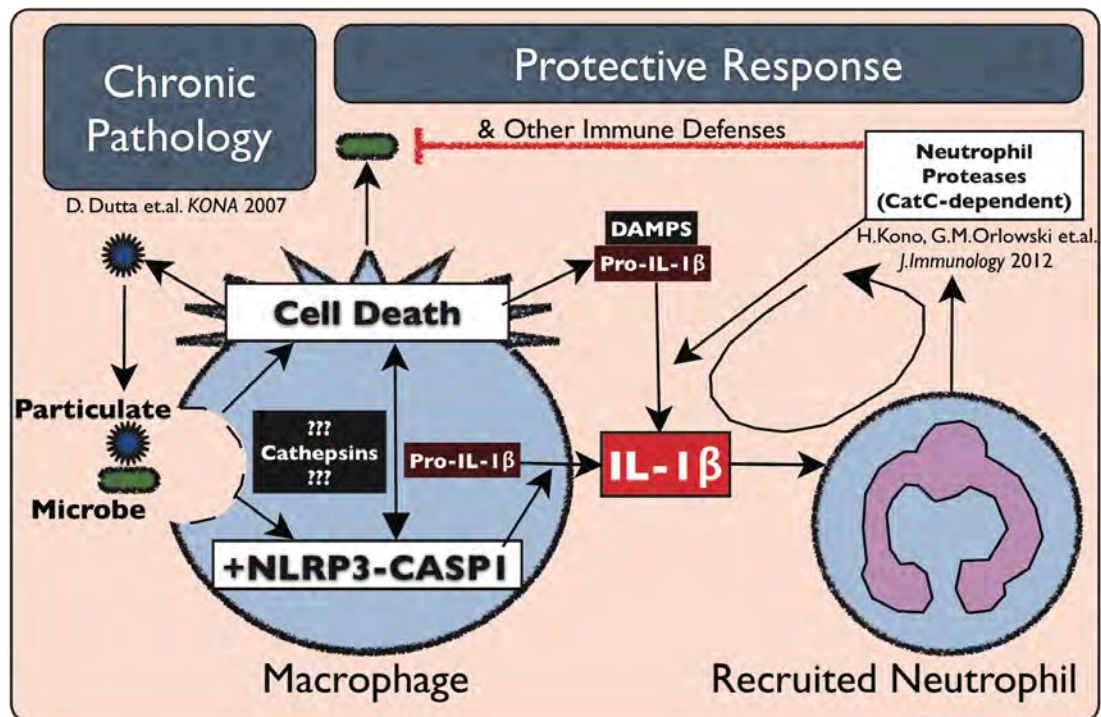
Other key questions that will be addressed in my investigation include the following:

- 1. Are cathepsins involved in particle-induced NLRP3-mediated IL-1 $\beta$  secretion?**
- 2. Are cathepsins involved in NLRP3-mediated IL-1 $\beta$  secretion induced by non-particulates?**
- 3. Are cathepsins involved in particle-induced cell death?**
- 4. How and which cathepsins?**
- 5. Which cathepsins are inhibited by Ca074Me and at what concentrations?**
- 6. What is the mechanism of NLRP3 activation by particulates and/or non-particulates?**
- 7. What organelles are involved?**
- 8. How is lytic cell death connected to NLRP3-mediated IL-1 $\beta$  secretion?**

9. How do cathepsin inhibitors/deficiencies compare to caspase inhibitors/inflammasome deficiencies?

**Chapter I, Figure 4:**

**Hypothetical model for the role of cathepsins in particle-induced sterile inflammatory pathology.** The figure shows a macrophage encountering a microbe or a sterile particle in the peripheral tissues. Upon ingestion (phagocytosis), the microbe or particle induces LMD, which leads to NLRP3/Caspase-1 activation. This results in IL-1 $\beta$  activation and secretion, which recruits neutrophils and other inflammatory cells to the site, as well as pyroptotic cell death. Cathepsins may be involved somehow in NLRP3/Caspase-1 activation or independently involved in promoting cell death. If this is the case, the cathepsin-dependent cell death can cause the release of DAMPs and pro-IL-1 $\beta$ , similar to the way pyroptosis does so, and these DAMPs may also recruit neutrophils. Neutrophils may then release cathepsin C-dependent serine proteases that can activate IL-1 $\beta$  extracellularly and drive the IL-1-dependent inflammatory response independently of NLRP3/Caspase-1. In the case of an invading microbe, this is most likely a protective response that releases the microbe attempting to invade the macrophage and recruits other immune defenses to kill this microbe. In contrast, when particles induce this response, cell death, inflammation and re-release of the particle leads to a cycle of chronic pathology. If cathepsins are driving these two responses to particles (cell death and IL-1 $\beta$  secretion), then cathepsins may be tractable therapeutic targets for the treatment of particle-induced sterile inflammatory diseases.



## **Chapter II: Materials and Methods**

## Chapter II: Materials and Methods

### **Reagents and Antibodies**

Abs for flow cytometry were against mouse Ly-6G (1A8; BD Biosciences), Ly-6C (7/4; AbD Serotec) and the dead cell marker was 7-AAD (LifeTechnologies). Antibodies for western blots were against mouse IL-1 $\beta$  (R&D Systems), caspase-1 p10 (sc-514; Santa Cruz Biotechnology),  $\beta$ -actin (C4; Santa Cruz Biotechnology) and GAPDH (6C5; EMD Millipore). ELISA kits were purchased for mouse IL-1 $\beta$  (BD Biosciences), pro-IL-1 $\beta$ , TNF- $\alpha$ , IL-6, MCP-1, and RANTES (eBioscience). Ultrapure LPS was from *Salmonella minnesota* (Invivogen). Poly(deoxyadenylic-deoxythymidylic) acid and nigericin were purchased from Sigma-Aldrich (St. Louis, MO). Silica crystals (MIN- U-SIL 15) were obtained from U.S. Silica (Frederick, MD). Cholesterol crystals were synthesized by acetone supersaturation and cooling(10), Alum (Imject alum adjuvant; a mixture of aluminum hydroxide and magnesium hydroxide) was from Pierce Biotechnology, and Leu-Leu-OMe $\square$ HCl was from Chem-Impex International. ZVAD-FMK, Ac-YVAD-CMK and Ca-074-Me were from Enzo Life Sciences and K777 was initially gifted to us by Stephanie A. Robertson and James H. McKerrow at UCSF, and further stocks obtained through services from the NHLBI's SMARTT Program. ABT199, ABT263 and AT406 were from Selleck Chemicals. Lipofectamine 2000, RNAiMax and all siRNA smart pools were from Life Technologies and Endoport was from Gene Tools.



### **Production and Measurement of Cytokines & Cell Death**

Peritoneal exudate cells were elicited by i.p. injection of 3 mL 1% thioglycollate and collected after 72 h by peritoneal lavage. Prior to experimentation, non-adherent cells were decanted, leaving primarily macrophages behind. Bone marrow-derived macrophages were generated as described(366). Bone marrow neutrophils were isolated from whole bone marrow, following RBC lysis, using the anti-Ly-6G Microbead Kit from Miltenyi Biotec. Purity was assessed to be 95–98% by flow cytometry. Murine bone marrow-derived mast cells were derived from whole bone marrow using murine rIL-3 (PeproTech), and purity was assessed to be 95% by toluidine blue(367). Cells were plated overnight in 96-well plates (ELISA), or 12-well plates (SDS-PAGE, cathepsin activity labeling with BMV109, and western blotting). Unless otherwise stated, the “Standard Protocol” followed herein is as follows: Priming in RPMI 1640 (or MC/9 medium for mast cells(367)) for 3h with LPS (200 ng/mL), with or without the addition of inhibitors after 2h of priming, followed by 6h of stimulation. Inhibitors were added in a final concentration of  $\leq 0.1\%$  DMSO, which has no effect on readouts compared to media alone. Supernatants were collected, with or without addition of Promega’s 10x lysis solution for measuring intracellular cytokines or LDH measurement by plate reader at OD<sub>490</sub> using Promega’s Cytox96 Non-radioactive cytotoxicity assay, and cytokine levels were analyzed by ELISA. In the same samples

assayed for IL-1 $\beta$  and TNF- $\alpha$ , cell death was assessed using either LDH or MTS assays as recommended by the manufacturer (Promega).

**Chapter II, Figure 1: Standard Assay for Understanding Inflammasomes.**

The figure shows the treatment schema following for the core assay used in most of the experiments described in this thesis. This approach is not conventionally used in the inflammasome field, but it includes controls that permit the simultaneous examination of at least 3 different cytokines (IL-1 $\beta$ , pro-IL-1 $\beta$  and TNF- $\alpha$ ) in the supernatants as well as intracellular cytokines (most importantly IL-1 $\beta$  & pro-IL-1 $\beta$ ) by using the LDH assay lysis buffer and measuring these cytokines by ELISA. Moreover, supernatants are used to measure cell death (LDH) and the cells left behind can be used to assess cell viability (MTS). By comparing the LDH (OD490) in lysed controls or MTS (OD490) in non-lysed controls, comparison of cell numbers between different genotypes can be compared to make sure that differences observed in the other readouts are not due to difference in cell number. Altogether, this system simultaneously measures several variables critical for interpreting and understanding inflammasome experiments.

## Standard Assay for Understanding Inflammasomes

	Controls (see below)				Experimental (n#)		
time (h)	1	2	3	4	5	6	Volume
0	LPS						50 µL
2	Media	Inhibitor	Media	Inhibitor	Media	Inhibitor	50 µL
3	Media		Media		Stimulus		50 µL
5.25	Lysis Buff.		Media				80 µL
6	Collect Supernatants & Add MTS						
Assay	MTS (OD490)	LDH (OD490)	IL-1β	pro-IL-1β	TNF-α		
Readouts	Cell Viability	Cell Death					

Controls	
1	0% Viability & 100% Cell Death Total Intracellular pro-IL-1 $\beta$ & IL-1 $\beta$ For each genotype, compare baseline cytokines and LDH(OD490) = Signal 1 & Cell# Indicators
2	Effect of Inhibitor on Intracellular pro-IL-1 $\beta$ & IL-1 $\beta$
3	100% Viability & 0% Cell Death Total LPS-induced pro-IL-1 $\beta$ & IL-1 $\beta$ secretion Compare Baseline Cytokines and LDH(OD490) for Each Genotype
4	Examine if inhibitor is toxic or stimulates cytokine secretion

### **Animal and Cell Lines**

Wild-type C57BL/6 mice were purchased from Jackson Laboratories. Caspase-1-deficient (Casp1<sup>-/-</sup>) mice(114) were previously described, NLRP3-deficient (Nlrp3<sup>-/-</sup>) mice(368) and ASC-deficient (ACS<sup>-/-</sup>) mice(368) were provided by Millennium Pharmaceuticals, and RIP3-deficient (RIP3<sup>-/-</sup>) mice were provided by Francis K. Chan (UMMS, Worcester). Caspase-1-deficient mice also lack caspase-11(73). Cathepsin S(369), L(275), and B(370) deficient mice were provided by Dr. Hal Chapman (UCSF, San Francisco) and Dr. Hidde Ploegh (Harvard Medical School), cathepsin C deficient mice(277) were provided by Dr. Christine Pham (Washington University School of Medicine, St. Louis) and all mice have been backcrossed to C57BL/6 background. All animal protocols were approved by the University of Massachusetts IACUC.

### **Generation of Bone Marrow Chimeras**

Adult wild-type (WT) C57BL/6 mice were lethally irradiated (1100 rads) and reconstituted for at least 8 weeks with bone marrow collected from age-matched WT or mutant donor mice 1-2 wks old. Some recipient mice in each group expressed the leukocyte marker Ly5.1 (CD45.1), while all donors expressed Ly5.2 (CD45.2), allowing confirmation of >90% chimerism to be determined by flow cytometric analysis of peripheral blood samples.

**(see Appendix 3 for an alternative method for collecting hematopoietic stem cells from fetal livers, which can be done by predicting the day of conception based on the weight of pregnant female mice.)**

### **siRNA Knockdowns**

Each targeting siRNA was compared with a control non-targeting siRNA pool (NT2) and used at a 50 nM final concentration (or control siRNA at 100 nM for double-knockdowns) after complexation in a mixture of Endoport (GeneTools) and RNAiMax (Life Technologies) at a ratio of 0.11  $\mu$ L:0.15  $\mu$ L in OptiMEM (Gibco), respectively, per 0.1 mL final volume (in 10% FCS). Complexes were combined with 10% FCS-containing RPMI at a ratio 1:10 (complexes:10% FCS) and added to cells for 96h. Media was supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 0.2 mM  $\beta$ -Me, 1x NEAAs and 100  $\mu$ g/mL ciprofloxacin.

**(see Appendix 4 for the detailed siRNA protocol used here and a demonstration of its superior knockdown in PMs and minimal toxicity from limiting the amount of RNAiMax required.)**

### **Immunoblotting & Live-cell Cathepsin Activity Labeling**

In 12-well plates, adherent macrophages were washed with RPMI, and incubated with or without LPS as indicated for 3h (inhibitors added after 2h) at which time BMV109(293) was added at a final concentration of 1  $\mu$ M. After 1h with BMV109, supernatants were collected, cells were washed with PBS and lysates made with Cell Extraction Buffer from Life Technologies with complete protease inhibitor cocktail from Roche. Supernatants were precipitated with chloroform/methanol and lysate protein concentration was equalized using the Pierce BCA Assay. At least 15  $\mu$ g was loaded for each sample and separated by 15% SDS-PAGE, and gels were analyzed with a Typhoon Trio phosphor-imager from GE, and protein transferred onto nitrocellulose membranes. Densitometry was performed using ImageJ. Images of gels or blots were cropped for the bands of interest and any contrast enhancement applied evenly throughout using iPhoto.

### **Neutrophil and Monocyte Recruitment to Peritoneal Cavity**

Quantification of recruited neutrophils and monocytes to the peritoneal cavity was described before(109). Mice were injected i.p. with 0.2 mg of silica crystals in 200  $\mu$ L PBS. After 4 of injection, the peritoneum was lavaged with 7 mL RPMI 1640 with 2% FCS, 3 mM EDTA, and 10 U/mL heparin. The absolute number of neutrophils (Ly- 6G+, 7/4+) and monocytes (Ly-6G-, 7/4+) in 100  $\mu$ L lavage was

counted using a flow cytometer equipped with a high throughput sampler (BD Biosciences).

### **K777 Treatment of Mice by Injection or Alzet Pump Infusion**

K777-HCl doses and formulations were as follows: DMSO (10%) / dextrose (5%) / water (85%) for i.v. (100  $\mu$ L = 62.5 mg/kg for ~20 g mice) or s.c. (200  $\mu$ L = 125 mg/kg for ~20 g mice), and Polyethyleneglycol-300 (25%) / Glycofurol (25%) / Cremophor ELP (25%) / Ethanol (15%) / Propylene Glycol (10%)(371) for Alzet pumps (Durect Corporation, Model 2001), which were surgically implanted s.c. on the backs of mice for 1 wk delivering drug or excipient formulation at a rate of 1  $\mu$ L/h for the indicated doses of K777. Prior to injection of PBS or silica i.p. on the 7<sup>th</sup> day of treatment, plasma samples were taken from mice and K777 concentration analyzed by mass spectrometry.

**(also, see Appendix 12 for K777 formulation)**

### **Real-time Measurement of LMD & MMP**

In black high-binding 96-well clear-bottom plates, 50  $\mu$ L of acridine orange (LifeTechnologies) in warm HBSS (with Ca<sup>2+</sup> & Mg<sup>2+</sup>) was added to cells in 100  $\mu$ L of RPMI containing 10% FCS to reach a final concentration of 3.75  $\mu$ g/mL and then incubated at 37°C for 15 min prior to washing 1x with 200  $\mu$ L HBSS. Cells



were then treated and stimulated as indicated in phenol red-free CO<sub>2</sub>-independent Leibovitz's medium. The same was done for TMRM (LifeTechnologies) with a final concentration of 1.25 µM and 45 min incubation. Fluorescence was measured in each well every 1-3 min using an incubated VictorX5 plate reader. Background fluorescence was subtracted from wells treated with dye-free HBSS.

**(also, see Appendix 5 for an example of controls that induce LMD or mitochondrial depolarization/hyperpolarization)**

**Chapter III:**

**Multiple Cathepsins**

**Promote Pro-IL-1 $\beta$  Synthesis**

**And**

**NLRP3-Mediated IL-1 $\beta$  Activation**

## **Attributions and Copyright Information**

The data described in this chapter comprises a publication currently in review at The Journal of Immunology in 2015.

All experiments and data were performed and analyzed by me (G.M.O.) and the other authors made significant technical, material, and intellectual contributions.

## **Chapter III: Multiple Cathepsins Promote Pro-IL-1 $\beta$ Synthesis and NLRP3-Mediated IL-1 $\beta$ Activation**

Gregory M. Orlowski,\* Jeff D. Colbert,\* Shruti Sharma,\*\* Matthew Bogoy,<sup>†,‡</sup>  
Stephanie A. Robertson,<sup>§</sup> and Kenneth L. Rock\*,<sup>¶</sup>

Departments of \*Pathology and \*\*Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA; <sup>†</sup>Department of Pathology and <sup>‡</sup>Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA; <sup>§</sup>Sandler Center for Drug Discovery, University of California, San Francisco, CA 94158, USA.

<sup>¶</sup>Corresponding author: Kenneth L. Rock, Phone: (508) 856-1090, Fax: (508) 856-1094, Email: [Kenneth.Rock@umassmed.edu](mailto:Kenneth.Rock@umassmed.edu)

## **Abstract**

Sterile particles induce robust inflammatory responses that underlie the pathogenesis of diseases like silicosis, gout and atherosclerosis. A key cytokine mediating this response is IL-1 $\beta$ . The generation of bioactive IL-1 $\beta$  by sterile particles is mediated by the NLRP3 inflammasome, although exactly how this occurs is incompletely resolved. Prior studies have found that the cathepsin B inhibitor, Ca074Me, suppresses this response, supporting a model whereby ingested particles disrupt lysosomes and release cathepsin B into the cytosol, somehow activating NLRP3. However, reports that cathepsin B-deficient macrophages have no defect in particle-induced IL-1 $\beta$  generation have questioned cathepsin B's involvement. Here, we examine the hypothesis that multiple redundant cathepsins (not just cathepsin B) mediate this process by evaluating IL-1 $\beta$  generation in murine macrophages, singly or multiply deficient in cathepsins B, L, C, S and X. Using an activity-based probe, we measure specific cathepsin activity in living cells, documenting compensatory changes in cathepsin-deficient cells, and Ca074Me's dose-dependent cathepsin inhibition profile is analyzed in parallel with its suppression of particle-induced IL-1 $\beta$  secretion. Also, we evaluate endogenous cathepsin inhibitors, cystatins C and B. Surprisingly, we find that multiple redundant cathepsins, inhibited by Ca074Me and cystatins, promote pro-IL-1 $\beta$  synthesis, and we provide the first evidence that cathepsin X plays a non-redundant role in non-particulate NLRP3 activation.

Finally, we find cathepsin inhibitors selectively block particle-induced NLRP3 activation, independently of suppressing pro-IL-1 $\beta$  synthesis. Altogether, we demonstrate that both small molecule and endogenous cathepsin inhibitors suppress particle-induced IL-1 $\beta$  secretion by inhibiting multiple cathepsins that contribute to pro-IL-1 $\beta$  synthesis and NLRP3 activation.

## **Introduction**

Sterile particles induce robust inflammatory responses that underlie the pathogenesis of many diseases. These pathogenic particles are diverse, and include silica(17, 130, 131, 144), which causes silicosis, monosodium urate(12), the etiologic agent in gout, and cholesterol crystals (CC)(10, 11), which are thought to contribute to the pathogenesis of atherosclerosis. Importantly, the sterile inflammatory response and resultant diseases caused by these particles all involve signaling through the interleukin-1 receptor, IL-1R1(109, 121). While IL-1R1 can be stimulated by either of two cytokines, IL-1 $\alpha$  or IL-1 $\beta$ , it has been shown that IL-1 $\beta$  plays a pivotal role in disease pathogenesis(372) because it not only directly stimulates IL-1R1-dependent inflammatory signaling, but is also needed for the secretion of IL-1 $\alpha$  from cells(122). Therefore, it is important to understand the exact mechanisms underlying the generation and secretion of active IL-1 $\beta$ . However, this process is still incompletely understood and the focus of the present report.

The generation of biologically active IL-1 $\beta$  is highly regulated and usually proceeds in two distinct steps(172, 185). The first step (Signal 1 or “priming”) is initiated when cells such as macrophages are stimulated by certain cytokines, pathogen-associated molecular patterns (PAMPs), or danger-associated molecular patterns (DAMPs). Signal 1 leads to the nuclear translocation of NF- $\kappa$ B, which then stimulates the synthesis of biologically inactive pro-IL-1 $\beta$  and,

among other things, NOD-like receptor containing a pyrin domain 3 (NLRP3), a protein important for IL-1 $\beta$  activation. The second step (Signal 2 or “activation”) induces the formation of a multimolecular complex, known as the inflammasome. Inflammasomes are composed of a sensor protein, an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and an executioner protease, caspase-1. Each inflammasome sensor detects distinct stimuli, thereby initiating multimerization and activating caspase-1, which then cleaves pro-IL-1 $\beta$  and facilitates the secretion of bioactive mature IL-1 $\beta$ . Among the known inflammasomes, the NLRP3 inflammasome is unique. While all inflammasomes rely on the availability of a newly synthesized pool of pro-IL-1 $\beta$ , basal levels of NLRP3 itself are limiting, making priming especially critical for *de novo* NLRP3 transcription and subsequent activation(67, 184). Moreover, the NLRP3 inflammasome is the exclusive mediator of IL-1 $\beta$  activation in response to sterile particles(10-12, 17, 130, 131, 144).

While the NLRP3 inflammasome is located in the cytosol, how this intracellular complex senses the presence of extracellular particles has been of considerable interest. It has been shown that internalization of particles by phagocytosis is a first essential step in activating the NLRP3 inflammasome(144). Multiple mechanisms have been proposed as to how particles in phagosomes then lead to NLRP3 inflammasome activation, including lysosomal membrane disruption (LMD)(17, 126, 128, 136, 140-153, 185), potassium efflux(15, 126-136, 154), and the generation of reactive oxygen



species (ROS)(127-130, 135, 137-140), among various other mechanisms (Reviewed (172)). All of these pathways may contribute to this process. In support of the LMD model, it has been shown that particles like silica, CC and the adjuvant alum can cause LMD(10, 11, 144), leading to the leakage of the lysosomal cysteine protease cathepsin B into the cytosol, where this protease is thought to activate NLRP3 through an as yet undescribed mechanism. Consistent with this model, particle-induced activation of the NLRP3 inflammasome is blocked by inhibitors of lysosomal acidification (cathepsins are optimally active in acidic conditions) and inhibitors of cathepsin B. However, the requirement for cathepsin B in this process is controversial.

A role for cathepsin B in NLRP3 activation is supported by a number of studies showing that Ca074Me, an inhibitor reported to be specific for cathepsin B, suppresses IL-1 $\beta$  activation induced by particulate and non-particulate stimuli(11, 16, 18, 126, 142, 144, 147, 160, 161, 163, 373-378). However, despite a few subsequent studies showing that cathepsin B or L-deficient macrophages show partial impairment of this response(10, 160, 161), several follow-up studies have found that responses are intact in these same mutant cells(15, 162, 163). Thus, it has become unclear whether the efficacy of Ca074Me is really a result of cathepsin B inhibition, or whether this is an off-target effect. Indeed, there are several reports demonstrating that Ca074Me inhibits other cathepsins as well(155-159). Therefore, one hypothesis proposed to explain the discrepancy between Ca074Me and genetic models is

that multiple cathepsins, which are a highly conserved family of proteases, play redundant roles in NLRP3 activation(370). Redundancy of cathepsins B and L has been demonstrated in a mouse model, where deficiency of both results in neonatal mortality, while deficiency of either alone does not(379). Similar redundancy also been observed in mouse cancer models showing upregulation of cathepsin X when cathepsin B is knocked out(380). However, the role of redundant cathepsins has not been examined in the context of NLRP3 activation and remains an open question.

Here, we utilize genetic inactivation of multiple cathepsins, together with exogenous and endogenous inhibitors of these proteases, and an activity-based probe to investigate the role of cathepsins in NLRP3-dependent particle-induced IL-1 $\beta$  secretion. This analysis reveals that multiple cathepsins indeed contribute to IL-1 $\beta$  secretion. Surprisingly, our data also demonstrate that cathepsins contribute, not only to the inflammasome-mediated cleavage of pro-IL-1 $\beta$  into mature IL-1 $\beta$  (Signal 2), but also, to the priming step of pro-IL-1 $\beta$  synthesis (Signal 1). In addition, we found a unique role for cathepsin X in nigericin-induced NLRP3 activation, a protease not previously implicated in the IL-1 response. Together, these data clarify the contribution of cathepsins to particle-induced IL-1 $\beta$  responses and define a previously unappreciated role for cathepsins and their inhibitors in regulating pro-IL-1 $\beta$  synthesis. In doing so, this study provides insight into the mechanistic regulation of IL-1 $\beta$  production and points to cathepsins as

unique therapeutic targets for controlling particle-induced sterile inflammatory responses.

## **Results**

### **Genetic and biochemical analysis of the impact of individual cathepsin deficiency on particle-induced IL-1 $\beta$ secretion**

The role of cathepsins in NLRP3 activation remains controversial. Some studies describe a role for cathepsin B or L(10, 160, 161), while others show no role for either cathepsin in particle-induced NLRP3 activation and IL-1 $\beta$  secretion(15, 162, 163). One interpretation of these data suggests that other cathepsins, besides B or L, may be the key players in this response. Therefore, we examined the impact that genetic deficiency of five closely-related individual cathepsins has on particle-induced IL-1 $\beta$  secretion. Unless noted otherwise, IL-1 $\beta$  secretion was induced with various stimuli following 3h of LPS priming. First, we examined peritoneal macrophages (PMs) elicited from mice lacking cathepsins B, L, S or C. However, these cathepsin-deficient PMs displayed no difference in IL-1 $\beta$  secretion in response to silica compared to PMs derived from wild-type (WT) mice (**Fig. 1a**). To examine the role of cathepsin X, we silenced cathepsin X in PMs by siRNA knockdown, and then these cells were stimulated with silica, the soluble NLRP3 activator nigericin, or the Absent In Melanoma 2 (AIM2) inflammasome activator poly(deoxyadenylic-deoxythymidylic) acid (dAdT) (**Fig. 1b**). We confirmed a 90-95% knockdown of cathepsin X mRNA by quantitative PCR (qPCR) (**Fig. 1c**) and noted a similar loss of cathepsin X activity using the fluorescent activity-based probe BMV109 (**Fig. 1d**), which binds covalently to

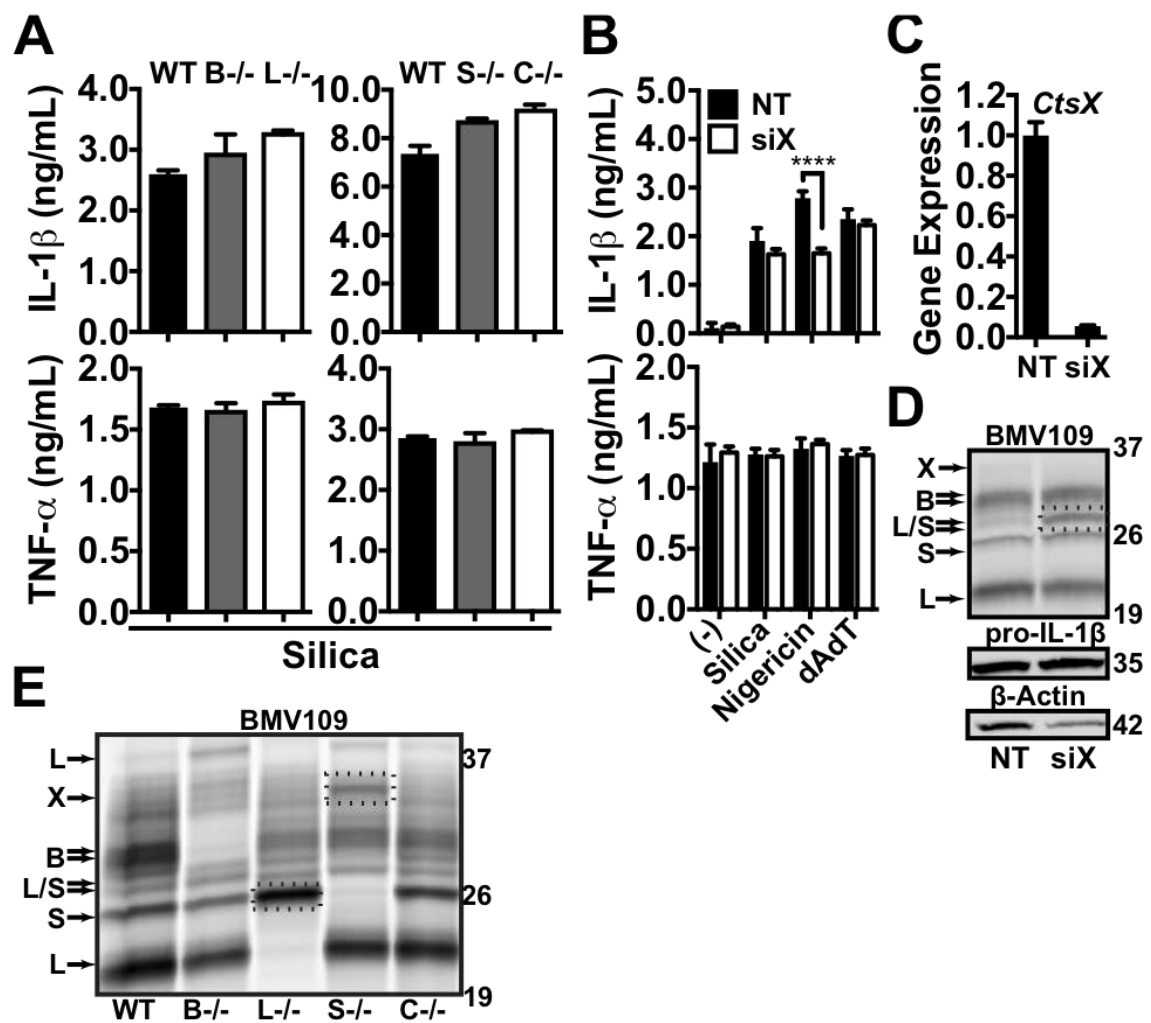
active cathepsins inside live cells(293). In lysates generated from these cells, the proteins were separated by SDS-PAGE, and then the activity of specific cathepsins was assessed in the gels using a laser phosphor-imager to analyze the degree of fluorescence for each cathepsin at the appropriate m.w.'s. Again, we noted no significant difference in IL-1 $\beta$  secretion between cathepsin X-sufficient and cathepsin X-deficient PMs in response to either silica or dAdT. Strikingly, cathepsin X deficiency significantly reduced IL-1 $\beta$  secretion in response to nigericin. In contrast, LPS-induced TNF- $\alpha$  secretion was unaffected by the loss of any of the cathepsins tested. Therefore, the individual cathepsins B, L, S, C and X are dispensable for silica-induced IL-1 $\beta$  secretion, but we found, unexpectedly, that in the response induced by nigericin, cathepsin X plays a non-redundant role.

Using cathepsin knockout animals to study IL-1 $\beta$  secretion could potentially be confounded if some cathepsins are upregulated in order to compensate for the deficiency of others(379, 381, 382). Using BMV109, we examined the activity of specific intracellular cathepsins in the LPS-primed WT and cathepsin-deficient PMs that were tested above in Fig. 1a and b. Indeed, knockdown of cathepsin X with siRNA resulted in an upregulation of cathepsin L and S activity (**Fig. 1d**). Moreover, PMs lacking cathepsin L showed increased cathepsin S activity, while those deficient in cathepsin S upregulated cathepsin X activity (**Fig. 1e**). Together, these data indicate that the cathepsins examined, including cathepsins B, L, S and X, are not essential for particle-induced IL-1 $\beta$

secretion, and they cannot be readily studied using genetic methods due to compensation issues upon knockdown.

### **Chapter III, Figure 1**

**Sterile particle-induced IL-1 $\beta$  secretion does not require cathepsins B, L, C, S or X, but nigericin is partially dependent on cathepsin X. (A)** LPS-primed PMs from WT mice or mice deficient for cathepsins B (B<sup>-/-</sup>), L (L<sup>-/-</sup>), S (S<sup>-/-</sup>) or C (C<sup>-/-</sup>), were stimulated with silica (40  $\mu$ g/mL). **(B)** PMs were treated with non-targeting (NT) control siRNA or siRNA targeting cathepsin X (siX) before priming with LPS and stimulating with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). IL-1 $\beta$  & TNF- $\alpha$  were measured in supernatants by ELISA. **(C)** PMs from “B” were analyzed for cathepsin X (CtsX) expression by qPCR following siRNA (siX) treatment and LPS priming; data are normalized to GAPDH expression and plotted relative to NT siRNA, or **(D)** cathepsin X activity was probed with BMV109; lysates were processed and pro-IL-1 $\beta$  &  $\beta$ -Actin analyzed by western blot; dashed box highlights upregulated cathepsin L/S activity; m.w. markers are on the right in kDa. **(E)** LPS-primed PMs from WT or cathepsin-deficient mice in “A” were probed for cathepsin activity with BMV109; dashed boxes highlight upregulated cathepsin S or X activity in L<sup>-/-</sup> and S<sup>-/-</sup> macrophages; m.w. markers are on the right in kDa. Error bars represent **(A)** range bars of technical duplicates, **(B)** S.D. of technical quadruplicates, and **(C)** S.D. of technical triplicates. **(B)** Statistical analysis was performed by Two-way ANOVA and Sidak’s multiple comparisons test; \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.



**Figure 1**



## **Analysis of small molecule cathepsin inhibitors**

The absence of a phenotype in cathepsin B-deficient macrophages, shown here and reported by others, contradicts the results reported with cathepsin B inhibitors(15, 162, 163). Despite several reports demonstrating that the cathepsin inhibitor Ca074Me inhibits multiple cathepsins in biochemical and cellular assays(155-159), Ca074Me is cited as a cathepsin B-specific inhibitor and used to implicate cathepsin B in NLRP3 activation in many studies(11, 16, 18, 126, 142, 144, 147, 160, 161, 163, 373-378). The non-selective pro-drug methyl ester, Ca074Me, is processed in lysosomes into the highly cathepsin B-selective free acid, Ca-074. However, this processing occurs slowly and allows time for Ca074Me to inhibit multiple cathepsins(155-159). Therefore, in the context of NLRP3 activation, Ca074Me's targets in intact cells have not yet been verified and closely examined as a function of inhibitor concentration. Here, we re-examine both Ca074Me and a newly described broad cathepsin inhibitor, K777 (N-methyl-piperazine-phenylalanyl-homophenylalanyl-vinylsulfone-phenyl), whose anti-inflammatory properties have not yet been tested. K777 inhibits cathepsins B, L, S, C, V and K in cell-free assays(383). Using Ca074Me or K777 in combination with the active site probe allowed us to correlate their effects on IL-1 $\beta$  secretion with the extent of inhibition of specific cathepsins as a function of concentration.

To examine the inhibition profile of K777, we treated PMs with K777 or solvent control (DMSO) for 1h (after 2h of LPS priming, unless stated otherwise), after which we probed for cathepsin activity in the intact cells with BMV109. As previously reported, K777 inhibited cathepsins B, L and S(383) over a titration range from 0.1 - 30  $\mu$ M (**Fig. 2a,b**). Interestingly, we also found that K777 inhibited cathepsin X at high concentrations, but unexpectedly increased cathepsin X activity at lower concentrations. These paradoxical effects can be explained by K777's greater potency towards cathepsin S, which fits with our data, in Fig. 1e above, showing that cathepsin S deletion causes an increase cathepsin X activity. Therefore, K777 inhibits cathepsin S at low concentrations, which likely causes a compensatory increase in cathepsin X activity.

In parallel to examining its effects on cathepsin activity, we also tested the effect of K777 on IL-1 $\beta$  secretion (**Fig. 2c**). PMs were primed with LPS and treated with K777 as done above (2h after LPS priming and 1h prior to stimulation) at which point they were exposed to various stimuli for an additional 6h of incubation; this is the "Standard Protocol" used for the rest of this study, unless stated otherwise. At concentrations where multiple cathepsins were inhibited, K777 suppressed silica-induced IL-1 $\beta$  secretion. In contrast to silica, K777 was much less effective at suppressing IL-1 $\beta$  secretion induced by nigericin. Presumably, this is because K777 has opposing effects on cathepsin X, which is uniquely required for the nigericin response, shown in Fig. 1b. Moreover, K777 had a negligible affect on the IL-1 $\beta$  response induced by dAdT. We also

confirmed that K777 is similarly selective and/or efficacious at suppressing IL-1 $\beta$  secretion induced by other particles, including alum and CC, and in other primary myeloid cell lines, including bone marrow-derived macrophages, mast cells and neutrophils (**Supp. Fig. 1a,b**). Importantly, K777 did not affect LPS-induced TNF- $\alpha$  production within the tested concentration range, suggesting specific inhibition of IL-1 $\beta$  secretion.

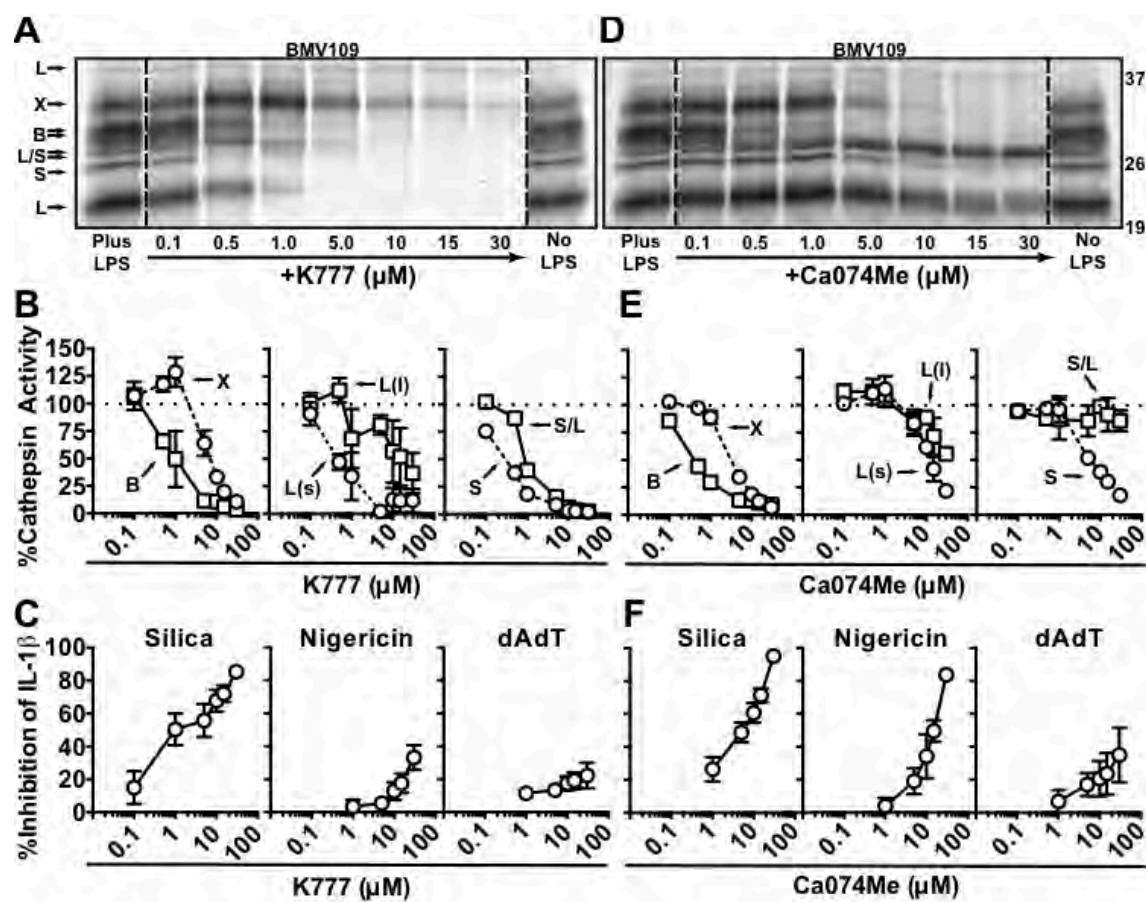
We performed similar analyses for Ca074Me (**Fig. 2d-f**). While Ca074Me was selective for cathepsin B at concentrations below 1  $\mu$ M, at higher concentrations (typically used in previous studies) it inhibited cathepsins broadly (**Fig. 2d,e**). Moreover, >10  $\mu$ M of Ca074Me was required to completely inhibit cathepsin B. Unlike K777, Ca074Me suppressed nigericin and silica-induced IL-1 $\beta$  secretion with similar potency, presumably because Ca074Me inhibits cathepsin X more potently than K777 (**Fig. 2f**). Interestingly, the concentration required to achieve and maximize these effects exceeds the range in which Ca074Me is selective for cathepsin B. In reviewing previous studies examining Ca074Me's effects on IL-1 $\beta$  responses, the concentrations used were also in the range that would inhibit multiple cathepsins (10-200  $\mu$ M)(11, 16, 18, 126, 142, 144, 147, 160, 161, 163, 373-378). Therefore, our findings likely explain the difference in results seen for the genetic loss of cathepsin B compared to small-molecule inhibitors of this protease. In summary, although both K777 and Ca074Me inhibit multiple cathepsins at concentrations required to suppress IL-1 $\beta$

secretion, K777 blocks particle-induced NLRP3 activation more selectively than Ca074Me.

To further investigate whether Ca074Me or K777 can inhibit IL-1 $\beta$  secretion in PMs from cathepsin-deficient mice, these cells were LPS primed and treated with inhibitors prior to stimulation. Indeed, K777 inhibited IL-1 $\beta$  secretion to the same extent in WT cells as in cathepsins B, L, S, or C-deficient cells (**Fig. 3a**). Moreover, across a titration range for both K777 and Ca074Me, the extent to which they suppressed IL-1 $\beta$  secretion was the same in both WT and cathepsin B-deficient PMs (**Fig. 3b**). Again, LPS-induced TNF- $\alpha$  secretion was relatively unaffected by cathepsin B deficiency or inhibitor treatments. Together, these data indicate that the individual cathepsins examined, including cathepsin B, are not essential for the activation of particle-induced IL-1 $\beta$  secretion or as targets for cathepsin inhibitors that suppress this response.

### **Chapter III, Figure 2**

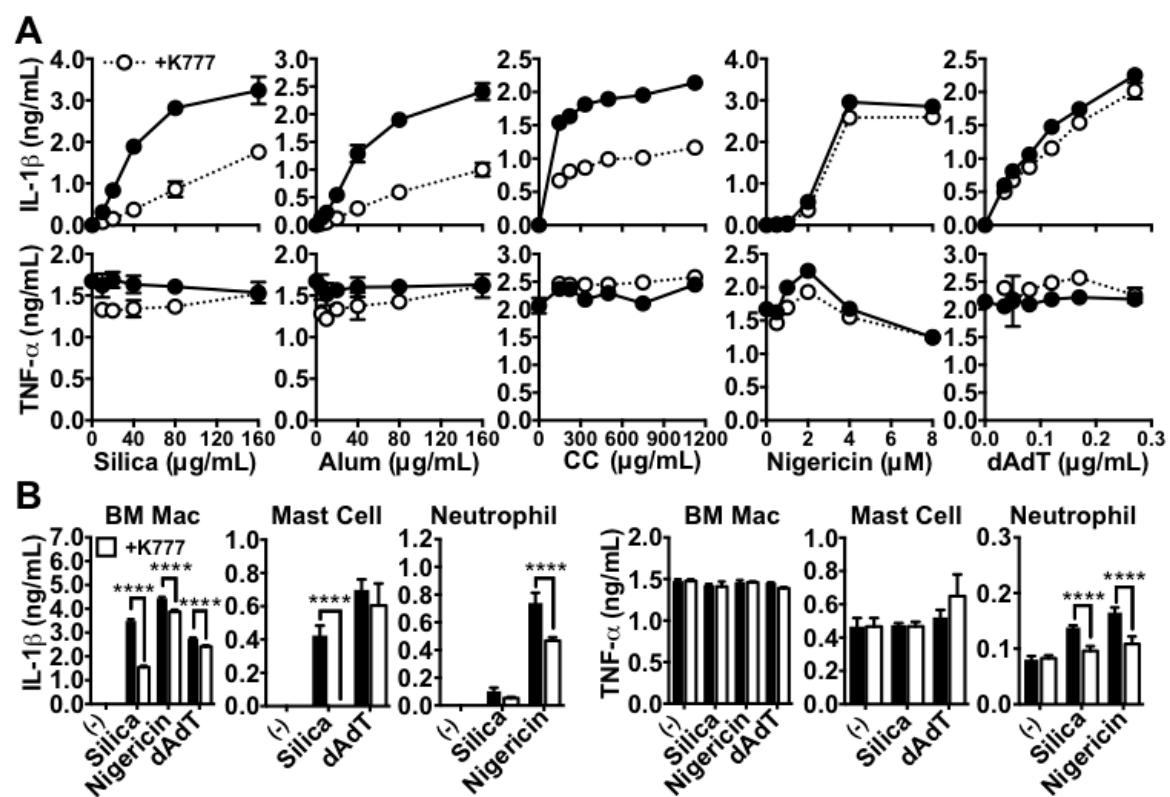
**Both Ca074Me and K777 inhibit multiple cathepsins at concentrations needed to block IL-1 $\beta$  secretion. (A)** PMs were given media control (No LPS) or LPS-primed (Plus LPS; +K777) and subsequently treated with media control (No LPS; Plus LPS) or the indicated concentrations of K777 (+K777), after which cathepsin activity was labeled with BMV109 in live cells before lysates were processed by SDS-PAGE and phosphor imaged; m.w. markers are on the right in kDa. **(B)** Concentration-dependent inhibition of cathepsin activity by K777 analyzed by densitometry of “A”: cathepsin B (square) and X (circle), large (L<sub>(l)</sub>; square) and small (L<sub>(s)</sub>; circle) m.w. isoforms of cathepsin L, cathepsin S (circle) and overlapping m.w. isoforms of S and L (S/L; square). **(C)** LPS-primed PMs were treated with media control or the indicated concentrations of K777 and stimulated with silica (40  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.5  $\mu$ g/mL); data shows percent inhibition of IL-1 $\beta$  secretion measured in supernatants compared to no inhibitor treatment. **(D-F)** Same as “A-C”, but with Ca074Me instead of K777. Error bars represent **(B)** S.E. of means from three independent experiments, **(C)** S.D. of means from four independent experiments (0.1-15  $\mu$ M) or S.D. of means from three independent experiments (30  $\mu$ M), **(E)** range bars of the means from two independent experiments, or **(F)** S.D. of the means from three independent experiments (0.5-15  $\mu$ M), or range bars of the means from two independent experiments (30  $\mu$ M).



**Figure 2**

### **Chapter III, Supplemental Figure 1**

**(A)** LPS-primed PMs were treated with media control (solid line) or K777 (+K777/dashed line; 15-20  $\mu$ M) and then stimulated with the indicated concentrations of silica, alum, CC, nigericin or dAdT; IL-1 $\beta$  (upper graphs) and TNF- $\alpha$  (lower graphs) were measured in supernatants. **(B)** LPS-primed bone marrow-derived macrophages (BM Mac), mast cells, or neutrophils were treated with media control (black bars) or K777 (+K777/white bars; 15  $\mu$ M) and then stimulated with media control (-), silica (40  $\mu$ g/mL; 100  $\mu$ g/mL for neutrophils), nigericin (2  $\mu$ M for BM Macs; 1  $\mu$ M for neutrophils) and/or dAdT (0.3  $\mu$ g/mL); IL-1 $\beta$  (graphs on left) and TNF- $\alpha$  (graphs on right) were measured in supernatants. Error bars represent **(A)** S.D. from technical triplicates (CC and dAdT) or range bars of technical duplicates (silica, alum and nigericin), **(B)** S.D. from technical triplicates (BM Mac for silica or nigericin; mast cells and neutrophils) or range bars from technical duplicates (BM Mac for dAdT). Statistical analysis was performed by **(B)** Two-way ANOVA and Sidak's multiple comparisons test; \*\*\*\* $P < 0.0001$ . All data are representative of at least three independent experiments.

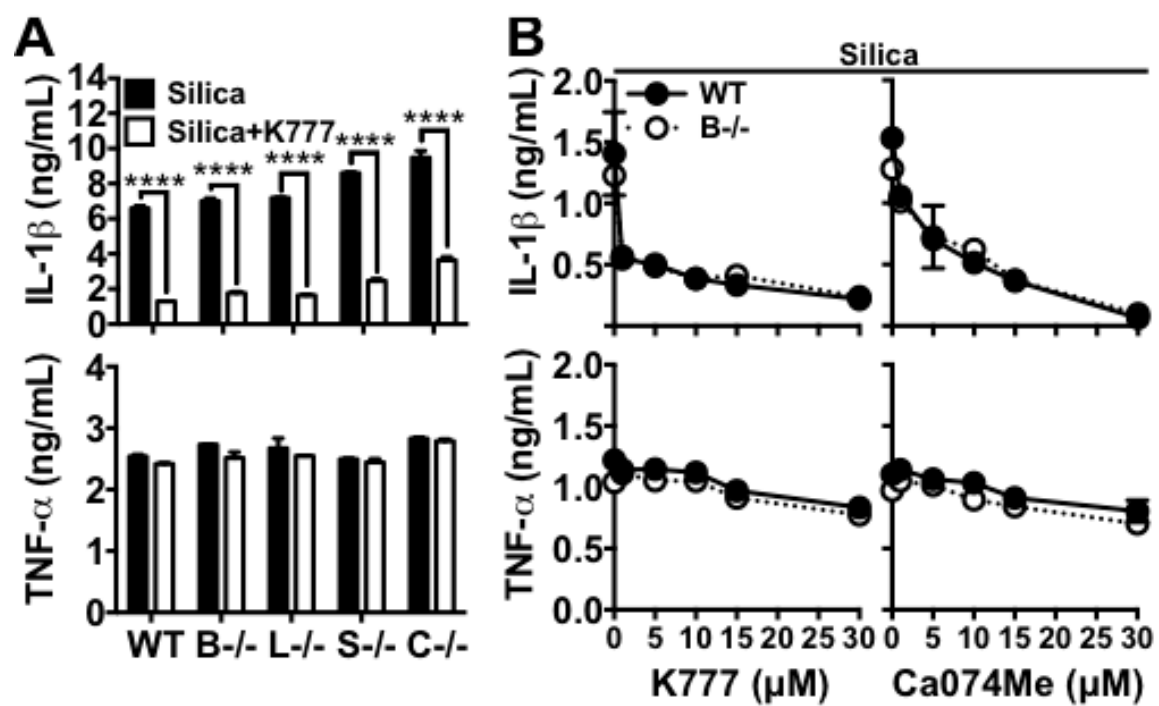


Supplemental Figure 1



### **Chapter III, Figure 3**

**Cathepsin inhibitors suppress particle-induced IL-1 $\beta$  secretion independently of individual cathepsins.** IL-1 $\beta$  (upper graphs) and TNF- $\alpha$  (lower graphs) were measured in supernatants. **(A)** LPS-primed WT PMs or those lacking cathepsins B (B $^{-/-}$ ), L (L $^{-/-}$ ), S (S $^{-/-}$ ) or (C $^{-/-}$ ) were treated with silica (black bars; 40  $\mu$ g/mL) or silica plus K777 (white bars; 15  $\mu$ M). **(B)** LPS-primed WT (closed circles, solid line) or cathepsin B-deficient (open circles, dashed line) PMs were treated silica (50  $\mu$ g/mL) or silica plus a range of K777 or Ca074Me concentrations (1, 5, 10, 15 or 30  $\mu$ M). Error bars represent range bars of technical duplicates. Statistical analysis was performed by Two-way ANOVA and Sidak's multiple comparisons test; \*\*\*\* $P$ <0.0001. Data are representative of two ("B" for Ca074Me) or three ("A"; "B" for K777) independent experiments.



**Figure 3**

## Analysis of compound cathepsin deficiencies

The analyses above suggest that multiple cathepsins likely play compensatory roles in particle-induced IL-1 $\beta$  secretion. This is in line with some genetic evidence that has shown partial or conditional involvement for cathepsin B or L in NLRP3 activation(10, 160, 161). In fact, these two cathepsins have been shown to compensate for one another in a study demonstrating that combined cathepsin B and L deficiency is neonatal lethal in mice, but deficiency of either protease alone is non-lethal(379). Therefore, a dual-deficiency of cathepsins B and L may have a greater effect on the IL-1 $\beta$  response(10).

To test this hypothesis, we bred mice lacking both cathepsins B and L. Since combined cathepsin B and L deficiency is neonatal lethal(379), we could not analyze responses directly in these animals. Instead, we harvested bone marrow from neonates and used it to reconstitute lethally irradiated adult WT mice. In these chimeric mice, cells of hematopoietic origin lack cathepsin B and L (B&L-/-). For comparison, we made similar chimeras with WT, cathepsin B-/- and cathepsin L-/- bone marrow. Then we elicited PMs from these chimeric mice, and treated them as above. We verified that the PMs collected from these chimeric mice lacked activity for the appropriate cathepsins using BMV109 (**Supp. Fig. 2a**). Again, we observed upregulation of cathepsin S activity upon loss of cathepsin L. However, cathepsin B-/-, L-/- or B&L-/- PMs showed no attenuation of IL-1 $\beta$  secretion in response to the lysosome-disrupting agent Leu-Leu-OMe

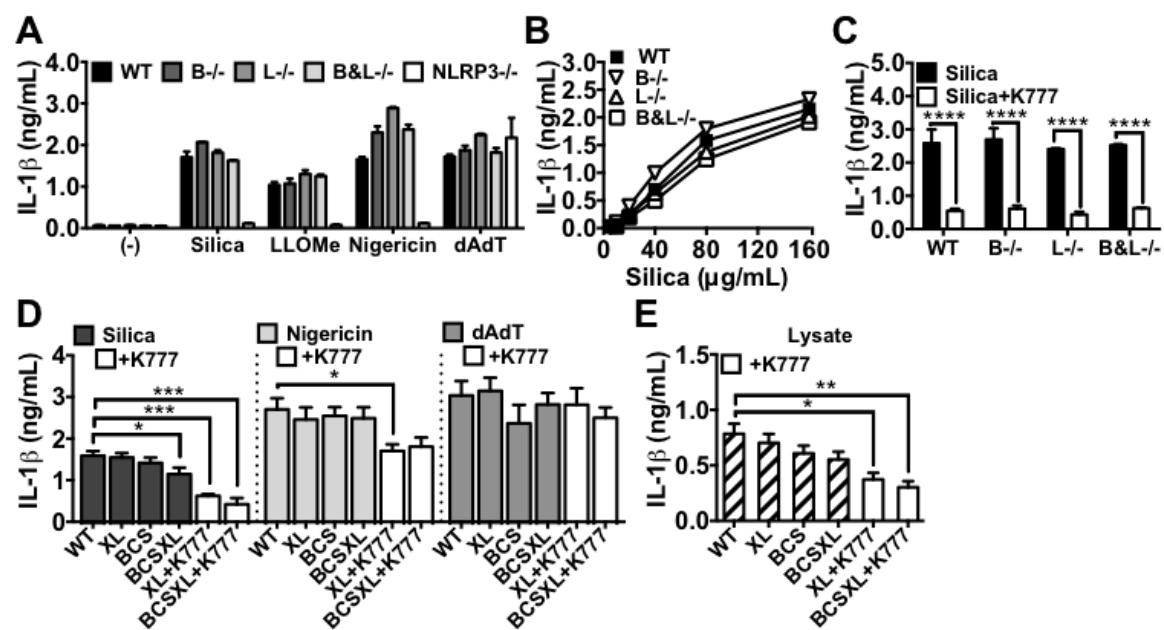
(LLOMe), silica, nigericin or dAdT (**Fig. 4a**). Moreover, there was no defect in IL-1 $\beta$  secretion over a broad titration of silica (**Fig. 4b**). Interestingly, K777 still suppressed silica-induced IL-1 $\beta$  secretion in the absence of cathepsins B and/or L (**Fig. 4c**), suggesting that other cathepsins potentially contribute to this response.

Since both K777 and Ca074Me inhibit more cathepsins than just B and L at the concentrations required to block particle-induced IL-1 $\beta$  secretion, we examined the particle-induced responses of macrophages genetically deficient in up to five cathepsins (**Fig. 4d,e**). To do this, we elicited PMs from WT mice or mice deficient in the three cathepsins B, C and S (BCS<sup>-/-</sup>), which are viable with no obvious physical or behavioral pathology. In addition, in both WT and BCS<sup>-/-</sup> macrophages, we silenced cathepsins X and L with siRNA (siXL; XL), or treated cells with non-targeting siRNA (WT). This resulted in a 90-95% reduction in mRNA of each targeted gene and reduction in enzyme activity, as assayed with BMV109 (**Supp. Fig. 2b,c**). Finally, PMs were primed with LPS, with media or K777 treatment, and stimulated with silica, nigericin or dAdT, as done above. Indeed, macrophages deficient in the five cathepsins B, C, S, X and L (BCSXL) showed a significant, though small, reduction in IL-1 $\beta$  secretion in response to silica, but not nigericin or dAdT (**Fig. 4d**). However, K777 was still effective at further suppressing IL-1 $\beta$  secretion in these macrophages. Interestingly, in the lysates of samples treated with LPS only, we observed a similar decrease in intracellular IL-1 $\beta$  levels, suggesting that lower levels of IL-1 $\beta$  synthesis may be

contributing to the reduction in IL-1 $\beta$  secretion seen for both BCSXL deficiency and K777 treatment (**Fig. 4e**). Again, we observed a compensatory upregulation of cathepsin activity, with cathepsin B and S activity upregulated in the cathepsin XL knockdown and increased cathepsin X activity in the cathepsin BCS-/- PMs (**Supp. Fig. 2c**). This may explain why nigericin was not significantly affected by knockdown of cathepsin X in combination with these other cathepsin deficiencies. As above, TNF- $\alpha$  secretion was unaffected, suggesting that compound cathepsin deficiency specifically impacts the IL-1 $\beta$  pathway (**Supp. Fig. 2d-h**). Thus, compound deficiency of cathepsins B, C, S, X and L demonstrates a reproducible, albeit minor, attenuation of particle-induced IL-1 $\beta$  secretion. However, the fact that cathepsin inhibitors have shown, yet again, more profound effects on IL-1 $\beta$  secretion than that caused by genetic deficiency, it remains possible that additional cathepsins might be involved in particle-induced IL-1 $\beta$  secretion.

#### **Chapter III, Figure 4**

**Compound cathepsin-deficiency causes a minor reduction in particle-induced IL-1 $\beta$  secretion.** IL-1 $\beta$  was measured in supernatants. **(A-C)** Lethally irradiated WT mice were reconstituted with bone marrow from WT, cathepsin B (B<sup>-/-</sup>), L (-/-), B and L (B&L<sup>-/-</sup>), or NLRP3 (NLRP3<sup>-/-</sup>)—deficient donor mice. LPS-primed PMs elicited from these mice were stimulated with **(A)** media control (-), silica (40  $\mu$ g/mL), LLOMe (0.75 mM), nigericin (2  $\mu$ M), or dAdT (0.4  $\mu$ g/mL), **(B)** a range of silica concentrations, **(C)** silica plus media (black bars) or silica plus K777 (white bars; 20  $\mu$ M). **(D)** PMs elicited from WT or mice deficient in the three cathepsins B, C and S (BCS) were treated with non-targeting siRNA (WT) or siRNA targeting both cathepsins X and L (“XL” when given to WT, or “BCSXL” when given to BCS) and subsequently LPS-primed and stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). XL and BCSXL macrophages were also treated with K777 (XL+K777 and BCSXL+K777; white bars; 15  $\mu$ M). Error bars represent **(A-C)** range bars of technical duplicates, or **(D)** S.E. of means from either five independent experiments (WT, XL, BCS, BCSXL) or three independent experiments (+K777). Statistical analysis was performed by **(A-C)** Two-way ANOVA and Sidak’s multiple comparisons test, or **(D-E)** Two-tailed Student’s t-test \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.

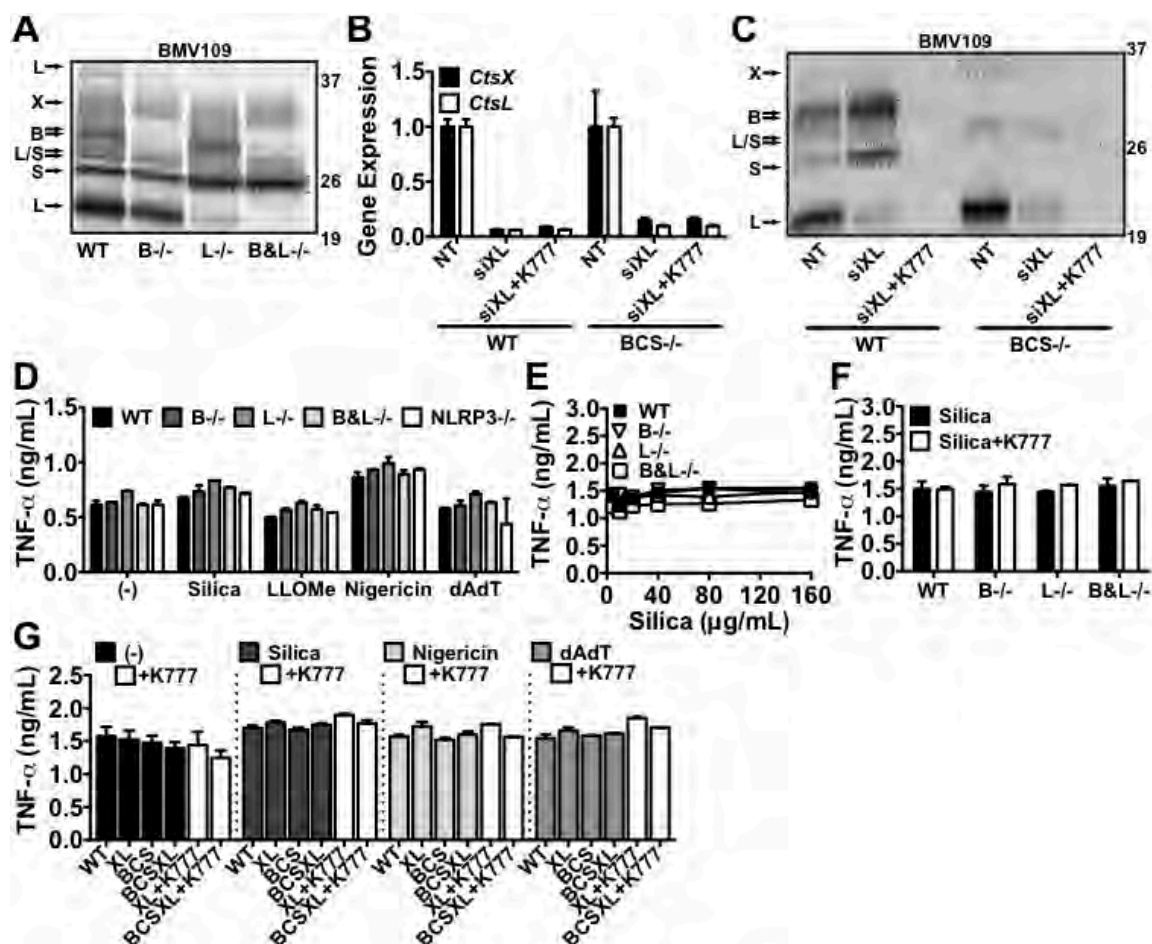


**Figure 4**

### **Chapter III, Supplemental Figure 2**

**(A,D-F)** Lethally irradiated WT mice were reconstituted with bone marrow from WT, cathepsin B (B<sup>-/-</sup>), L (L<sup>-/-</sup>), B and L (B&L<sup>-/-</sup>), or NLRP3 (NLRP3<sup>-/-</sup>)–deficient donor mice. PMs elicited from these mice were LPS-primed and **(A)** probed for cathepsin activity with BMV109 in live cells; lysates were processed by SDS-PAGE and phosphor imaged; dashed boxes highlight upregulated cathepsin S activity for L<sup>-/-</sup> & B&L<sup>-/-</sup> and m.w. markers are on the right in kDa, **(D)** stimulated with media control (-), silica (40 µg/mL), LLOMe (0.75 mM), nigericin (2 µM), or dAdT (0.4 µg/mL), **(E)** stimulated with a range of silica concentrations, or **(F)** stimulated with silica plus media (black bars) or silica plus K777 (white bars; 20 µM). **(B,C,G)** PMs elicited from WT or mice deficient in the three cathepsins B, C and S (BCS) were treated with non-targeting siRNA (WT) or siRNA targeting both cathepsins X and L (“XL” when given to WT, or “BCSXL” when given to BCS) and stimulated with media control (-), silica (80 µg/mL), nigericin (1.5 µM), or dAdT (0.5 µg/mL). XL and BCSXL macrophages were also treated with K777 (XL+K777 and BCSXL+K777; white bars; 15 µM). Knockdown (siXL) was verified by **(B)** cathepsin X (*CtsX*) and L (*CtsL*) expression analysis by qPCR; data are normalized to GAPDH expression and plotted relative to non-targeting siRNA (NT), and **(C)** Labeling of cathepsin activity with BMV109 in live cells, as done in “A”; dashed boxes highlight upregulation of cathepsins B & S for siXL and cathepsin X for BCS<sup>-/-</sup> treated with NT; m.w. markers are on the right in kDa. **(D-G)** TNF-α was measured in supernatants. Error bars represent **(A,D-F)** range bars of technical duplicates **(G)** S.E. of means of either five (WT, XL, BCS, BCSXL) or three (+K777) independent experiments. Statistical analysis was performed by **(A,D-F)** Two-way ANOVA and Sidak’s multiple comparisons test or **(G)** Two-tailed Student’s t-test; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, \*\*\*\**P*<0.0001. All data are representative of at least three independent experiments.





Supplemental Figure 2

## Analysis of endogenous cathepsin inhibitors

While technical limitations prevent us from genetically deleting all potentially relevant cathepsin activity, these proteases are specifically inhibited by a family of endogenous regulators called cystatins(384). Therefore, we examined the effects of genetically disabling the activity of the endogenous cathepsins inhibitors, cystatin C and B, on particle-induced IL-1 $\beta$  secretion.

We used siRNA to silence cystatin C, B or C and B in PMs to investigate their role in IL-1 $\beta$  secretion (**Fig. 5**). In all cases, we achieved ~95% knockdown of cystatin expression (**Supp. Fig. 3a**). Indeed, cystatin C deficiency alone caused a significant increase in silica and nigericin-induced IL-1 $\beta$  secretion, but not following stimulation with dAdT (**Fig. 5a**). Moreover, combined deficiency of cystatin B and C synergistically enhanced IL-1 $\beta$  secretion for all stimuli tested. In the absence of these cystatins, K777 selectively reduced silica-induced IL-1 $\beta$  secretion. Therefore, cystatins C and B appear to non-specifically regulate the level of IL-1 $\beta$  secretion, while cystatin C preferentially affects particulate and NLRP3-activating stimuli.

Surprisingly, knockdown of cystatin C and/or B caused an upregulation of pro-IL-1 $\beta$  transcription induced by LPS priming, and an increase in the level of mature IL-1 $\beta$  and pro-IL-1 $\beta$  detected in lysates; mature IL-1 $\beta$  detected in lysates by ELISA after LPS priming directly reflects levels of pro-IL-1 $\beta$  (**Fig. 5b-e**). While this effect is more prominent with cystatin C deficiency, knockdown of both

cystatin C and B synergistically enhances pro-IL-1 $\beta$  synthesis. Assessment of cellularity by detergent-induced LDH release (OD<sub>490</sub>) indicated that the elevation in pro-IL-1 $\beta$  levels was not a result of enhanced proliferation during knockdown (**Supp. Fig. 3b**). Interestingly, the observed elevation in pro-IL-1 $\beta$  synthesis was proportional to increases observed in IL-1 $\beta$  secretion following stimulation with silica, nigericin or dAdT. Moreover, K777 suppressed the increase in pro-IL-1 $\beta$  synthesis and IL-1 $\beta$  secretion resulting from cystatin C and B knockdown, specifically for silica. The fact that K777 reduced pro-IL-1 $\beta$  synthesis more effectively than it reduced IL-1 $\beta$  secretion induced by nigericin and dAdT may reflect that intracellular levels of pro-IL-1 $\beta$  were not limiting for these stimuli and/or that there are kinetic differences in pro-IL-1 $\beta$  induction with the different stimuli. Alternatively, cathepsins may also play a selective role in particle-induced NLRP3 activation (Signal 2) as originally proposed.

Given that cathepsins are not known to play a role in Signal 1 (LPS priming), our finding that cystatins regulate pro-IL-1 $\beta$  synthesis is surprising. However, this is consistent with our observation that the multiply-deficient BCSXL PMs have a lower level of IL-1 $\beta$  detected in the lysate that seems proportional to the reduction in IL-1 $\beta$  secretion. In fact, this indicates that previous findings of lower IL-1 $\beta$  secretion from cathepsin-deficient macrophages may be a direct result of depressed pro-IL-1 $\beta$  synthesis. Indeed, careful examination revealed that cathepsin B&L<sup>-/-</sup> or BCS<sup>-/-</sup> macrophages have partial but significant reductions in intracellular IL-1 $\beta$ /pro-IL-1 $\beta$  detected in lysates after LPS priming by

either ELISA or western blot (**Fig. 5f,g**). The fact that we did not see a significant reduction in secreted IL-1 $\beta$  corresponding to the reduction in intracellular IL-1 $\beta$ /pro-IL-1 $\beta$  is presumably because the reduced pro-IL-1 $\beta$  levels were not below the threshold required to limit the response. Importantly, no single-cathepsin deficiency significantly reduced intracellular IL-1 $\beta$  levels (**Supp. Fig. 3c,d**). Therefore, this effect was not responsible for the reduction in the response to nigericin after silencing cathepsin X (**Supp. Fig. 3d and Fig. 1d**). In any case, our data indicate that cathepsins do indeed play a role in pro-IL-1 $\beta$  synthesis. Notably, LPS-induced TNF- $\alpha$  secretion is relatively unaffected, suggesting that the impact of cystatin deficiency or K777 treatment on pro-IL-1 $\beta$  synthesis does not apply to all NF- $\kappa$ B-dependent cytokines (**Supp. Fig. 3e**). Our data indicate a previously unreported and significant role for cathepsins and their endogenous inhibitors in pro-IL-1 $\beta$  synthesis and that cystatins C and B regulate particle-induced IL-1 $\beta$  secretion by suppressing multiple cathepsins involved in mediating pro-IL-1 $\beta$  synthesis.

### **Chapter III, Figure 5**

**Endogenous cathepsin inhibition by Cystatins C & B regulates particle-induced IL-1 $\beta$  secretion and LPS-induced pro-IL-1 $\beta$  synthesis.** In all experiments, PMs were LPS-primed and treated with media control or K777 (+K777; white bars; 15  $\mu$ M) prior to stimulation or analysis. **(A-E)** PMs were transfected with non-targeting (NT), cystatin C (siCstC), cystatin B (siCstB), or both cystatin C and B (siCstC&B) siRNA. **(A)** PMs were stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL), and IL-1 $\beta$  measured in supernatants. **(B-E)** After priming, PMs were treated with media control for 6h. **(B)** IL-1 $\beta$  or **(C)** Pro-IL-1 $\beta$  were measured in cell lysates by ELISA. **(D)** IL-1 $\beta$  (*IL1b*) expression was analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to NT siRNA. **(E)** Lysates were processed, then pro-IL-1 $\beta$  and  $\beta$ -Actin analyzed by western blot; m.w. markers are on the right in kDa. **(F,G)** PMs from WT mice and cathepsin BCS $^{-/-}$  mice, or chimeric WT mice lethally irradiated and reconstituted with WT or cathepsin BL $^{-/-}$  bone marrow. PMs were treated with media for 6h after LPS priming. **(F)** IL-1 $\beta$  (hatched bars) was measured in lysates by ELISA; data are normalized to LDH (OD<sub>490</sub>) and plotted as fold-change in IL-1 $\beta$  relative to WT controls. **(G)** lysates were processed and analyzed for pro-IL-1 $\beta$  and  $\beta$ -Actin by western blot (measured by densitometry); data are plotted as pro-IL-1 $\beta$  levels normalized to  $\beta$ -Actin and relative to WT controls. Error bars represent **(A)** S.D. of technical quadruplicates, **(B,C)** range bars of technical duplicates, **(D)** S.D. of technical triplicates, **(F)** S.E. of means from nine (WT vs. BCS $^{-/-}$ ) or twelve (WT vs. BL $^{-/-}$ ) independent experiments, **(G)** S.E. of means from five (WT vs. BCS $^{-/-}$ ) or four (WT vs. BL $^{-/-}$ ) independent experiments. Statistical analysis was performed by **(A)** Two-way ANOVA and Dunnett's multiple comparisons test, **(B-D)** One-way ANOVA and Sidak's multiple comparisons test, or **(F,G)** Two-tailed Student's t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.

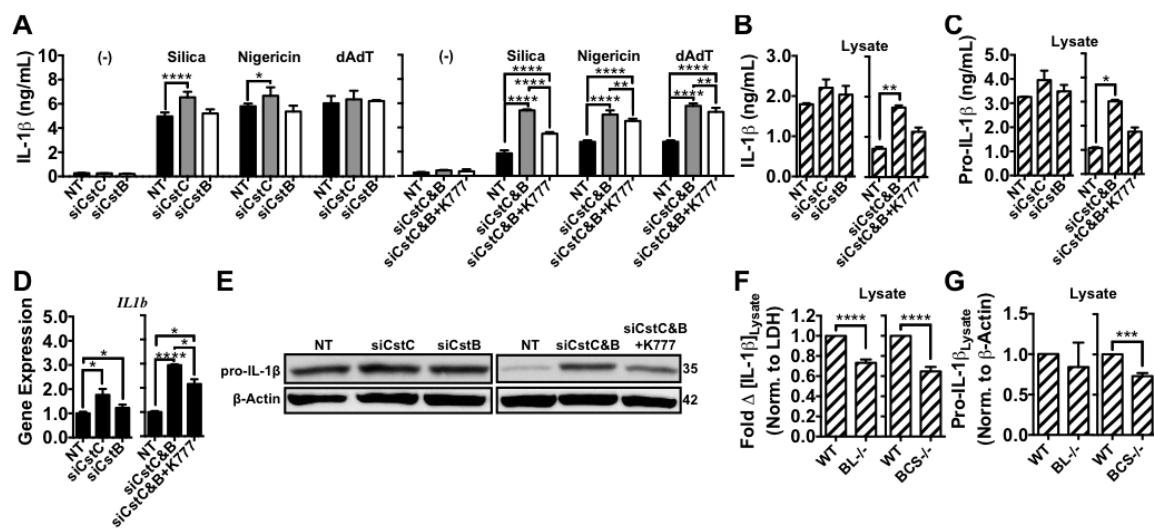
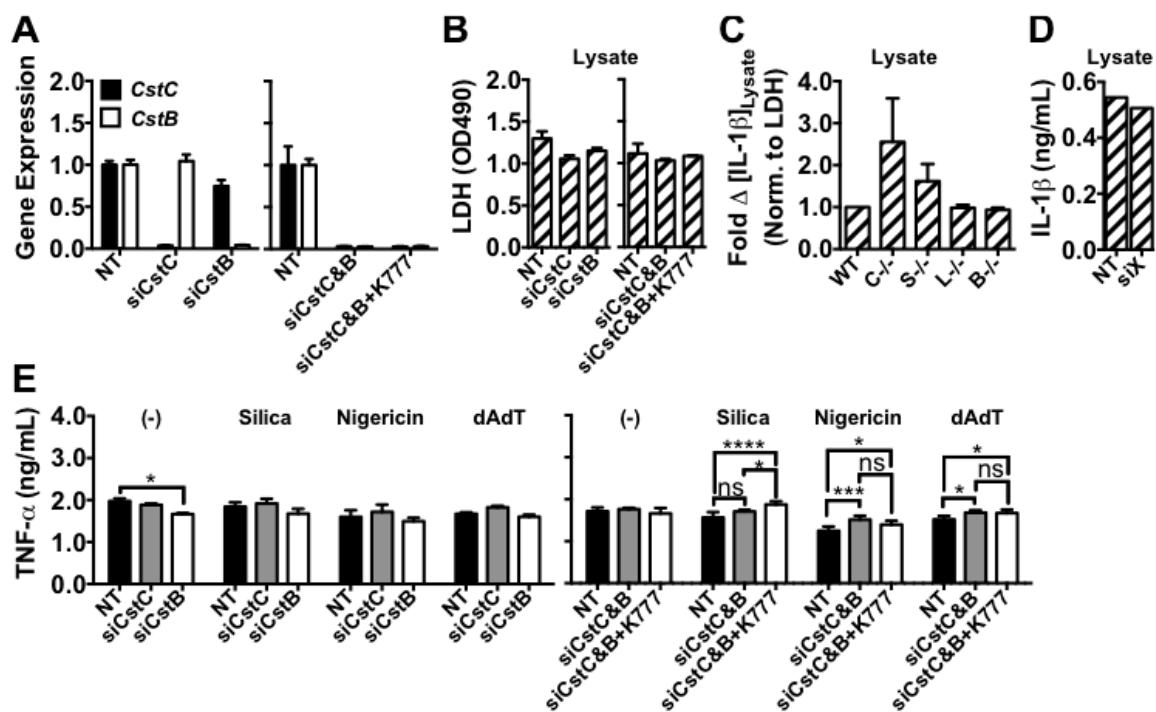


Figure 5

### **Chapter III, Supplemental Figure 3**

In all experiments, PMs were LPS-primed and treated with media control or K777 (+K777; white bars; 15  $\mu$ M) prior to stimulation or analysis. **(A,B,E)** PMs were transfected with non-targeting (NT), cystatin C (siCstC), cystatin B (siCstB), or both cystatin C and B (siCstC&B) siRNA. **(A)** Cystatin C (CstC; black bars) or cystatin B (CstB; white bars) expression was analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to non-targeting (NT) siRNA. **(B)** After priming, PMs were treated with media control for 6h and LDH (OD<sub>490</sub>; hatched bars) was measured in the lysates with a plate reader. **(E)** PMs were stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL) and TNF- $\alpha$  was measured in supernatants. **(C)** LPS-primed PMs from WT mice or mice deficient in either cathepsin C (C<sup>-/-</sup>), S (S<sup>-/-</sup>), L (L<sup>-/-</sup>), or B (B<sup>-/-</sup>) were treated with media for 6h after LPS priming and IL-1 $\beta$  (hatched bars) was measured in lysates by ELISA; data are normalized to LDH (OD<sub>490</sub>), measured with a plate reader, and plotted as fold-change in IL-1 $\beta$  relative to WT controls. **(D)** PMs were transfected with NT siRNA or siRNA targeting cathepsin X (siX), then primed with LPS, treated with media for 6h, and IL-1 $\beta$  (hatched bars) was measured in lysates by ELISA. Error bars represent **(A)** S.D. of technical triplicates, **(B)** range bars of technical duplicates, **(C)** S.E. of means from three (C<sup>-/-</sup> and S<sup>-/-</sup>) or twelve (L<sup>-/-</sup> and B<sup>-/-</sup>) independent experiments, or **(E)** S.D. of technical quadruplicates. Statistical analysis was performed by **(E)** Two-way ANOVA and Dunnett's multiple comparisons test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.



Supplemental Figure 3



## Analyzing the effect of small molecule cathepsin inhibitors on pro-IL-1 $\beta$ synthesis

We demonstrated that cathepsin deficiency attenuates pro-IL-1 $\beta$  synthesis, while cathepsin deregulation by cystatin C and B knockdown enhances pro-IL-1 $\beta$  synthesis. These data indicate that cathepsin inhibitors may suppress IL-1 $\beta$  secretion by affecting pro-IL-1 $\beta$  synthesis. However, if this is true, it is surprising that K777 and Ca074Me did not similarly suppress dAdT-induced IL-1 $\beta$  secretion in previous experiments. However, the kinetics of LPS priming is an important variable when considering the effect of inhibitors on IL-1 $\beta$  secretion, and influences on priming seem to be selective for NLRP3-dependent stimuli compared to those activating other inflammasomes(184). Therefore, we examined whether cathepsin inhibitors affect pro-IL-1 $\beta$  synthesis and how the timing of inhibitor treatment affects their specificity.

To test the effect of cathepsin inhibitors on pro-IL-1 $\beta$  synthesis, we varied the timing of inhibitor treatment relative to LPS priming using an “Early versus Late Inhibitor Treatment Protocol” (**Fig. 6a-c**). First, we treated PMs with K777, Ca074Me or the pan-caspase inhibitor ZVAD immediately prior to LPS priming. In a parallel sample set, we added these inhibitors just prior to stimulation, 3h after LPS priming, and examined how treatment with inhibitors at this time point compares with the former. K777 or Ca074Me treatment prior to LPS priming suppressed both pro-IL-1 $\beta$  in macrophage lysates (**Fig. 6a**) and IL-1 $\beta$  secretion

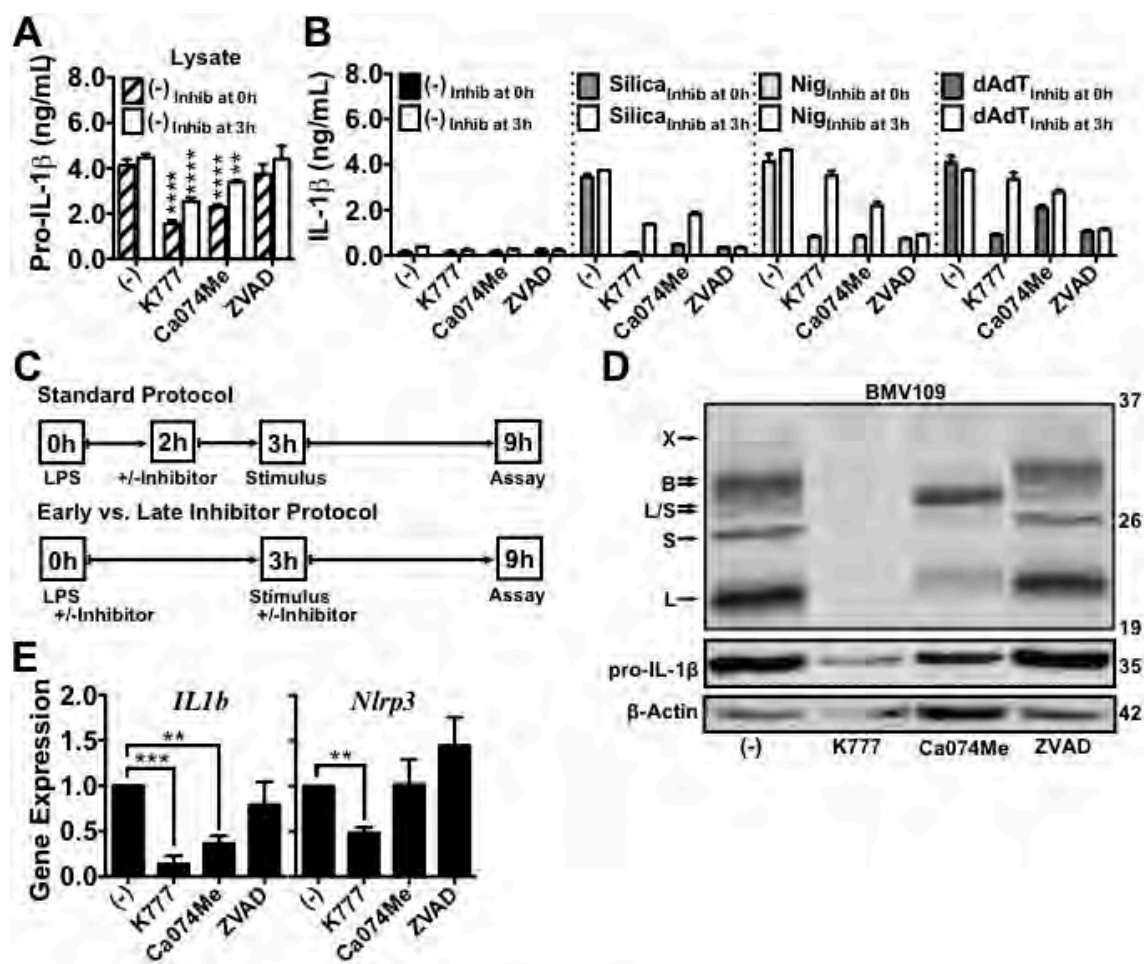
by silica, nigericin and dAdT (**Fig. 6b**). Moreover, these effects were greater for inhibitor treatment just prior to priming. K777 or Ca074Me treatment 3h after LPS priming (just before stimulation) had no effect on dAdT and, as shown earlier, Ca074Me had a more potent effect on nigericin-induced IL-1 $\beta$  secretion (**Fig. 6b**).

To determine whether the reductions in IL-1 $\beta$  secretion that we previously observed were also a reflection of reduced pro-IL-1 $\beta$  levels, we re-tested K777 and Ca074Me using the “Standard Protocol” described for these earlier experiments and examined their effects on pro-IL-1 $\beta$  synthesis (**Fig. 6c-e**). Indeed, treatment with K777 or Ca074Me after only 2h of LPS priming reduced pro-IL-1 $\beta$  levels in lysates (**Fig. 6d**) and also reduced pro-IL-1 $\beta$  transcription (**Fig. 6e**). In fact, K777 even suppressed NLRP3 transcription, although the reduction in NLRP3 transcription caused by Ca074Me was not significant. In contrast to the near complete inhibition of IL-1 $\beta$  secretion by all stimuli, ZVAD treatment had no effect on intracellular IL-1 $\beta$  or pro-IL-1 $\beta$  levels detected in LPS-primed macrophage lysates (**Fig. 6a-e, Supp. Fig. 4a**). Moreover, ZVAD did not suppress pro-IL-1 $\beta$  and NLRP3 transcription or cathepsin activity. Again, under all these conditions above, TNF- $\alpha$  secretion remained unaffected (**Supp. Fig. 4b**). Therefore, cathepsin inhibitors suppressed the synthesis of pro-IL-1 $\beta$  and not TNF- $\alpha$ . When added just prior to LPS priming, cathepsin inhibitors also attenuated NLRP3-independent IL-1 $\beta$  secretion, yet they maintained some selectivity for NLRP3-dependent IL-1 $\beta$  secretion (**Fig. 6b**). These findings are

consistent with a previous study finding that several inhibitors, which also affect Signal 1, preferentially affect NLRP3-dependent stimuli(184). Indeed, the persistent selectivity of cathepsin inhibitors for NLRP3-dependent stimuli may reflect a unique dependence of these responses on Signal 1, based on the requirement for *de novo* NLRP3 transcription or some other factor yet to be defined. However, this is less likely a reflection of differences in Signal 2 kinetics, which are similar for silica and dAdT (**Supp. Fig. 4c,d**).

### **Chapter III, Figure 6**

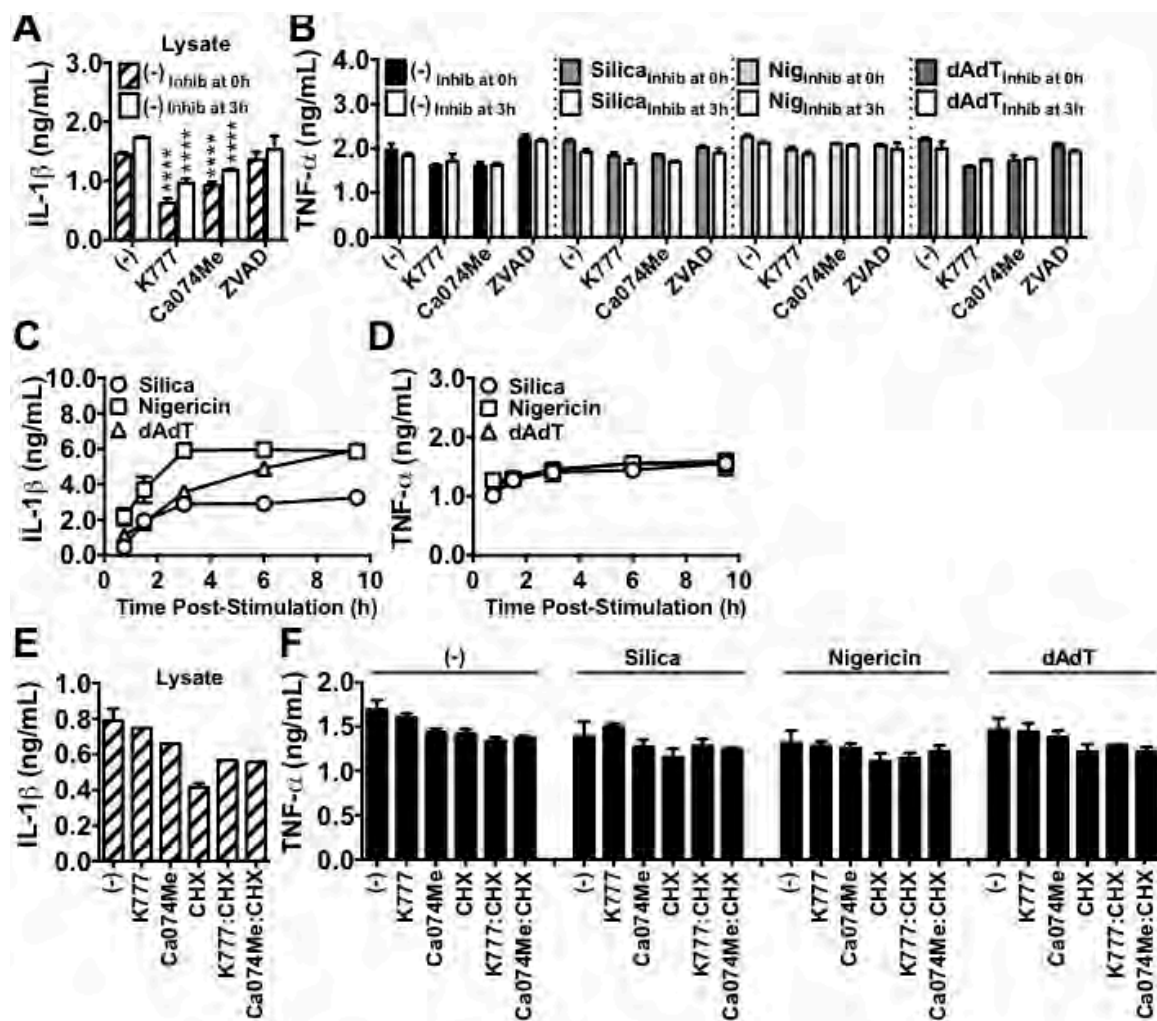
**Small-molecule cathepsin inhibitors suppress pro-IL-1 $\beta$  synthesis.** In all experiments, PMs were primed with LPS for 3h, and then treated with media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M), or ZVAD (10  $\mu$ M) at the indicated time points. **(A,B)** Inhibitors were added at the same time as LPS (Inhib at 0h; hatched or filled bars) or 3h after LPS (Inhib at 3h; white bars) prior to the addition of media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL) for an additional 6h, at which point **(A)** pro-IL-1 $\beta$  was measured in lysates, or **(B)** IL-1 $\beta$  was measured in supernatants by ELISA. **(C)** Comparison of the inhibitor protocol followed in prior figures and “D and E” (Standard Protocol) with the protocol used in “A” and “B” (Early vs. Late Inhibitor Protocol). **(D,E)** Inhibitors were added 2h after LPS priming for 1h, as in the Standard Protocol, then cells were treated with media for 4h. **(D)** Cathepsin activity was probed with BMV109 in live cells; lysates were processed by SDS-PAGE and phosphor imaged, or analyzed for pro-IL-1 $\beta$  and  $\beta$ -Actin by western blot; m.w. markers are on the right in kDa. **(E)** IL-1 $\beta$  (*IL1b*) or NLRP3 (*Nlrp3*) expression was analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to media controls (-). Error bars represent **(A,B)** S.D. of technical triplicates (-), **(B)** duplicates (silica, nigericin, dAdT), **(E)** S.E. of means from three independent experiments. Statistical analysis was performed by **(A)** Two-way ANOVA and Dunnett’s multiple comparisons test, or **(E)** Two-tailed Student’s t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.



**Figure 6**

### **Chapter III, Supplemental Figure 4**

**(A,B)** LPS-primed PMs were treated with media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M), or ZVAD (10  $\mu$ M), which were added at the same time as LPS (Inhib at 0h; hatched or filled bars) or 3h after LPS (Inhib at 3h; white bars) prior to stimulation with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL) for an additional 6h. **(A)** IL-1 $\beta$  was measured in lysates, or **(B)** TNF- $\alpha$  was measured in supernatants by ELISA. **(C,D)** LPS-primed PMs were stimulated with silica (40  $\mu$ g/mL; circle), nigericin (2  $\mu$ M; square) or dAdT (0.3  $\mu$ g/mL; triangle); IL-1 $\beta$  & TNF- $\alpha$  were measured in supernatants after 0.75, 1.5, 3, 6, or 9h of stimulation. **(E,F)** PMs were primed with LPS for 5.5h and treated with either media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M), CHX (1  $\mu$ M), K777 combined with CHX, or Ca074Me combined with CHX for another 0.5h, and then treated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL) for another 3h. **(E)** IL-1 $\beta$  (hatched bars) was measured in lysates, or **(F)** TNF- $\alpha$  (filled bars) was measured in supernatants by ELISA. Error bars represent **(A,B)** S.D. of technical triplicates (-), **(B)** duplicates (silica, nigericin, dAdT), **(C,D)** S.D. of technical duplicates, **(E)** S.D. of technical triplicates, **(F)** S.D. of technical triplicates (media or CHX), duplicates (K777 & Ca074Me  $\pm$ CHX), sextuplicates (silica, nigericin, dAdT  $\pm$ CHX), or triplicates (silica, nigericin, dAdT with K777 & Ca074Me  $\pm$ CHX). Statistical analysis was performed by **(A)** Two-way ANOVA and Dunnett's multiple comparisons test; \*\*\*\* $P < 0.0001$ . Data are representative of **(A-D)** at least three independent experiments, or **(E,F)** two independent experiments.



Supplemental Figure 4

## **Analyzing the effect of cathepsin inhibitors on Signal 2 of NLRP3 activation**

We found that cathepsin inhibition by both small molecules and endogenous regulators suppresses pro-IL-1 $\beta$  synthesis. However, we expected that these effects on pro-IL-1 $\beta$  synthesis would affect all stimuli equally, but cathepsin inhibition had a greater impact on silica-induced IL-1 $\beta$  secretion compared to nigericin or dAdT. Moreover, this selectivity cannot be completely explained by kinetics. Therefore, it was important to determine whether cathepsin inhibitors suppress IL-1 $\beta$  secretion by blocking NLRP3 activation, independently of their effects on pro-IL-1 $\beta$  synthesis.

To determine whether cathepsin inhibition blocks NLRP3-dependent IL-1 $\beta$  secretion (Signal 2) independently of suppressing pro-IL-1 $\beta$  synthesis, we examined the effect of K777 or Ca074Me treatment on IL-1 $\beta$  responses in macrophages with a pool of preexisting pro-IL-1 $\beta$  (**Fig. 7a-c**). Following an extended priming protocol, we primed PMs with LPS for 5.5h to build up an intracellular pool of pro-IL-1 $\beta$ , at which time we added K777, Ca074Me, cycloheximide (CHX), or CHX combined with K777 or Ca074Me, and stimulated 30 min later with silica, nigericin or dAdT for an additional 3h; CHX blocked new IL-1 $\beta$  synthesis so that we could isolate and analyze the effect of the protease inhibitors on the processing of pro-IL-1 $\beta$ . K777 and Ca074Me had minimal effect on IL-1 $\beta$  or pro-IL-1 $\beta$  protein levels in LPS-primed macrophage lysates at this



late time point, and also had no additional effect when combined with CHX compared to CHX alone (**Fig. 7a, Supp. Fig. 4e**). Importantly, K777 and Ca074Me still attenuated silica-mediated IL-1 $\beta$  secretion, both alone and in the presence of CHX, while only Ca074Me affected nigericin-induced activation of the pathway. Again, neither K777 nor Ca074Me blocked dAdT-induced IL-1 $\beta$  secretion, and TNF- $\alpha$  secretion was unaffected (**Fig. 7b, Supp. Fig. 4f**).

To determine whether K777 selectively attenuates particle-induced NLRP3 activation, we examined caspase-1 cleavage in response to silica, CC, nigericin or dAdT (**Fig. 7c,d**). Following our standard protocol, we treated PMs with media or K777, 2h after LPS priming and 1h prior to stimulation with silica, CC, nigericin or dAdT (**Fig. 7c**). After 6h of stimulation, we examined caspase-1 cleavage by western blot. Interestingly, while K777 reduced pro-IL-1 $\beta$  levels in lysates of LPS-primed macrophages, K777 also suppressed caspase-1 activation and mature IL-1 $\beta$  secretion only after stimulation with silica or CC, and not with nigericin or dAdT (**Fig. 7d**). Therefore, in addition to suppression of pro-IL-1 $\beta$  synthesis, both K777 and Ca074Me can also independently suppress NLRP3 activation, while K777 does so selectively for particles.

Taken together, our data suggests a hitherto unrecognized role for cathepsins in inflammasome-mediated IL-1 $\beta$  responses to sterile particles. Furthermore, our study implicates a complex role for cathepsins and their endogenous regulators, cystatins, in regulating not only IL-1 $\beta$  secretion but also

IL-1 $\beta$  induction, highlighting a multi-step involvement of this family of proteases during particle-induced inflammation.

### **Chapter III, Figure 7**

**Cathepsin inhibitors also suppress NLRP3 activation independently of effects on pro-IL-1 $\beta$  synthesis.** **(A,B)** PMs were primed with LPS for 5.5h and treated with either media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M), CHX (1  $\mu$ M), K777 combined with CHX, or Ca074Me combined with CHX for another 0.5h, and then treated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL) for another 3h. **(A)** Pro-IL-1 $\beta$  (hatched bars) was measured in lysates, or **(B)** IL-1 $\beta$  (filled bars) was measured in supernatants by ELISA. **(C)** Comparison of the inhibitor protocol followed in prior figures (Standard Protocol) with the protocol used in “A” and “B” (Extended-Priming Protocol). **(D)** PMs were either unprimed or primed with LPS and treated with K777 (20  $\mu$ M) 2h after LPS priming, as in the Standard Protocol, and cells were treated 1h later with media control (-), silica (40  $\mu$ g/mL), CC (100  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.4  $\mu$ g/mL) for an additional 6h, then lysates were processed by SDS-PAGE and analyzed for pro-caspase-1, active caspase-1 (p-10), pro-IL-1 $\beta$ , active IL-1 $\beta$  (p-17) and GAPDH by western blot; m.w. markers are on the right in kDa. Error bars represent **(A)** S.D. of technical triplicates, **(B)** S.D. of technical triplicates (media or CHX), duplicates (K777 & Ca074Me  $\pm$ CHX), sextuplicates (silica, nigericin, dAdT  $\pm$ CHX), or triplicates (silica, nigericin, dAdT with K777 & Ca074Me  $\pm$ CHX). Statistical analysis was performed by **(B)** Two-way ANOVA and Dunnett's multiple comparisons test; \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. Data are representative of two **(A,B)** or at least three **(D)** independent experiments.

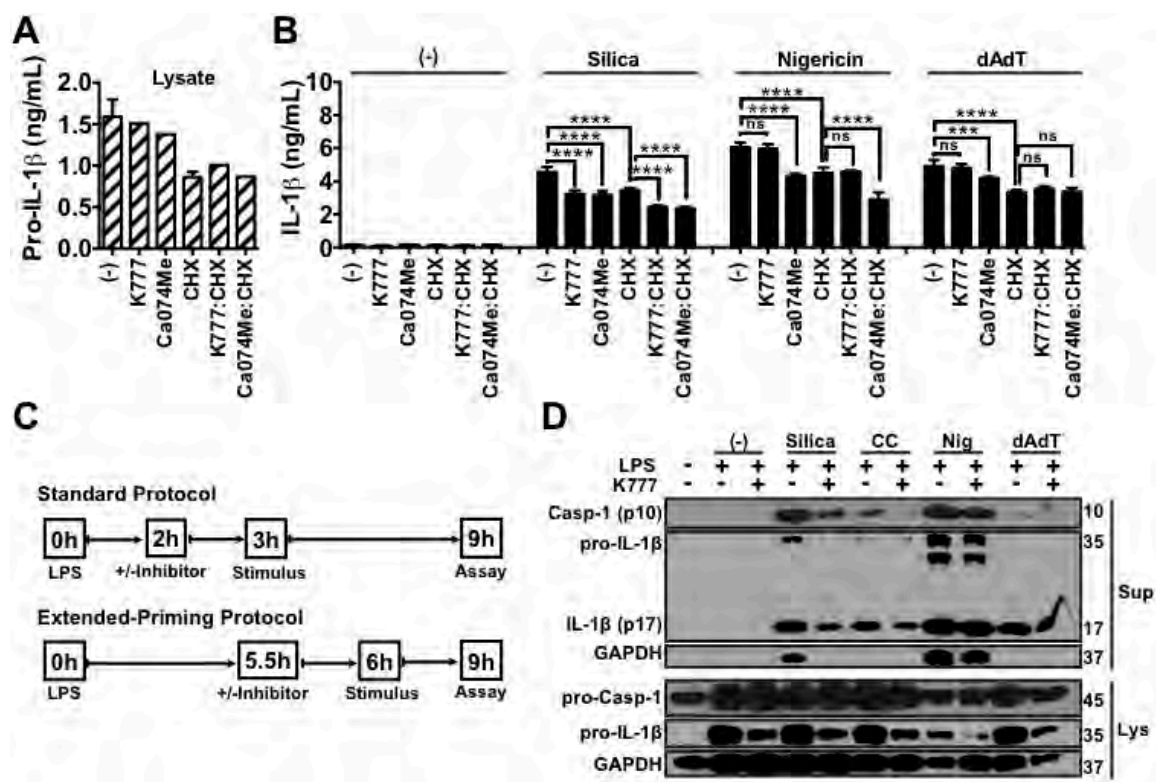


Figure 7

## **Discussion**

Cathepsin B has been implicated in the activation of NLRP3 inflammasomes by particulate stimuli. In this report, we show that contrary to earlier suggestions, multiple cathepsins are involved redundantly in the production of IL-1 $\beta$  induced by sterile particles. These data address and potentially reconcile earlier controversies on the role of cathepsins. Surprisingly, we also found that cathepsins not only play a role in the NLRP3-dependent maturation of pro-IL-1 $\beta$ , but they also have a substantial role in the priming phase of this response.

Given the controversial role of cathepsins in NLRP3-dependent IL-1 $\beta$  responses(11, 15, 16, 18, 126, 142, 144, 147, 160-163, 373-378), it was important to clarify their contribution by performing a rigorous analysis of two confounding variables that have likely influenced prior results and caused confusion. First, we found that the loss of certain cathepsins causes a compensatory upregulation in the activity of other cathepsins. Since the cysteine cathepsin family shares considerable homology and broad substrate specificities(259), functional redundancy may obscure the contribution of any one cathepsin. Therefore, the lack of a phenotype in any single cathepsin knockout does not rule out the involvement of that cathepsin or other cathepsins.

Second, as we show here, the inhibitor Ca074Me actually inhibits multiple cathepsins in living cells at the concentrations used in prior studies of NLRP3 activation(11, 16, 18, 126, 142, 144, 147, 160, 161, 163, 373-378). In fact, we

found that, at doses where Ca074Me is cathepsin B-specific, it does not block NLRP3-dependent IL-1 $\beta$  secretion; at higher doses where it inhibits multiple cathepsins, its blockade of IL-1 $\beta$  secretion increases. Indeed, Ca074Me suppresses IL-1 $\beta$  secretion in cathepsin B-deficient macrophages, and we found similar results with the other cathepsin knockouts as well. Concomitant testing with K777, an orally bioavailable broad inhibitor of cathepsins(385-391), yielded comparable results to Ca074Me. Given this new evidence, it is now clear that the broad specificity of cathepsin inhibitors (Ca074Me and K777) is concordant with a role for multiple cathepsins in particle-induced IL-1 $\beta$  secretion. Moreover, even if it plays an important role in NLRP3 activation under some conditions, our data indicate that cathepsin B is not essential for this response.

Importantly, we document these two confounding variables above using a recently developed activity-based probe, BMV109(293). Although a separate report has shown that Ca074Me can inhibit cathepsins B, S and L in live cells with a similar probe(155), this is the first time that the concentration-dependent inhibition of these cathepsins, or the compensatory upregulation of cathepsin activity, has been demonstrated in parallel with an examination of IL-1 $\beta$  secretion. Moreover, BMV109 labels cathepsin X, which allowed us to investigate the role of this cathepsin in IL-1 $\beta$  secretion.

It is critical to note that, of the five cathepsins tested herein, cathepsin X was the only one that played a non-redundant role in IL-1 $\beta$  secretion. Cathepsin X appeared to be uniquely required for the IL-1 $\beta$  response to nigericin. In fact, we

show that Ca074Me potently inhibits cathepsin X, and this likely accounts for its ability to strongly suppress nigericin-induced IL-1 $\beta$  secretion. Unlike Ca074Me, K777 inhibits cathepsins S at low concentrations, and deficiency of cathepsins S upregulates cathepsin X activity. Thus, this may explain why K777 is less effective against nigericin than Ca074Me, and how its broader specificity for cathepsins paradoxically makes it a more selective inhibitor of particle-induced responses. Therefore, pharmacological suppression of IL-1 $\beta$  secretion induced by particular stimuli likely depends on, not only on how many but, which cathepsins are inhibited and at what concentrations.

While Ca074Me and K777 could have non-cathepsin off-target effects responsible for their suppression of particle-induced IL-1 $\beta$  secretion, we strongly favor the interpretation that they are achieving this effect by inhibiting multiple functionally redundant cathepsins. Although we observed a minor but insignificant reduction of particle-induced IL-1 $\beta$  secretion in the cathepsin BL-/- PMs, and a small but significant reduction in the pentuple cathepsin BCSXL-/- PMs, we believe that the residual cathepsin activity in these cells, as shown by BMV109 labeling, could be sufficient to mediate NLRP3 activation. In fact, a recent study demonstrated that inflammasome activation is an “all-or-none” response(77), which gives credence to earlier proposals that only a few molecules of active cathepsins may be sufficient to reach a minimum threshold for inflammasome activation(162). Whether this is true or not remains to be

demonstrated. However, we did find more robust genetic evidence supporting an unexpected role for cathepsins in regulating the priming phase of IL-1 $\beta$  secretion. Because we could not genetically suppress cathepsin activity to the same extent as inhibitors, which further reduced IL-1 $\beta$  secretion by these genetically deficient cells, we adopted an alternative strategy. Instead of examining cathepsin deficiency, we evaluated the effect of cathepsin deregulation by silencing two broadly active endogenous cathepsin inhibitors, cystatins C and B. Like the cathepsin family(392), the cystatin family is large(384), as might be expected of regulators of a large family of proteases. Moreover, individual cystatins specifically regulate multiple cysteine cathepsin proteases, including B, L and S(384). Indeed, knockdown of cystatin C and B synergistically enhanced IL-1 $\beta$  secretion, but did so for all stimuli tested. Further analyses revealed that the increase in IL-1 $\beta$  secretion we observed was directly proportional to the upregulation of pro-IL-1 $\beta$  transcript and protein synthesis. In fact, reexamination of the compound cathepsin knockouts (BL-/- & BCS-/-) also showed that multiple redundant cathepsins play a partial, but significant, role in LPS-induced pro-IL-1 $\beta$  synthesis. As far as we know, these findings are among the first to implicate and clarify the role of endogenous cathepsin inhibitors, cystatins, in regulating IL-1 $\beta$  responses.

While an association between cystatins and inflammation has been widely reported, the mechanism underlying this association has not been established. Given this context, our evidence that both cystatin B and especially cystatin C



play a role in the IL-1 $\beta$  response is enlightening. In fact, lower serum levels of cystatin C, considered the “dominant” cystatin(393), are associated with numerous inflammatory conditions(384), including sterile inflammatory arterial disease(394). Furthermore, cystatin B deficiency in mice exacerbated LPS-induced sepsis and elevated IL-1 $\beta$  levels in the serum(395). This latter study demonstrated higher caspase-1 and/or -11 activity and mitochondrial ROS, suggesting that loss of cystatin B increased inflammasome activation(395). However, the authors noted that there were no signs of LMD or elevated cathepsin activity in the cytosol, and effects on pro-IL-1 $\beta$  were not measured. Thus, our data demonstrating that cystatin deficiency increases pro-IL-1 $\beta$  synthesis offers a different perspective that may help to explain these results. In this context, it is interesting that other studies have shown that cystatin B interacts with cathepsin L in the nucleus(396), and that cathepsin L can play a role in NF- $\kappa$ B activation(397). Moreover, cystatin B-deficient macrophages have lower IL-10 expression(398), and IL-10 transcriptionally downregulates IL-1 $\beta$  synthesis(239).

While unexpected, our data with cystatins shed further light on the mechanism by which small molecule cathepsin inhibitors may impact IL-1 $\beta$  secretion by modulating pro-IL-1 $\beta$  synthesis. Indeed, we directly demonstrated that exogenous cathepsin inhibitors also suppress LPS-induced pro-IL-1 $\beta$  synthesis, and that this effect contributes substantially to their suppression of IL-1 $\beta$  secretion by inflammasome-activating particulates and non-particulates.

Importantly, K777 and Ca074Me reduce pro-IL-1 $\beta$  synthesis in response to LPS priming alone, prior to any IL-1 $\beta$  being secreted, and they do not affect TNF- $\alpha$  secretion. Thus, it is unlikely that inhibitors are reducing the autocrine-like priming of pro-IL-1 $\beta$  synthesis simply by suppressing TNF- $\alpha$  or IL-1 $\beta$  secretion upon stimulation. Together, these findings reiterate the importance of examining both Signal 1 and 2 when interpreting inflammasome studies. In fact, a recent paper emphasized this point by demonstrating that several ROS inhibitors thought to suppress NLRP3 activation actually affect Signal 1(184). We also find that the timing of inhibitor treatment relative to LPS priming can confirm this phenomenon. If inhibitors are added earlier with respect to LPS priming, effects on priming become more pronounced and less NLRP3-specific. In some contexts, this may actually be a therapeutically advantageous characteristic.

Our findings are consistent with a prior study demonstrating that a cathepsin B inhibitor, Z-FA-fmk, suppresses LPS signaling(399). Finding discordant results with cathepsin B-deficient cells, the authors suggested this was a non-cathepsin off-target effect. Similarly, we cannot completely exclude the possibility that the various exogenous and endogenous cathepsin inhibitors are reducing IL-1 $\beta$  responses through off-target effects. However, given our results, it is likely that redundant cathepsins compensated for the loss of cathepsin B, and even more likely that Z-FA-fmk is non-specific for cathepsin B. Moreover, since we observed concordant results with two chemically distinct cathepsin inhibitors, Ca074Me and K777, as well as the endogenous cathepsin

inhibitors, we favor the idea that the common effect of these inhibitors on pro-IL-1 $\beta$  synthesis is attributable to their common cathepsin targets.

Importantly, Ca074Me and K777 were consistently more effective against NLRP3-mediated IL-1 $\beta$  secretion compared to that mediated by AIM2 via dAdT, and the effects of cystatin deficiencies were similarly biased. Therefore, it appeared that cathepsins may indeed have a role in mediating stimulus-specific/priming-independent NLRP3 activation. While this is one interpretation, others would predict that NLRP3-mediated IL-1 $\beta$  secretion is particularly sensitive to the levels of pro-IL-1 $\beta$  or that the levels of NLRP3 itself are significantly impacted by inhibitor treatment. Given the importance of LPS priming kinetics, deducing priming-independent effects on IL-1 $\beta$  secretion can be achieved via prolonged priming and/or concomitant inhibition of protein synthesis. Indeed, by inhibiting further pro-IL-1 $\beta$  synthesis with CHX following a prolonged period of LPS priming, we show that subsequent treatment with K777 and Ca074Me affects Signal 2, independently of Signal 1. This indicates that cathepsins may also play a role in NLRP3 activation, as originally proposed.

Whether cathepsins play a role in Signal 1 or Signal 2, it is likely that the proteolytic activity of cathepsins is necessary, given the efficacy of inhibitors; if true, the substrate involved remains to be elucidated. Importantly, both TLR4 and NLRP3, which sequentially mediate the priming and activation of IL-1 $\beta$  secretion, respectively, have large leucine-rich repeats (LRRs). It is presumed the LRRs act as autoinhibitory motifs that block activation until induction of structural changes

or ligand binding. In fact, cathepsin inhibitors have been used to demonstrate that cleavage of the LRRs for TLRs 3, 7 and 9 is necessary for optimal activation(400, 401). Moreover, it has also been shown that NLRP1 activation can be directly mediated by proteolytic cleavage of its LRR(402, 403), and that expression of a transgenic NLRP3 protein lacking an LRR motif makes it constitutively active(404). Although this is still all speculation, LRR-targeted cleavage of TLR4 and NLRP3 by cathepsins remains an intriguing possibility that might explain our findings.

Together, this study identifies a previously unappreciated role for cathepsins and cystatins in the regulation of pro-IL-1 $\beta$  synthesis (as well as IL-1 $\beta$  secretion), and provides compelling evidence that cathepsins play redundant and compensatory roles in these processes. Furthermore, we have re-confirmed that Ca074Me inhibits multiple cathepsins and demonstrate conclusively that cathepsin B is not the sole target of this agent that mediates its effect on IL-1 $\beta$  secretion. Moreover, we identified cathepsin X as a previously unappreciated player in nigericin-induced NLRP3 activation, and raised important questions as to the relative importance of cathepsins in mediating Signal 1 and 2 during particle-induced NLRP3 activation and IL-1 $\beta$  secretion. Finally, we have characterized a cathepsin inhibitor, K777, which selectively reduces particle-induced induced IL-1 $\beta$  responses and possesses pharmacological properties warranting its investigation as a potential anti-inflammatory therapeutic(385-391). Indeed, cathepsins are tractable targets for the development of small molecule

inhibitors. Our data predict that inhibitors that broadly inhibit cathepsins, like K777, might have potential as therapeutic inhibitors of particle-induced sterile inflammation.

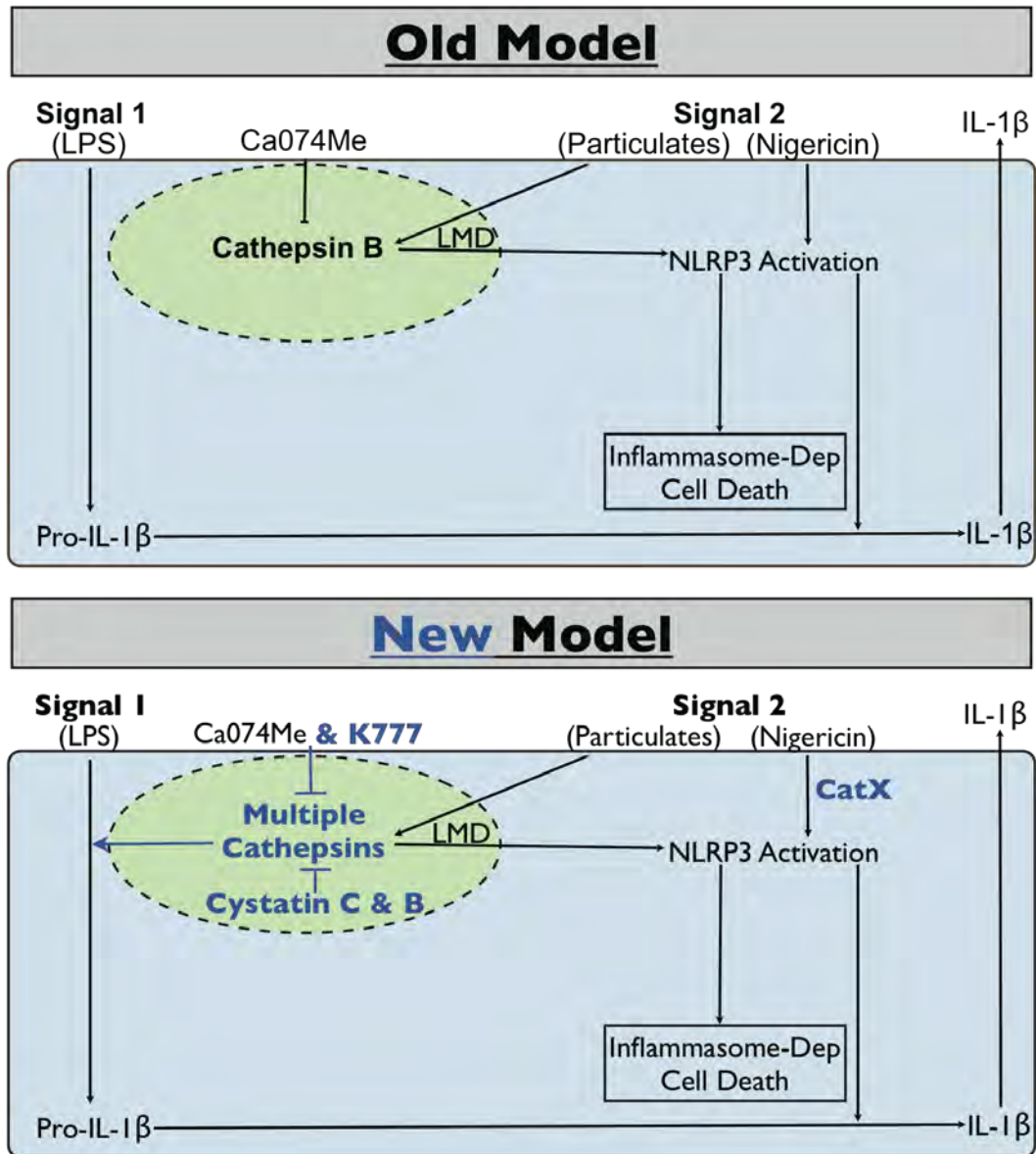
### **Acknowledgements**

We thank Havisha Karnam from the Brown and Khvorova labs as well as Myriam Aouadi from the Czech lab at  $\mu$ MMS for help in the optimization of siRNA knockdowns, as well as Kate Fitzgerald, Douglas Golenbock and Eicke Latz for advice and reagents.

### **Grant Support**

This work was supported by an R01 (Grant# 5R01AI078287-05) to K.L.R., a T32 (Grant# T32AI095213-01), and services from the NHLBI, NIH, DHHS, and the Science Moving Towards Research Translation and Therapy (SMARTT) program via the following contract HHSN268201100015C.

### Summary of Major Contributions in Chapter III



## **Chapter IV:**

**Lysosomal disruption induces  
cell death that depends on  
multiple cathepsins  
and  
requires intact mitochondria  
to promote  
NLRP3-mediated IL-1 $\beta$  secretion.**



## **Attributions and Copyright Information**

The data described in this chapter comprises a study that is currently in preparation for publication.

All experiments and data were performed and analyzed by me (G.M.O.) and the other authors made significant technical, material, and intellectual contributions.

## **Chapter IV: Lysosomal disruption induces cell death that depends on multiple cathepsins and requires intact mitochondria to promote NLRP3-mediated IL-1 $\beta$ secretion.**

Gregory M. Orlowski,\* Shruti Sharma,\*\* Jeff D. Colbert,\* Matthew Bogyo,<sup>†,‡</sup> Stephanie A. Robertson,<sup>§</sup> Hiroshi Kataoka,\* Francis K. Chan\* and Kenneth L. Rock\*,<sup>¶</sup>

Departments of \*Pathology and \*\*Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA; <sup>†</sup>Department of Pathology and <sup>‡</sup>Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA; <sup>§</sup>Sandler Center for Drug Discovery, University of California, San Francisco, CA 94158, USA.

<sup>¶</sup>Corresponding author: Kenneth L. Rock, Phone: (508) 856-1090, Fax: (508) 856-1094, Email: [Kenneth.Rock@umassmed.edu](mailto:Kenneth.Rock@umassmed.edu)

## **Abstract**

Sterile particles are known to cause a number of chronic inflammatory diseases characterized by cycles of particle phagocytosis and inflammatory cell death that releases these particles back into the local environment. These particles are known to activate IL-1 $\beta$  secretion via NLRP3 inflammasome. However, caspase-1-deficient mice often show similar IL-1-dependent particle-induced inflammatory responses compared to WT mice *in vivo*, suggesting that caspase-1-independent processes are promoting inflammation in this setting. While it is often assumed that particles induce cell death through caspase-1-dependent pyroptosis in LPS-primed macrophages, one group has recently reported that lysosomal membrane disruption (LMD) and the particle alum cause inflammasome-independent necrosis, which antagonizes NLRP3 activation. Although multiple reports have shown that the cathepsin inhibitor Ca074Me (which is thought to block inflammasome activation initiated by the lysosomal protease cathepsin B) suppresses particle-induced cell death, cathepsin B knockouts do not reproduce this effect. Therefore, the involvement of cathepsin B in particle-induced NLRP3 activation and cell death has been controversial. Our most recent study demonstrated a substantial redundancy in the contribution of cathepsins to pro-IL-1 $\beta$  synthesis. Here, we find similar redundancy for cathepsins in promoting particle-induced cell death. Moreover, we find that particles induce inflammasome-independent cell death and that severe LMD antagonizes NLRP3

activation by disrupting mitochondrial membrane potential (MMP), which we show to be essential for pro-IL-1 $\beta$  synthesis and particle-induced NLRP3 activation downstream of cathepsins. Finally, we find that a cathepsin inhibitor suppresses particle-induced IL-1-dependent peritonitis, suggesting that cathepsins, and not caspase-1, play fundamental roles in particle-induced inflammatory responses *in vivo*.

## **Introduction**

Particle-induced sterile inflammation underlies the pathogenesis of a number of common and often chronic diseases(2). Silica particles cause silicosis, the most prevalent work-associated pneumoconiosis in the world(17, 130, 131, 144). Monosodium urate crystals (MSU) cause gout, a prevalent inflammatory joint disease(12). Moreover, cholesterol crystals (CC) have been recently implicated in the development of atherosclerosis, which is the leading cause of cardiac death worldwide(10, 11). In the current report, we focus on the mechanisms inciting this inflammatory pathology on a cellular level in macrophages.

Two events occur concomitantly during the leukocyte response to particles: cell death and the generation of inflammatory mediators(2). The death of cells that have ingested pathogens may be beneficial to the host by helping to limit infections (22-24). However, for sterile particles, which cannot replicate or be destroyed by immune defenses, cell death is not thought to be beneficial but instead results in pathology(9, 25, 26). In fact, chronic pathology in response to sterile particles has been described as a repeating cycle of particle ingestion, inflammatory cell death, and the release of particles back into the environment for re-ingestion by other cells(29). In addition, phagocytes are stimulated by particles, and also by dying cells (e.g. those killed by ingestion of particles), to produce mediators that cause inflammation(9-11, 109). Although the initial inflammatory response to particles has been shown to depend on signaling

through the IL-1 receptor (IL-1R1), the exact mechanisms responsible for the production and release of IL-1 $\beta$  are not completely understood(95, 109, 121). Therefore, a better understanding of the processes regulating both cell death and IL-1 $\beta$  secretion is important and may help identify tractable drug targets for suppressing particle-induced sterile inflammation.

In macrophages *in vitro*, the induction of IL-1 $\beta$  secretion by sterile particles requires two steps. The first step, often referred to as “priming”, occurs when macrophages are stimulated through a receptor, such as TLR4, that leads to the activation of the transcription factor NF- $\kappa$ B, which then drives the transcription of pro-IL-1 $\beta$  and NOD-like Receptor containing a Pyrin domain 3 (NLRP3)(67, 184). In the second step, ingestion of particles by macrophages stimulates the assembly and activation of the NLRP3 inflammasome, which includes NLRP3, the adaptor apoptosis-associated speck-like protein with a CARD domain (ASC), and the effector caspase-1(10, 12, 64, 65, 144). Upon NLRP3 activation, caspase-1 converts pro-IL-1 $\beta$  into active IL-1 $\beta$  for secretion. In addition, caspase-1 can also drive a lytic form of inflammatory programmed cell death, called pyroptosis(115), and this has often been assumed to be the mechanism through which ingestion of particles kills primed macrophages. Therefore, activation of the NLRP3 inflammasome can lead to both cell death and mature IL-1 $\beta$  secretion.

One of the models proposed for particle-induced NLRP3 activation (and lytic cell death) is that phagocytosed particles cause lysosome membrane

disruption (LMD) that then initiates inflammasome activation and cell death (10, 11, 144). According to this model, a lysosomal cysteine protease, cathepsin B, is released into the cytosol and somehow activates NLRP3 leading to pyroptosis(10, 11, 144). In support of this model, an inhibitor that had been assumed to be specific for cathepsin B, Ca074Me, was shown to suppress cell death and IL-1 $\beta$  secretion during NLRP3 activation induced by particulate and lysosome disrupting stimuli(155, 163, 376, 405). However, the dependence of cell death on cathepsin B during particle-induced NLRP3 activation has not been demonstrated using genetic tools, such as cathepsin B-deficient cells. The latter kind is important because several reports have now shown that Ca074Me inhibits multiple cathepsins at concentrations used to block NLRP3 activation in prior studies(155-159, 406). Moreover, studies examining genetic deficiency of cathepsin B and/or L in these responses have yielded conflicting results, with some showing a partial role for both of these proteases in NLRP3 activation(10, 160, 161), and most others showing no role for either(15, 162, 163). A confounding factor in these genetic experiments is that loss of one cathepsin can result in a compensatory upregulation of other functionally redundant cathepsins (379, 380, 406). More recently, we have found that multiple redundant cathepsins participate in particle-stimulated IL-1 $\beta$  production and do so by both enhancing IL-1 $\beta$  priming and NLRP3-dependent pro-IL-1 $\beta$  cleavage. Whether multiple cathepsins are also involved in particle-induced cell death, remains an open question.

*In vivo*, IL-1 $\beta$  has been shown to be a critical mediator of particle-induced sterile inflammatory diseases(407-410). However, in contrast to the absolute requirement for inflammasome-dependent caspase 1 activation for IL-1 $\beta$  responses *in vitro*, several studies have shown that caspase-1-deficient mice have, on average, <50% reduction in particle or dead cell-induced responses compared to WT animals; in other studies, these responses were not reduced at all(10, 12, 121, 130). Two explanations were proposed for these findings. One explanation proposed that an unidentified inflammasome-independent pathway drives particle and dead cell-induced IL-1 $\beta$  activation by intact cells *in vivo*(121). As far as we know, there is currently no evidence for this. The second explanation proposed that this response is driven by caspase-1-independent lytic cell death, which releases IL-1 $\alpha$ , pro-IL-1 $\beta$  and various other pro-inflammatory danger-associated molecular patterns (DAMPs) into an extracellular environment where cathepsin C-dependent neutrophil proteases can further process pro-IL-1 $\beta$ (121). Indeed, there is some evidence that sterile particles induce inflammasome-independent cell death during NLRP3 activation(155, 163). Therefore, the considerable caspase-1-independent component of the *in vivo* response suggests that understanding the caspase-1-independent mechanisms involved in particle-induced IL-1 production and cell death may be of considerable value to our understanding inflammatory diseases caused by particles.



In the current study, we examine whether particle-induced cell death during NLRP3 activation depends on inflammasomes or cathepsins using both inhibitors and genetic models. We find that particle-induced cell death is not inflammasome dependent, as has often been assumed, but instead depends on multiple redundant cathepsins. Furthermore, we investigate the relationship between particle-induced cell death and IL-1 $\beta$  secretion, and find that the Bcl-2 family influences both responses, presumably through their effects on mitochondria. Interestingly, although pro-apoptotic Bcl-2 family members can cause mitochondrial disruption, and some have suggested that mitochondrial disruption can stimulate NLRP3, we find that a substantial disruption of the mitochondrial membrane potential (MMP) actually antagonizes NLRP3-mediated IL-1 $\beta$  secretion by interrupting the priming of pro-IL-1 $\beta$  and NLRP3 synthesis. Therefore, this study highlights the requirement for intact mitochondria during particle-induced IL-1 $\beta$  secretion and distinguishes the IL-1 $\beta$ -promoting function of LMD from the IL-1 $\beta$ -antagonizing role of mitochondrial outer membrane permeabilization (MOMP).

## **Results**

### **Particle-induced cell death is inflammasome-independent**

Sterile particles ingested by macrophages stimulate inflammasome activation and cell death(2, 10-12). The mechanism of particle-induced cell death has often been assumed to be inflammasome-dependent pyroptosis that is initiated by activated caspase-1. Contrary to this assumption, two recent studies reported that lysosome disruption and alum particles actually elicit inflammasome-independent necrosis in LPS-primed macrophages, although this was not examined side-by-side with IL-1 $\beta$  responses(155, 163). Therefore, we examined whether other particle-induced cell death depends on inflammasomes in LPS-primed macrophages.

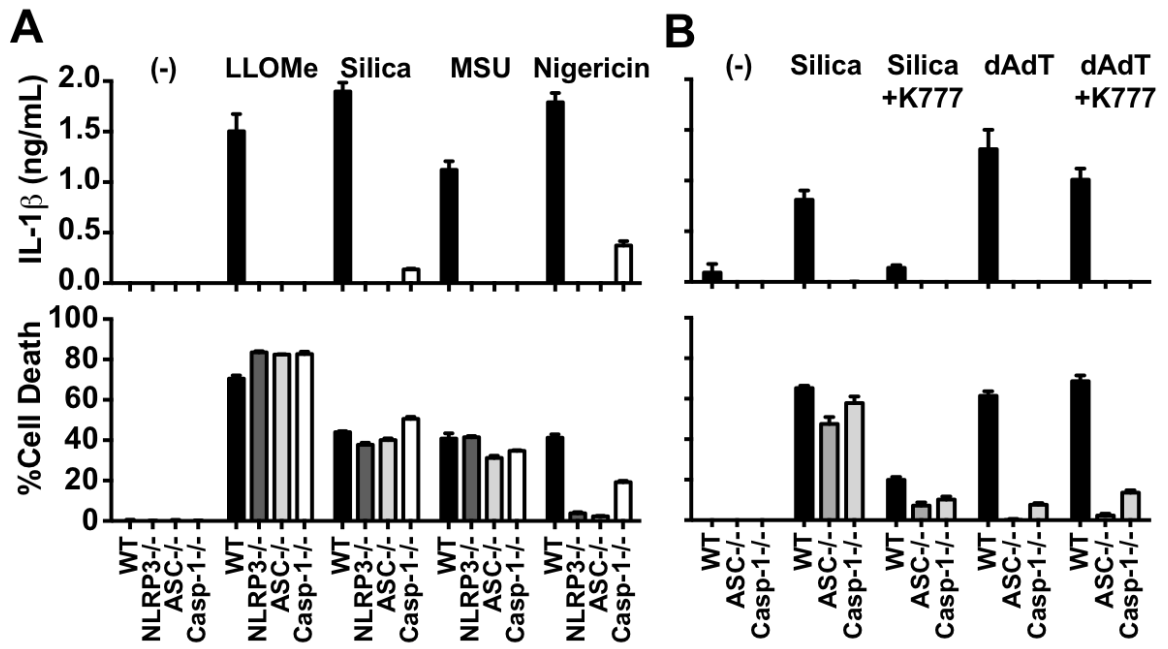
In all the following experiments, unless otherwise noted, we primed peritoneal macrophages (PMs) with LPS and followed a “Standard Protocol”: LPS priming for 2h → Add Inhibitors or Media Control for 1h → Add Stimuli for 6h → Analysis. Here, we stimulated PMs from WT mice or mice deficient in the inflammasome components NLRP3, ASC or caspase-1 with various NLRP3-activators: a lysosomotropic detergent (LLOMe), particles (silica & MSU) or the soluble potassium ionophore (nigericin) (**Fig. 1a**). The cell death induced by LLOMe, silica, and MSU was inflammasome-independent, although the IL-1 $\beta$  measured by these same stimuli was completely inflammasome-dependent. In

contrast, nigericin-induced cell death and IL-1 $\beta$  production were both inflammasome-dependent. Interestingly, the broad cathepsin inhibitor K777 suppressed silica-induced cell death in PMs lacking inflammasome components. In contrast, cell death and IL-1 $\beta$  responses induced by dAdT (a stimulator of Aim2 inflammasomes) were both dependent on inflammasome components, whereas the cell death and most of the IL-1 $\beta$  response induced by this stimulus were not blocked by the cathepsin inhibitor K777 (**Fig 1b**). In all the experiments above, TNF- $\alpha$  secretion was unaffected by K777, demonstrating that this agent is selectively affecting cell death and IL-1 $\beta$  (**Supp. Fig. 1a-c**). These data suggest that despite being absolutely critical for IL-1 $\beta$  secretion *in vitro*, inflammasomes are not essential for particle-induced cell death in LPS-primed macrophages, and point instead to a role for cathepsins in this process.

#### **Chapter IV, Figure 1**

**Particle-induced cell death is inflammasome-independent in LPS-primed macrophages.** In all cases, PMs were primed with LPS. **(A)** PMs from WT, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp1<sup>-/-</sup>) stimulated with media control (-), LLOMe (1 mM), silica (50 µg/mL), MSU (250 µg/mL) or nigericin (2 µM). **(B)** PMs from WT, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp-1<sup>-/-</sup>) mice treated with K777 (30 µM) before stimulation with silica (80 µg/mL) or dAdT (1 µg/mL). **(A,B)** S.D. of technical triplicates. All data are representative of at least three independent experiments.

**Figure 1**



## **Particle-induced cell death and IL-1 $\beta$ secretion are co-dependent on cathepsins and the Bcl-2 family**

Sterile particles have been shown to induce LMD during NLRP3 activation(10, 11, 144). Moreover, previous studies have shown that LMD releases cathepsins into the cytosol, where these proteases can activate Bid and inactivate Bcl-2, and thereby tip the balance between pro-survival and pro-death Bcl-2 family members in favor of cell death(200). In the context of NLRP3 activation, it has been shown that Ca074Me inhibits cleavage of Bid induced by dsRNA, a stimulus that can activate NLRP3 as well as other pattern recognition receptors (377). Moreover, two studies showed that Bcl-2 overexpression reduced cell death and IL-1 $\beta$  secretion induced by particulate and non-particulate stimuli(173, 218), and one of these studies showed that Bcl-2 knockdown enhances cell death and IL-1 $\beta$  secretion induced by the soluble NLRP3 activator ATP(218). Therefore, we examined whether cathepsins and the Bcl-2 family are involved in particle-induced cell death and IL-1 $\beta$  secretion in LPS-primed macrophages.

First, we examined the effects cathepsin inhibitors on particle-induced cell death during NLRP3 activation and compared them to the standard pyroptosis blocking caspase inhibitors. We treated PMs with either YVAD (caspase-1 inhibitor), ZVAD (pan-caspase inhibitor), Ca074Me (cathepsin B-selective inhibitor at  $\leq 1 \mu\text{M}$ ) or K777 (broad cathepsin inhibitor) and then stimulated with silica, CC, nigericin or the AIM2 inflammasome activator poly(deoxyadenylic-

deoxythymidylic) acid (dAdT) (**Fig 2a**). K777 and Ca074Me suppressed both particle-induced cell death and IL-1 $\beta$  secretion, but Ca074Me was more effective than K777 against these responses induced by the soluble NLRP3 activator, nigericin. Neither K777 nor Ca074Me had much effect on dAdT; the small effect shown here is likely due to their suppression of pro-IL-1 $\beta$  synthesis (406). Conversely, though YVAD and ZVAD blocked IL-1 $\beta$  secretion induced by all stimuli tested, they did not suppress cell death induced by silica or CC. Surprisingly, they also did not block cell death induced nigericin or dAdT either, suggesting that these inhibitors may be having toxic off-target effects that interfere with their suppression of pyroptosis. Therefore, inhibitors of cathepsins, and not caspases, suppress both particle-induced IL-1 $\beta$  secretion and cell death.

As this is the first report examining the effects of K777 on cell death during NLRP3 activation, we further characterized this inhibitor. In addition to suppressing particle-induced cell death and IL-1 $\beta$  secretion in PMs, K777 also did so for these responses induced by the LMD agent LLOMe, without affecting TNF- $\alpha$  (**Supp Fig 1d**). Consistent with our previous report, demonstrating K777's selective suppression of particle-induced IL-1 $\beta$  secretion(406), K777 selectively inhibited cell death across a broad range of silica and alum concentrations while having no effect on cell death induced by nigericin or dAdT at any concentration tested (**Supp Fig 1e**). Moreover, these effects were not limited to PMs. K777 also selectively suppressed particle-induced cell death in bone marrow-derived macrophages and mast cells, although K777 did not suppress neutrophil cell

death (**Supp. Fig 1f**). Importantly, our most recent study demonstrated that the timing of cathepsin inhibitor treatment relative to LPS priming affects their selectivity, since they can affect priming (406). Using these same protocols for K777 and Ca074Me, addition of inhibitors at the start of LPS-priming or 3h later at the time of stimulation with silica, nigericin or dAdT, did not affect their selectivity for NLRP3 stimuli or K777's selectivity for particulates (**Supp. Fig 1g**). Moreover, to further verify priming-independent effects of cathepsin inhibitors on particle-induced cell death, we followed an extended LPS-priming protocol (priming 5.5h instead of only 2h before adding inhibitors). This allowed time for pro-IL-1 $\beta$  and NLRP3 synthesis before we blocked new translation and subsequently examined the effects of cathepsin inhibitors on the translation/priming-independent response. To do this, K777 and Ca074Me were added alone or combined with the protein translation inhibitor cycloheximide before stimulating with silica, nigericin, or dAdT. Again, we found that suppression of cell death by these inhibitors was independent of translation-dependent priming (**Supp. Fig 1h**). Therefore, these data suggest that K777 is a selective inhibitor of particle-induced IL-1 $\beta$  secretion and inflammasome-independent cell death, which likely depends on cathepsins.

Next, we examined the role of the Bcl-2 family on particle-induced cell death during NLRP3 activation. PMs were treated with YVAD as a control or Bax Inhibitory Peptide V (BaxIP5)(411), an inhibitor of the pro-death Bcl-2 family protein Bax, then stimulated with silica, nigericin or dAdT. Similar to cathepsin

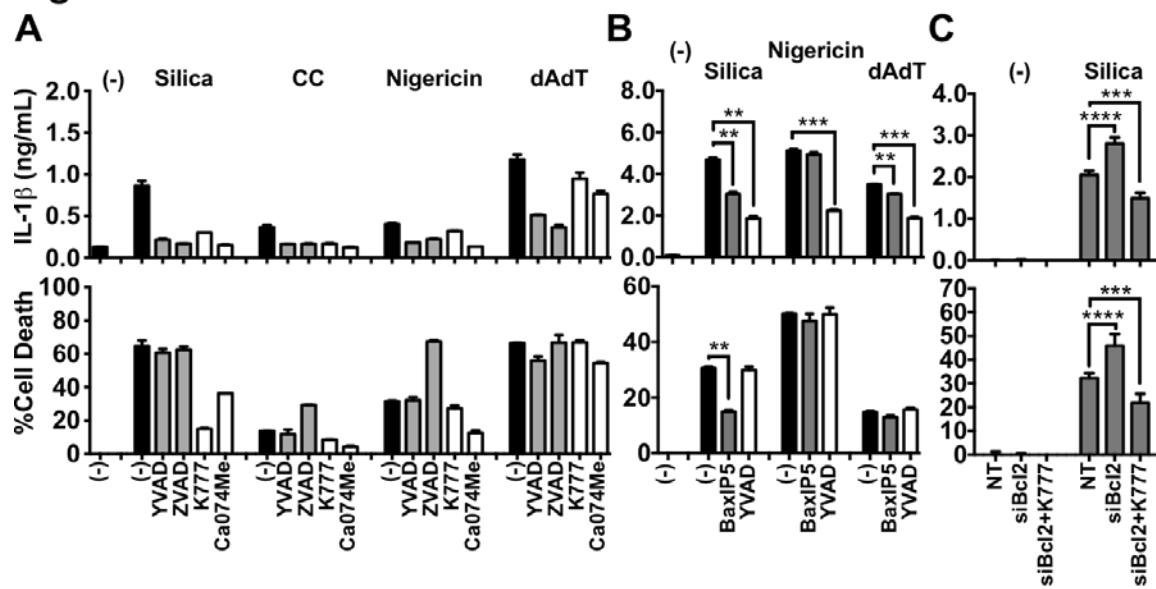


inhibitors, BaxIP5 selectively suppressed both cell death and IL-1 $\beta$  secretion induced by silica, while YVAD affected only IL-1 $\beta$  (**Fig 2b**). In contrast, nigericin and dAdT-induced responses were only mildly affected by BaxIP5 or not at all. Next, in PMs we silenced pro-survival Bcl-2 with siRNA (~80% knockdown by qPCR (**Supp. Fig. 2b**)), then primed them with LPS and stimulated with silica. Consistent with our findings with the Bax inhibitor, Bcl-2 deficiency had the opposite effect to BaxIP5; it increased both silica-induced cell death and IL-1 $\beta$  secretion, and both of these responses were suppressed by K777 (**Fig 2c**). In the above experiments, TNF- $\alpha$  was not significantly affected (**Supp. Fig 2a,c**). Together, these data indicate that cathepsins and the Bcl-2 family specifically modulate particle-induced cell death and IL-1 $\beta$  secretion during NLRP3 activation.

## **Chapter IV, Figure 2**

**Particle-induced cell death and IL-1 $\beta$  secretion are co-dependent on cathepsins and the Bcl-2 family during NLRP3 activation.** In all cases, PMs were primed with LPS. **(A)** PMs were treated with media control (black bars; (-)), caspase-1 inhibitors (gray bars; YVAD (20  $\mu$ M), ZVAD (10  $\mu$ M)) or cathepsin inhibitors (white bars; K777 (20  $\mu$ M), Ca074Me (20  $\mu$ M)) before stimulation with media control (-), silica (50  $\mu$ g/mL), CC (75  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). **(B)** PMs treated with media control (-), BaxIP5 (200  $\mu$ M) or YVAD (20  $\mu$ M) stimulated with media control (-), silica (50  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.3  $\mu$ g/mL); cell death analyzed by MTS assay. **(C)** PMs treated with NT control siRNA or siRNA targeting Bcl-2 (siBcl2) were primed with LPS and treated with media control or K777 (15  $\mu$ M) as indicated and stimulated with silica (80  $\mu$ g/mL). Error bars represent **(B)** range bars of technical duplicates, or **(C)** S.D. of technical quadruplicates. Statistical analysis was performed by **(B)** Two-tailed Student's t-test or, **(C)** Two-way ANOVA and Dunnett's multiple comparisons test, or; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. Data are representative of **(A)** three or, **(B,C)** two independent experiments.

**Figure 2**



#### **Chapter IV, Supplementary Figure 1**

In all cases, cells were primed with LPS. **(A)** PMs treated with media control (black bars; (-)), caspase-1 inhibitors (gray bars; YVAD (15  $\mu$ M), ZVAD (10  $\mu$ M)) or cathepsin inhibitors (white bars; K777 (20  $\mu$ M), Ca074Me (20  $\mu$ M)) before stimulation with media control (-), silica (50  $\mu$ g/mL), CC (75  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). **(B)** PMs from WT, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp1<sup>-/-</sup>) stimulated with media control (-), LLOMe (1 mM), silica (50  $\mu$ g/mL), MSU (250  $\mu$ g/mL) or nigericin (2  $\mu$ M). **(C)** PMs from WT, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp-1<sup>-/-</sup>) mice treated with K777 (30  $\mu$ M) before stimulation with silica (80  $\mu$ g/mL) or dAdT (1  $\mu$ g/mL). **(D)** PMs treated with media control or K777 (white bars; 30  $\mu$ M) and stimulated with silica (50  $\mu$ g/mL), LLOMe (0.75 mM), or dAdT (0.5  $\mu$ g/mL). **(E)** PMs treated with media control (solid line) or K777 (dashed line; 15  $\mu$ M) before stimulation with a titration of silica, alum, nigericin or dAdT. **(F)** Bone marrow derived macrophages (BMDM), neutrophils (N $\phi$ ) or mast cells treated with K777 (white bars; 15  $\mu$ M) and then stimulated with silica (40  $\mu$ g/mL for BMDMs and mast cells, 100  $\mu$ g/mL for N $\phi$ ), nigericin (1  $\mu$ M) or dAdT (0.3  $\mu$ g/mL). **(G)** PMs treated with media control (-) or inhibitors (15  $\mu$ M K777 & Ca074Me, 10  $\mu$ M ZVAD) at the same time as LPS (Inhib at 0h; filled bars) or 3h after LPS (Inhib at 3h; white bars) before stimulation with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL) for an additional 6h. **(H)** PMs were primed with LPS for 5.5h and treated with either media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M), CHX (1  $\mu$ M), K777 combined with CHX, or Ca074Me combined with CHX for another 0.5h, and then treated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL) for another 3h. Error bars represent **(A,D,E)** range bars of technical duplicates, and **(B,C,F)** S.D. of technical triplicates. **(G)** S.D. of technical triplicates (-) or range bars of duplicates (silica, nigericin, dAdT), and **(H)** S.D. of technical triplicates (media or CHX; silica, nigericin, dAdT with K777 & Ca074Me  $\pm$ CHX), sextuplicates (silica, nigericin, dAdT  $\pm$ CHX), or range bars of duplicates (K777 & Ca074Me  $\pm$ CHX). All data are representative of at least **(A-G)** three or **(H)** two independent experiments.

**A** (-) Silica CC Nigericin dAdT

TNF- $\alpha$  (ng/mL)

YVAD ZVAD K777 Ca074Me

**B** (-) LLOMe Silica MSU Nigericin

TNF- $\alpha$  (ng/mL)

WT NLRP3 $^{-/-}$  ASC $^{-/-}$  Casp-1 $^{-/-}$

**C** (-) Silica Silica +K777 dAdT dAdT +K777

TNF- $\alpha$  (ng/mL)

WT ASC $^{-/-}$  Casp-1 $^{-/-}$

**D** +K777

IL-1 $\beta$  (ng/mL)

%Cell Death

Silica ( $\mu$ g/mL) Alum ( $\mu$ g/mL) Nigericin ( $\mu$ M) dAdT ( $\mu$ g/mL)

**E** +K777

%Cell Death

Silica ( $\mu$ g/mL) Alum ( $\mu$ g/mL) Nigericin ( $\mu$ M) dAdT ( $\mu$ g/mL)

**F** BMDM N $\Phi$  Mast Cell

%Cell Death

Silica Alum dAdT

**G** Silica LLOMe dAdT

%Cell Death

TNF- $\alpha$  (ng/mL)

**H** Silica Nigericin dAdT

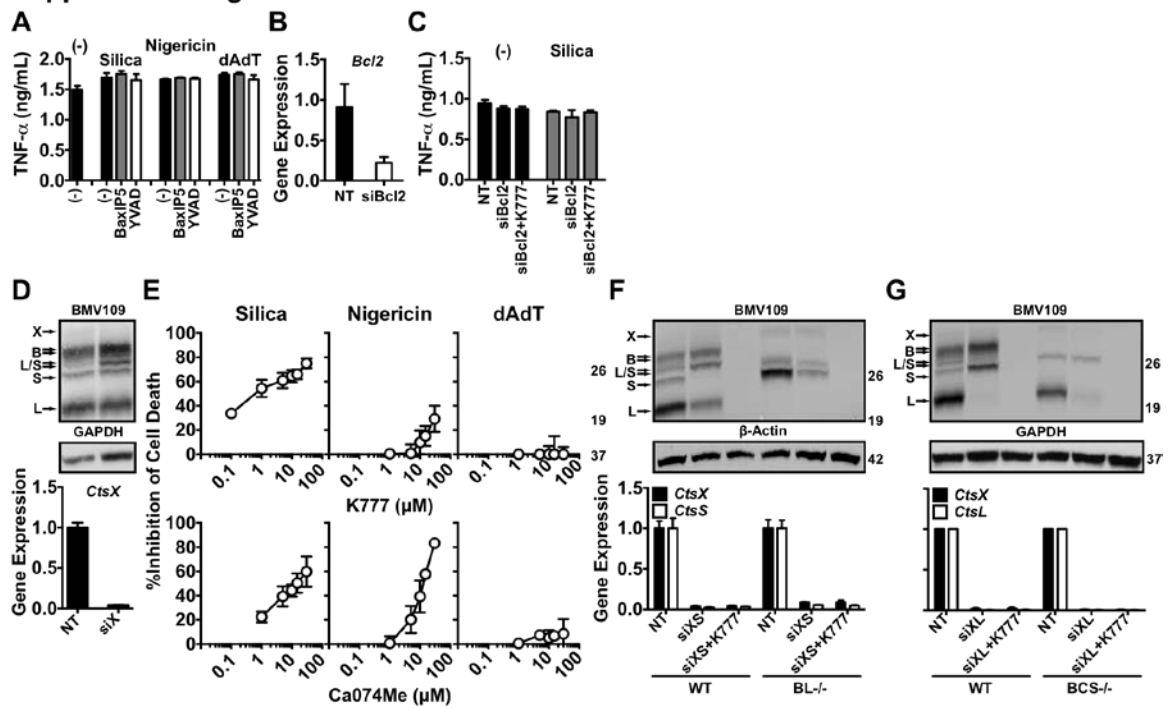
%Cell Death

TNF- $\alpha$  (ng/mL)

#### **Chapter IV, Supplemental Figure 2**

In all cases, PMs were primed with LPS. **(A)** PMs treated with media control (-), BaxIP5 (200  $\mu$ M) or YVAD (20  $\mu$ M) stimulated with media control (-), silica (50  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.3  $\mu$ g/mL). **(B)** PMs treated with NT control siRNA or siRNA targeting Bcl-2 (siBcl2) were primed with LPS and treated with media control or K777 (15  $\mu$ M) and Bcl-2 (*Bcl2*) expression analyzed by qPCR 3h after priming. **(C)** PMs treated with NT control siRNA or siRNA targeting Bcl-2 (siBcl-2) were primed with LPS, treated with media control or K777 (15  $\mu$ M) as indicated before stimulation with silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). **(D,F,G)** PMs treated with media control or K777 (15  $\mu$ M) where indicated, and after 3h of LPS priming **(top)** cathepsin activity labeled with BMV109; lysates were processed and  $\beta$ -Actin or GAPDH analyzed by western blot; arrows on the left indicate the bands marking activity of specific cathepsins and m.w. markers are on the right in kDa, or **(bottom)** gene expression (cathepsin X = *CtsX*, cathepsin S = *CtsS*, cathepsin L = *CtsL*) analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to non-targeting (NT) siRNA. **(D)** PMs treated with NT control siRNA or siRNA targeting cathepsin X (siX), or **(F)** PMs from WT mice irradiated and reconstituted with bone marrow neonatal WT or cathepsin B and L-deficient (BL<sup>-/-</sup>) mice treated with NT siRNA or siRNAs targeting cathepsins X and S (siXS), or **(G)** PMs from WT mice or cathepsin B, C, and S-deficient (BCS<sup>-/-</sup>) mice treated with NT siRNA or siRNAs targeting cathepsins X and L (siXL). **(E)** PMs were treated with media control or the indicated concentrations of K777 (top figures) or Ca074Me (bottom figures) and stimulated with silica (40  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.5  $\mu$ g/mL); data shows percent inhibition of cell death compared to media control. Error bars represent **(A)** range bars from technical duplicates representing (silica and dAdT) three or (nigericin) two independent experiments, **(B)** S.D. of technical triplicates representing two independent experiments, **(C)** S.D. of technical quadruplicates representing two independent experiments, **(D,F,G; bottom)** S.D. of technical triplicates representing three independent experiments or, **(E)** S.D. of means from four independent experiments (K777 from 0.1-15  $\mu$ M), S.D. of means from three independent experiments (Ca074Me from 0.1-15  $\mu$ M; K777 at 30  $\mu$ M), range bars from two independent experiments (Ca074Me at 30  $\mu$ M).

## Supplemental Figure 2



## **Particle-induced cell death is dependent on multiple redundant cathepsins.**

Since cathepsin inhibitors blocked particle-induced cell death, we next sought to test whether loss of individual cathepsins similarly inhibited this process. Deficiency of cathepsin B, L, C or S failed to significantly attenuate cell death in response to silica (**Fig. 3a**). K777 attenuated cell death induced by silica equally well in cells sufficient or deficient for any one of these cathepsins, indicating that K777 does not suppress this response by inhibiting only one of these cathepsins. To examine the role of cathepsin X, we silenced cathepsin X in PMs with siRNA. We confirmed a 90-95% knockdown of cathepsin X mRNA by qPCR and saw a similar loss of enzyme activity was observed with the fluorescent cathepsin activity-based probe BMV109(293) (**Supp. Fig. 2d**). After silencing cathepsin X, we stimulated these cells with silica, nigericin or dAdT. There was no significant difference between cathepsin X-sufficient or cathepsin X-deficient macrophages in the amount of cell death induced by either silica or dAdT (**Fig. 3b**). Similar to our previous observations for the nigericin-induced IL-1 $\beta$  response, deficiency of cathepsin X significantly reduced nigericin-induced cell death (406). Therefore, these data suggest that cathepsin X plays a previously unrecognized non-redundant role in nigericin-induced NLRP3-dependent cell death, while the individual cathepsins examined, including cathepsins B, L, C, S and X, are not essential for particle-induced inflammasome-independent cell death during



NLRP3 activation.

Next, we determined the concentrations of Ca074Me and K777 necessary to inhibit cell death induced by silica, nigericin or dAdT, and compared this to the cathepsin inhibition profile of these inhibitors that has been established in this cell type(406). This analysis revealed that at the concentrations of these agents that were needed to inhibit cell death, they were inhibiting multiple cathepsins (**Supp Fig. 2e**); these results were similar to what we recently observed for the concentrations needed to block IL-1 $\beta$  responses(406). Similar results were obtained when these cathepsin inhibitors were titrated with cathepsin B-deficient macrophages (**Fig. 3c,d**). In these experiments K777 again selectively inhibited particle-induced cell death, while Ca074Me inhibited nigericin-induced cell death as well. These data indicate that Ca074Me likely inhibited other cathepsins, not just cathepsin B, at the concentrations used to inhibit particle-induced cell death during NLRP3 activation in other studies(155, 163, 376, 405).

Finding that particle-induced cell death does not require any of the single cathepsins examined and that cathepsin inhibitors only blocked responses at doses that inhibited multiple cathepsins suggested that multiple cathepsins might play redundant roles in particle-induced cell death. To test this hypothesis, we bred mice deficient for both cathepsins B and L. Because mice doubly deficient in cathepsin B and L die within the first few weeks of life(379), we could not directly analyze PMs from these animals. To circumvent this limitation, we harvested bone marrow from these neonatal double knockout mice and used it to

reconstitute lethally irradiated adult WT mice. In these chimeric mice, cells of hematopoietic origin lack cathepsin B and L (B&L<sup>-/-</sup>) activity(406). For comparison, we also made chimeras with bone marrow from neonatal WT, cathepsin B<sup>-/-</sup> and cathepsin L<sup>-/-</sup> mice. We elicited PMs from these various chimeric mice and then stimulated as done above. Again, cathepsin B, L or B&L-deficient macrophages showed no attenuation of cell death induced by LLOMe, silica titration, nigericin or dAdT (**Fig. 3e,f**). K777 treatment still attenuated silica-induced cell death in the absence of these cathepsins (**Fig. 3g**), suggesting that K777's suppresses this response by inhibiting more cathepsins at once than just B and L.

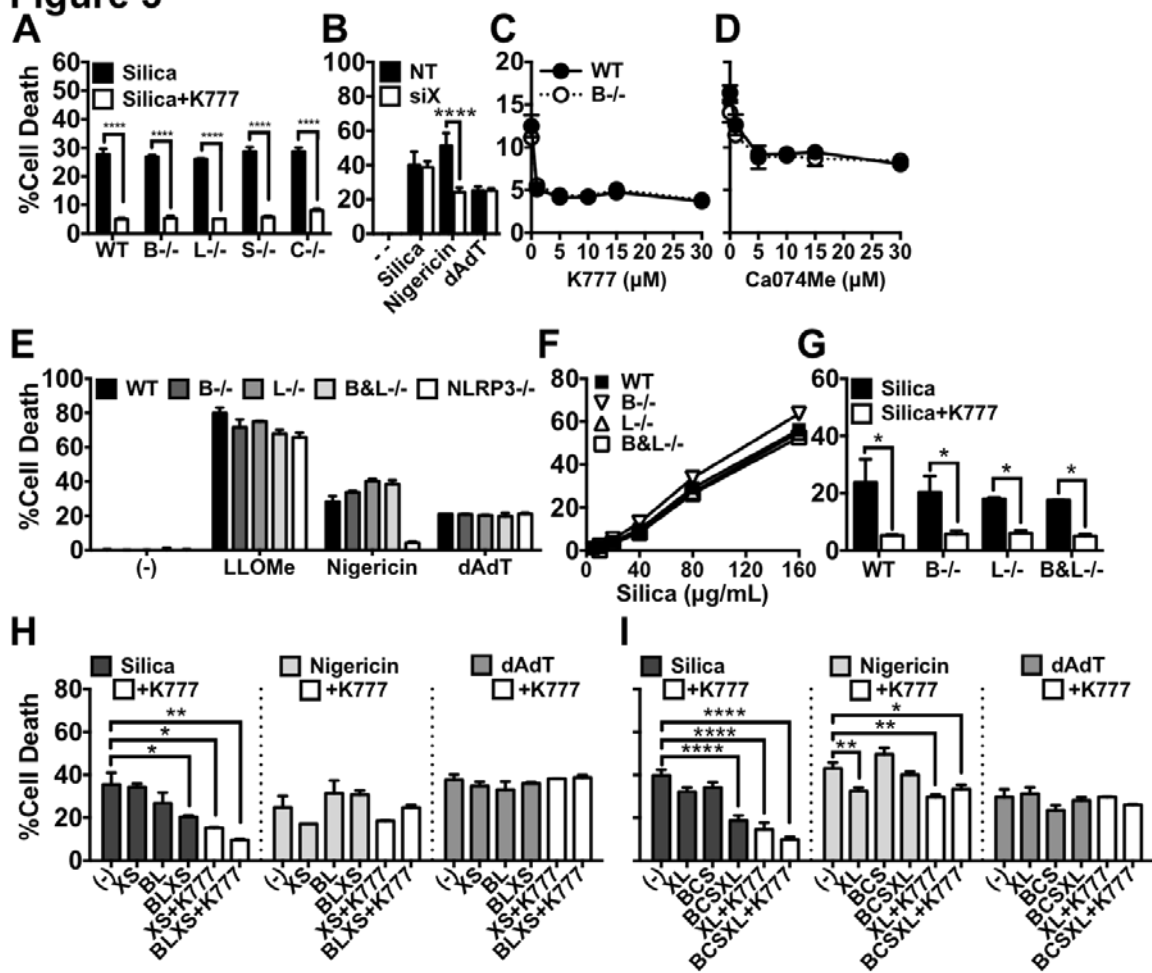
Since we have shown that K777 and Ca074Me inhibit more cathepsins than just B and L at concentrations required to block particle-induced cell death, we examined the particle-induced response of PMs deficient in up to four or five cathepsins. To do this, we examined the PMs deficient for both cathepsins B&L that were described above, and also elicited PMs from WT mice or mice deficient for the three cathepsins B, C & S (BCS<sup>-/-</sup>); these mice were viable with no obvious physical or behavioral pathology. In the WT or B&L<sup>-/-</sup> (BL) macrophages, we silenced cathepsins X and S with siRNA. Similarly, in WT or BCS<sup>-/-</sup> (BCS) macrophages, we silenced cathepsins X and L with siRNA. In both cases, treatment with siRNA resulted in a >90% reduction in the mRNA of each gene and a corresponding reduction in cathepsin activity, as assayed with BMV109 (**Supp. Fig. 2f,g**). Indeed, compound deficiency of cathepsins B, L, X and S

(BLXS) or cathepsins B, C, S, X and L (BCSXL) both resulted in a specific and significant attenuation of cell death induced by silica and not by nigericin or dAdT (**Fig. 3h,i**). Importantly, K777 was able to further attenuate particle-induced cell death in the BLXS and BCSXL macrophages, suggesting that other and/or residual cathepsins may still be involved. While cathepsin XL deficiency significantly reduced nigericin-induced cell death, the other combinations of cathepsin X-deficiency examined did not. This is likely because of the unique dependency of nigericin-induced responses on cathepsin X activity, and the fact that cathepsin X is upregulated in cathepsin S-deficient macrophages (406). Altogether, these data suggest that multiple cathepsins play redundant roles in particle-induced cell death during NLRP3 activation.

### **Chapter IV, Figure 3**

**Particle-induced cell death is initiated by multiple redundant cathepsins, and does not require cathepsin B, during NLRP3 activation.** In all cases, cells were primed with LPS. **(A)** WT PMs or those lacking cathepsins B (B<sup>-/-</sup>), L (L<sup>-/-</sup>), S (S<sup>-/-</sup>) or (C<sup>-/-</sup>) treated with silica (black bars; 40 µg/mL) or silica plus K777 (white bars; 15 µM). **(B)** PMs treated with non-targeting (NT) control siRNA or siRNA targeting cathepsin X (siX) before priming with LPS and stimulating with media control (-), silica (80 µg/mL), nigericin (1.5 µM) or dAdT (0.5 µg/mL). **(C,D)** WT (closed circles, solid line) or cathepsin B-deficient (open circles, dashed line) PMs treated a range of K777 or Ca074Me concentrations (1, 5, 10, 15 or 30 µM) before stimulation with silica (50 µg/mL). **(E-G)** Lethally irradiated WT mice were reconstituted with bone marrow from WT, cathepsin B (B<sup>-/-</sup>), L (-/-), B and L (B&L<sup>-/-</sup>), or NLRP3 (NLRP3<sup>-/-</sup>)—deficient donor mice. LPS-primed PMs elicited from these mice were stimulated with **(E)** media control (-), silica (40 µg/mL), LLOMe (0.75 mM), nigericin (2 µM), or dAdT (0.4 µg/mL), **(F)** a range of silica concentrations, **(G)** silica plus media (black bars) or silica plus K777 (white bars; 20 µM). **(H,I)** PMs elicited from **(H)** chimeric WT or knockout mice from “E-G”, or **(I)** WT mice or mice deficient in the three cathepsins B, C and S (BCS), were treated with non-targeting siRNA (WT) or siRNA targeting both **(H)** cathepsins X and S (“XS” when given to WT, or “BLXS” when given to B&L<sup>-/-</sup> labeled as “BL”), or **(I)** cathepsins X and L (“XL” when given to WT, or “BCSXL” when given to BCS), and subsequently LPS-primed and stimulated with media control (-), silica (80 µg/mL), nigericin (1.5 µM), or dAdT (0.5 µg/mL). XL, BCSXL, BL, BLXS macrophages were also treated with K777 (white bars; 15 µM). Error bars represent **(A,C,D,E-G)** range bars of technical duplicates, **(B)** S.D. of technical quadruplicates, **(H)** S.E. of means from either two independent experiments, or **(I)** five independent experiments (WT, XL, BCS, BCSXL) or three independent experiments (+K777). Statistical analysis was performed by **(A,B,G)** Two-way ANOVA and Sidak’s or **(H,I)** Dunnett’s multiple comparisons test; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, \*\*\*\**P*<0.0001. Data are representative of **(A,C,B-G,I)** at least three, or **(D,H)** two independent experiments.

**Figure 3**



## **Bcl-2 inhibition induces inflammasome & RIP3-independent cell death and IL-1 $\beta$ secretion.**

Finding that the Bcl-2 family regulates particle-induced cell death and IL-1 $\beta$  secretion during NLRP3 activation, we sought to better understand this connection. MOMP is the major mechanism described for cell death mediated by the Bcl-2 family. MOMP results in the generation of mitochondrial reactive oxygen species (ROS) production and the eventual loss of MMP(200). Importantly, common terminal events proposed to activate NLRP3 involve a combination of MOMP and mitochondrial ROS(173), which lead to the liberation of oxidized mitochondrial DNA(174, 218) or cardiolipin(220) into the cytosol to be sensed by NLRP3(172). This pathway could potentially lead to cell death upstream of NLRP3 activation, which would be consistent with the cathepsin-dependent mechanism described above. Therefore, we examined whether pharmacologically promoting the activity of pro-death Bcl-2 family members following LPS-priming can initiate NLRP3-dependent or independent cell death and/or IL-1 $\beta$  secretion.

First, we stimulated WT or NLRP3<sup>-/-</sup> PMs with a Bcl-2-specific inhibitor (ABT199)(412, 413), and compared this response to responses induced by silica, nigericin, or dAdT. As an inflammasome-independent control, we also tested a Smac mimetic drug (AT406)(253) since drugs in this class have been shown to induce RIP3-dependent cell death and IL-1 $\beta$  secretion(253). ABT199 and the

other stimuli tested induced both IL-1 $\beta$  secretion and cell death (**Fig 4a**). Although we found above that Bcl-2 knockdown did not result in IL-1 $\beta$  secretion and cell death with LPS priming alone, it is likely that the sudden or non-specific inhibition of the anti-apoptotic Bcl-2 family with ABT199 caused more of a disruption in the balance with pro-apoptotic Bcl-2 family members. As expected, both of these outcomes were NLRP3-independent for AT406 and dAdT-induced responses. Again, IL-1 $\beta$  secretion induced by silica and nigericin depended on NLRP3, while only nigericin-induced cell death required NLRP3. Surprisingly, ABT199-induced IL-1 $\beta$  secretion and cell death was NLRP3-independent, suggesting that NLRP3 activation is not downstream of the Bcl-2 family.

Next, we tested whether other inflammasomes are involved in ABT199-induced cell death and IL-1 $\beta$  secretion, and whether cathepsin inhibition can block these processes. We stimulated PMs from either WT, ASC<sup>-/-</sup> or caspase1&11<sup>-/-</sup> mice with ABT199, AT406, or dAdT. As expected, IL-1 $\beta$  secretion and cell death induced by AT406 were largely inflammasome-independent, while these responses induced by dAdT were inflammasome-dependent (**Fig. 4b**). Again, ABT199 induced these responses independently of inflammasomes. Interestingly, K777 partially reduced cell death induced by ABT199, which may suggest that cathepsins could be also downstream of ABT199 possibly from enhancement of lysosomal disruption. Moreover, K777 suppressed ABT199 and AT406-induced IL-1 $\beta$  secretion, and to some extent reduced IL-1 $\beta$  secretion induced by dAdT as well. This is consistent with our

most recent study showing that cathepsins are important for pro-IL-1 $\beta$  synthesis (406). To examine this more directly, we looked at the effects of Ca074Me and K777 on responses induced by ABT199 and AT406. After 6h of stimulation, lysates were analyzed by western blot. Indeed, K777 and Ca074Me suppressed IL-1 $\beta$  secretion induced by these inflammasome/NLRP3-independent stimuli primarily by reducing pro-IL-1 $\beta$  synthesis (**Fig. 4c**).

Finally, given that ABT199 may be inducing MOMP and the release of Smac from the mitochondria, we also examined whether cell death and IL-1 $\beta$  secretion induced by ABT199 may be dependent on RIP3, as has been shown for other Smac mimetics(414). We stimulated PMs from either WT or RIP3 $^{-/-}$  mice with silica, ABT199, AT406 or dAdT. Importantly, silica-induced cell death and IL-1 $\beta$  secretion was RIP3-independent (**Fig. 4d**). As expected, this was also the case for dAdT. Conversely, AT406-induced cell death and IL-1 $\beta$  secretion depended largely on RIP3, confirming findings from a recent study(414). Surprisingly, ABT199-induced cell death and IL-1 $\beta$  secretion were RIP3-independent. Moreover, similar results were observed across a wide range of stimuli concentrations (**Supp. Fig. 3a**). Again, TNF- $\alpha$  is not significantly affected in all experiments above (**Supp. Fig. 3a-d**). Therefore, ABT199 initiates a pathway of concomitant cell death and IL-1 $\beta$  secretion that does not depend on any of the known IL-1 $\beta$  activating pathways. Moreover, these data indicate that by blocking pro-IL-1 $\beta$  synthesis, cathepsin inhibition can suppress, not only cell

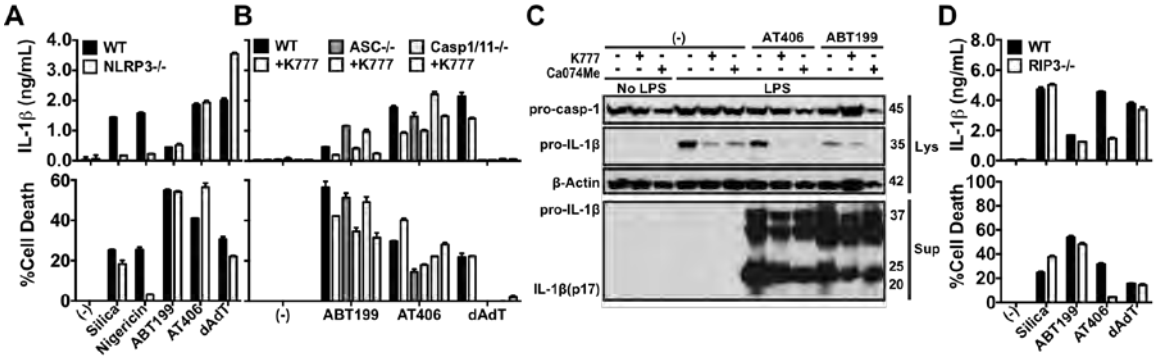


death by particulate stimuli, but also, IL-1 $\beta$  secretion by diverse stimuli regardless of the pathways involved.

#### **Chapter IV, Figure 4**

**Bcl-2 inhibition induces inflammasome and RIP3-independent cell death and IL-1 $\beta$  secretion, but cathepsin inhibition still suppresses IL-1 $\beta$  secretion.** In all cases, except part of “C”, PMs were primed with LPS. **(A)** PMs from WT or NLRP3<sup>-/-</sup> mice were stimulated with silica (40  $\mu$ g/mL), nigericin (2  $\mu$ M), ABT199 (5  $\mu$ M), AT406 (7.5  $\mu$ M), or dAdT (0.4  $\mu$ g/mL). **(B)** PMs from WT, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp1<sup>-/-</sup>) mice were treated with media control or K777 (30  $\mu$ M) before stimulation with ABT199 (5  $\mu$ M), AT406 (10  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). **(C)** PMs were primed (LPS) or not primed (No LPS) prior to treatment with K777 (15  $\mu$ M) or Ca074Me (15  $\mu$ M) then stimulated with AT406 (15  $\mu$ M) or ABT199 (15  $\mu$ M); lysates and supernatants were processed and pro-caspase-1, pro-IL-1 $\beta$ , mature IL-1 $\beta$  (p17), and  $\beta$ -actin analyzed by western blot; m.w. markers are on the right in kDa. **(D)** PMs from WT or RIP3<sup>-/-</sup> mice were stimulated with silica (50  $\mu$ g/mL), ABT199 (5  $\mu$ M), AT406 (10  $\mu$ M) or dAdT (0.3  $\mu$ g/mL). Error bars represent **(A,B)** range bars of technical duplicates or **(D)** S.D. of technical triplicates. Data are representative of **(A for ABT199; C)** two or **(A,B,D)** three independent experiments.

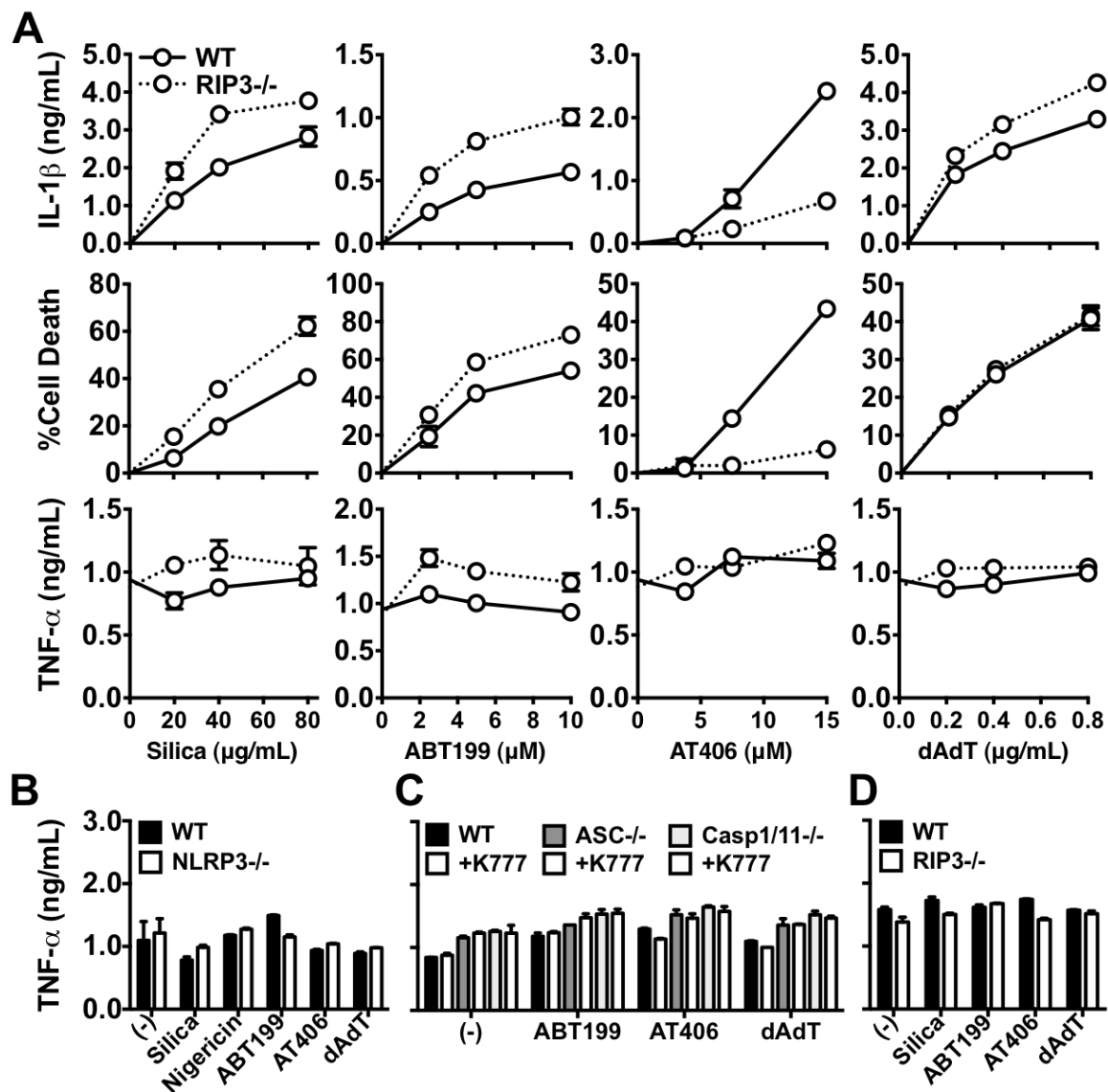
**Figure 4**



### **Chapter IV, Supplemental Figure 3**

In all cases, PMs were primed with LPS. **(A)** PMs from WT or RIP3<sup>-/-</sup> mice were stimulated with titrations of silica, ABT199, AT406 or dAdT. **(B)** PMs from WT or NLRP3<sup>-/-</sup> mice were stimulated with silica (40 µg/mL), nigericin (2 µM), ABT199 (5 µM), AT406 (7.5 µM), or dAdT (0.4 µg/mL). **(C)** PMs from WT, ASC<sup>-/-</sup> or Caspase-1<sup>-/-</sup> (Casp1<sup>-/-</sup>) mice were treated with media control or K777 (30 µM) before stimulation with ABT199 (5 µM), AT406 (10 µM) or dAdT (0.5 µg/mL). **(D)** PMs from WT or RIP3<sup>-/-</sup> mice were stimulated with silica (50 µg/mL), ABT199 (5 µM), AT406 (10 µM) or dAdT (0.3 µg/mL). Error bars represent **(A-C)** range bars of technical duplicates or **(D)** S.D. of technical triplicates. Data are representative of **(B for ABT199)** two or **(A-D)** three independent experiments.

# Supplemental Figure 3



## **Particle-induced IL-1 $\beta$ secretion is antagonized by mitochondrial disruptive cell death.**

MOMP and the release of mitochondrial DAMPs into the cytosol is thought to be a terminal event leading to NLRP3 inflammasome activation(172-174, 218, 220). Despite our data suggesting that the Bcl-2 family modulates particle-induced inflammasome-dependent IL-1 $\beta$  secretion, direct Bcl-2 inhibition induced IL-1 $\beta$  secretion via a pathway that depended on neither inflammasomes nor RIP3. Therefore, we examined whether Bcl-2 inhibition might positively (or negatively) modulate NLRP3-dependent IL-1 $\beta$  secretion induced by sterile particles.

First, we stimulated PMs with ABT199 in the presence or absence of particulate NLRP3 activators (silica, CC, MSU), soluble NLRP3 activators (ATP, nigericin), or the AIM2 activator dAdT. Surprisingly, ABT-199 did not enhance IL-1 $\beta$  secretion by any of these stimuli. Instead, ABT199 strongly enhanced cell death induced by particulates and dAdT (**Fig. 5a**). This is in line with the possibility that ABT199 causes a more pronounced disturbance in the balance between pro- and anti-apoptotic Bcl-2 family members. However, ABT199 did not enhance cell death induced by ATP or nigericin, likely because they had already induced a high level of cell death. Next, we stimulated PMs with an inhibitor of pro-survival Bcl-2, Bcl-xL and Bcl-w (ABT263)(415), alone or in combination with silica, nigericin, or dAdT. Like ABT199, ABT263 alone induced IL-1 $\beta$  secretion and cell death (**Supp. Fig. 4a**). However, similar to ABT199, ABT263 strongly

enhanced cell death induced by silica, and also by nigericin and dAdT, but failed to enhance IL-1 $\beta$  secretion. In the experiments above, TNF- $\alpha$  was not significantly affected (**Supp. Fig 4b, c**). Together, these data indicate that Bcl-2 inhibitors do not act cooperatively with particle-induced IL-1 $\beta$  secretion, but instead, they strongly promote cell death and may even interfere with pathways promoting IL-1 $\beta$  secretion.

Our data showing that Bcl-2 inhibition, which should induce MOMP, did not promote particle-induced IL-1 $\beta$  secretion (and that it induced IL-1 $\beta$  secretion in NLRP3-/- macrophages) seems inconsistent with the hypothesis that MOMP induces NLRP3-dependent IL-1 $\beta$  secretion. However, the Bcl-2 family has been shown to influence the integrity of both the mitochondrial and the lysosomal membranes(172). Therefore, which organelle Bcl-2 inhibition is affecting may be important for interpreting these results. In fact, a recent study reported that robust LMD induced by LLOMe also promotes cell death and antagonizes NLRP3-mediated IL-1 $\beta$  secretion(155). If LMD is the mechanism whereby ABT199, LLOMe and silica promote a form of inflammasome-independent cell death that antagonizes IL-1 $\beta$  secretion, then inhibiting cell death induced by these stimuli may paradoxically promote sustained IL-1 $\beta$  secretion. Therefore, we compared the effects of Bcl-2 inhibition, LLOMe, and silica on lysosomal and mitochondrial integrity.

To examine the effects of stimuli on lysosomes and mitochondria in real-time, PMs were incubated with either the lysosomotropic dye acridine orange

(AO) or the MMP indicator tetramethylrhodamine (TMRM), primed with LPS, then stimulated with LLOMe, silica, ABT199 or dAdT; changes in fluorescence were measured at short intervals (**Fig 5b**). Deviations in baseline fluorescence of the resultant traces were interpreted using positive controls; AO green fluorescence increased upon LMD induced with LLOMe or Bafilomycin A (BafA) (**Supp. Fig. 4d**) and TMRM red fluorescence increased upon depolarization of MMP with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and decreased upon hyperpolarization with oligomycin A (OMA) (**Supp. Fig. 4e**). It should be noted that, after hyperpolarization, OMA causes the depolarization and collapse of MMP. Interestingly, LLOMe and ABT199 both caused LMD (**Fig 5b; top**) and MMP depolarization (**Fig 5b; bottom**). However, LLOMe induced both of these processes early and robustly, while ABT199 caused MMP depolarization first and LMD later. ABT199 eventually caused LMD, which is consistent with our finding that K777 partially reduced the resultant cell death, as shown in Fig. 4b, and indicates that cathepsins are likely involved in cell death downstream of ABT199-induced LMD. Indeed, reagents that influence MMP, like the electron transport chain (ETC) uncouplers used above, FCCP and OMA, as well as antimycin A (AntA), also disrupted lysosomal pH gradients (**Supp. Fig. 4d,f**). As expected, dAdT did not induce either LMD or MMP depolarization, suggesting that neither of these events are required for initiating inflammasome-dependent pyroptosis. Importantly, silica caused a mild, gradual disruption of lysosomes without



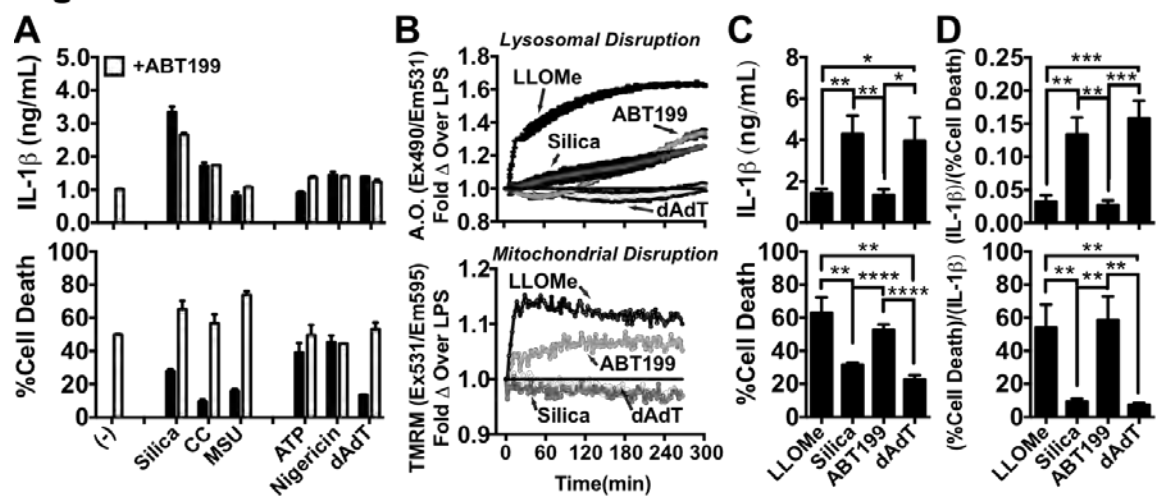
affecting MMP, suggesting that silica-induced NLRP3 activation does not involve robust MOMP.

To compare the proportion of cell death induced by these stimuli to the resultant IL-1 $\beta$  secretion, we stimulated PMs with the reagents above. Importantly, stimulation with LLOMe or ABT199, which both caused early and robust disruption of MMP, both generated significantly more cell death and less IL-1 $\beta$  secretion compared to silica or dAdT (**Fig. 5c**). These results were recapitulated when expressed either as the amount of IL-1 $\beta$  secreted per unit of cell death, which was significantly lower for LLOMe or ABT199, or as the amount of cell death per unit of IL-1 $\beta$  secreted, which was significantly higher for LLOMe and ABT199 (**Fig. 5d**). Although MOMP has been proposed as a terminal event activating NLRP3, these data suggest that disruption of the mitochondria, and not necessarily LMD, promotes a pathway of cell death that actually antagonizes NLRP3-mediated IL-1 $\beta$  secretion.

#### **Chapter IV, Figure 5**

**Particle-induced cell death and IL-1 $\beta$  secretion do not require mitochondrial disruption, which actually antagonizes IL-1 $\beta$  secretion.** In all cases, PMs were primed with LPS. **(A)** PMs stimulated with media control (-), ABT199 (white bars; 5  $\mu$ M) or silica (40  $\mu$ g/mL), CC (100  $\mu$ g/mL), MSU (200  $\mu$ g/mL), ATP (2 mM), nigericin (2  $\mu$ M) or dAdT (0.4  $\mu$ g/mL) combined with media control (black bars) or ABT199 (white bars). **(B)** PMs stained with A.O. (top; increasing values = disruption of lysosomes/pH gradient) or TMRM (bottom; increasing values = disruption of mitochondrial membrane potential) were stimulated with LLOMe (1 mM), silica (50  $\mu$ g/mL), ABT199 (5  $\mu$ M), or dAdT (0.4  $\mu$ g/mL); fluorescence traces monitored by plate reader at short intervals and plotted as fold change over LPS. **(C)** PMs stimulated with LLOMe (0.75-1 mM), Silica (40-50  $\mu$ g/mL), ABT199 (5  $\mu$ M), or dAdT (0.4-0.5  $\mu$ g/mL). **(D)** Data from “C” are plotted as (top) “IL-1 $\beta$  (ng/mL) per % Cell Death”, or (bottom) “% Cell Death per IL-1 $\beta$  (ng/mL)”. Error bars represent **(A,B)** range bars of technical duplicates, or **(C,D)** S.E. of means from eight independent experiments. Statistical analysis was performed by **(C,D)** Two-tailed Student’s t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. All data are representative of at least three independent experiments.

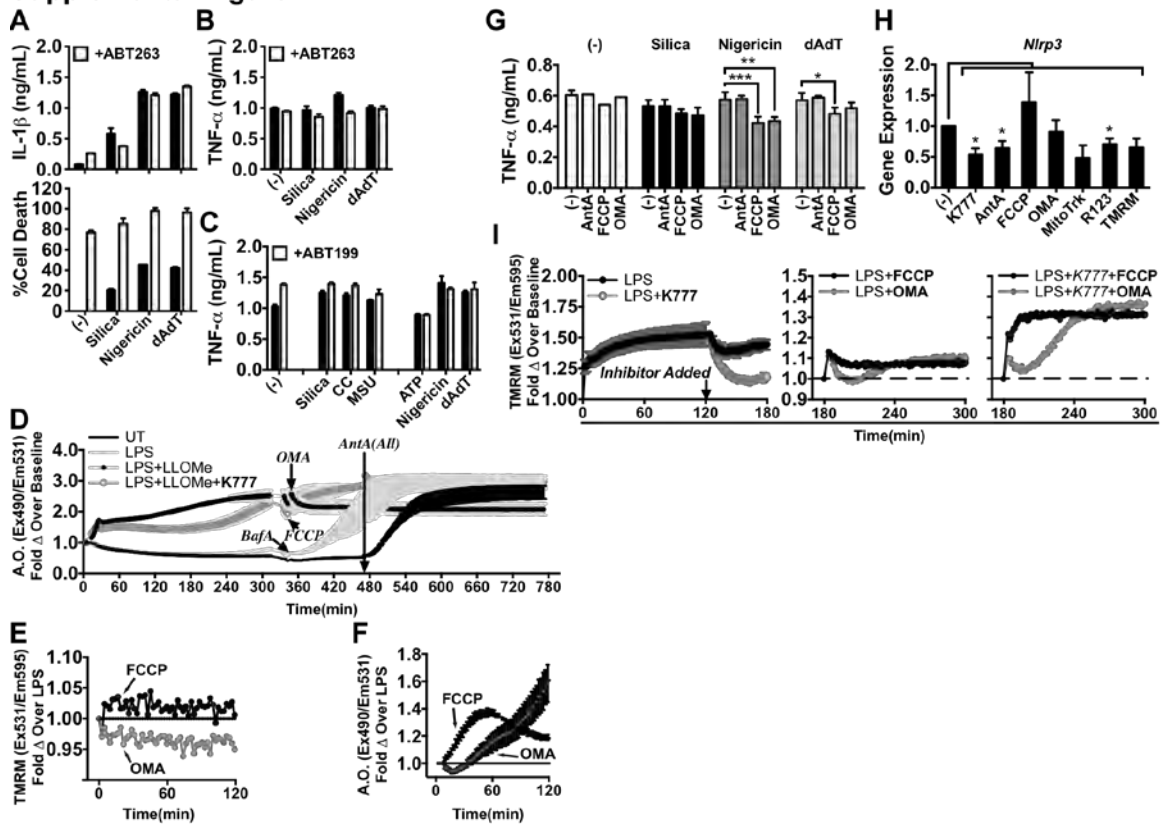
**Figure 5**



#### **Chapter IV, Supplemental Figure 4**

**(A,B)** LPS-primed PMs stimulated with media control (-), ABT263 (white bars; 15  $\mu$ M) or silica (40  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.4  $\mu$ g/mL) combined with media control (black bars) or ABT263 (white bars). **(C)** LPS-primed PMs stimulated with media control (-), ABT199 (white bars; 5  $\mu$ M) or silica (40  $\mu$ g/mL), CC (100  $\mu$ g/mL), MSU (200  $\mu$ g/mL), ATP (2 mM), nigericin (2  $\mu$ M) or dAdT (0.4  $\mu$ g/mL) combined with media control (black bars) or ABT199 (white bars). **(D)** PMs stained with A.O. (increasing values = disruption of lysosomes/pH gradient) were treated with media control (untreated = UT) or primed with LPS before treatment with media control or K777 (15  $\mu$ M) prior to stimulation with media control or LLOMe (1 mM); fluorescence traces monitored by plate reader at short intervals and plotted as fold change over baseline; after ~6h, samples from the indicated traces were treated with media control (UT), Bafilomycin A (BafA; 50 nM), FCCP (2  $\mu$ M) or OMA (5  $\mu$ g/mL); and after ~8h, samples from all traces treated with AntA (All; 5  $\mu$ M). **(E,F)** PMs stained with **(E)** TMRM (increasing values = disruption of mitochondrial membrane potential), or **(F)** A.O., were primed with LPS, then treated with FCCP (2  $\mu$ M) or OMA (5  $\mu$ g/mL); fluorescence monitored as above and plotted as fold change over LPS. **(G)** LPS-primed PMs treated with media control (-), AntA (5  $\mu$ M), FCCP (10  $\mu$ M) or OMA (50 nM) and stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). **(H)** PMs were primed with LPS for 2h, and treated with media control (-), K777 (15  $\mu$ M), AntA (5  $\mu$ M), FCCP (10  $\mu$ M), OMA (50 nM), MitoTrk (3  $\mu$ M), R123 (25  $\mu$ M), or TMRM (5  $\mu$ M) for an additional 4h; NLRP3 (*Nlrp3*) expression analyzed by qPCR and plotted relative to media control (-). **(I)** PMs stained with TMRM, as above, were primed with LPS for 2h, then (left) treated with media control or K777 (15  $\mu$ M) for 1h, then (middle) LPS or (right) LPS+K777 treated samples were treated again with either FCCP (2  $\mu$ M) or OMA (5  $\mu$ g/mL) for another 2h; fluorescence monitored as above. Error bars represent **(A-D,F,G,I)** range bars of technical duplicates, **(H)** S.E. of means from three independent experiments. Statistical analysis was performed by **(G)** Two-way ANOVA and Dunnett's multiple comparisons test, or **(H)** Two-tailed Student's t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001. Data are representative of **(I)** two or **(A-H)** at least three independent experiments.

**Supplemental Figure 4**



### **Particle-induced IL-1 $\beta$ secretion and pro-IL-1 $\beta$ synthesis require an intact MMP.**

Our findings suggest that silica-induced cell death and NLRP3 activation are downstream of LMD, and not MOMP. As shown with LLOMe, robust LMD is likely required to induce cell death through downstream MOMP. Instead of activating NLRP3, LLOMe or ABT199-induced MOMP seems promote a mode of cell death that antagonizes or prevents NLRP3 activation. By inducing MOMP well before it causes LMD, ABT199 appear to preclude LMD-mediated NLRP3 activation altogether. Indeed, it has been shown that complete loss of MMP antagonizes NLRP3 activation induced by several RNA viruses(224). Therefore, we sought to understand whether MMP is also required for particle-induced NLRP3 activation.

We primed PMs with LPS before treating them with media control, K777, or electron transport chain (ETC) uncoupling agents, and then stimulated with silica, nigericin or dAdT. We found that ETC uncoupling with FCCP, OMA or AntA selectively antagonized IL-1 $\beta$  secretion induced by silica, but had no significant effect on cell death (Fig. 6a). FCCP caused the most pronounced reduction in IL-1 $\beta$  secretion, which was still selective for silica compared to its more minor effect on nigericin and dAdT-induced IL-1 $\beta$  secretion. There was no significant effect on TNF- $\alpha$  (**Supp. Fig. 4g**). In addition, we also examined the effects of these inhibitors on pro-IL-1 $\beta$  synthesis in LPS-primed PMs. As shown in our most recent study (406), K777 suppressed pro-IL-1 $\beta$  synthesis, which was

evident at the level of the transcript (**Fig. 6b**) and protein (**Fig. 6c**). Surprisingly, ETC uncouplers or high concentrations of MMP-dependent dyes (MitoTracker Red CMXRos (MitoTrk), Rhodamine 123 (R123) or TMRM) also suppressed pro-IL-1 $\beta$  transcript levels/protein synthesis (**Fig 6b,c**) and there was a similar but less pronounced suppression of NLRP3 transcript (**Supp. Fig. 4h**). Interestingly, ABT199, which also induced the rapid loss of MMP, as shown in Fig. 5b, also reduced pro-IL-1 $\beta$  levels, as shown above in Fig. 4c. The fact that these effects were selective for silica compared to nigericin and dAdT may reflect that pro-IL-1 $\beta$  levels are not limiting for their response under these conditions. Alternatively, an intact MMP may be more important for NLRP3 activation induced by particles/LMD.

Our finding that both cathepsin inhibitors and ETC uncouplers inhibit pro-IL-1 $\beta$  and NLRP3 synthesis suggests that lysosomes and mitochondria play important roles in macrophage priming. To further test this, we incubated PMs with either AO or TMRM, primed them with LPS, but measured fluorescence levels during LPS priming instead of during the subsequent stimulation with particles or other stimuli. LPS priming alone caused a low level of gradual LMD and mitochondrial depolarization (**Fig 6d**), but to a lesser extent than that caused by LLOMe or ABT199, shown in Fig. 5b. Importantly, K777 did not depolarize, but instead, repolarized the MMP (**Supp. Fig. 4i**). This indicates that, instead of destroying MMP, K777 counteracts LPS-induced depolarization. Deviation in the TMRM fluorescence trace was interpreted according to controls, as done before

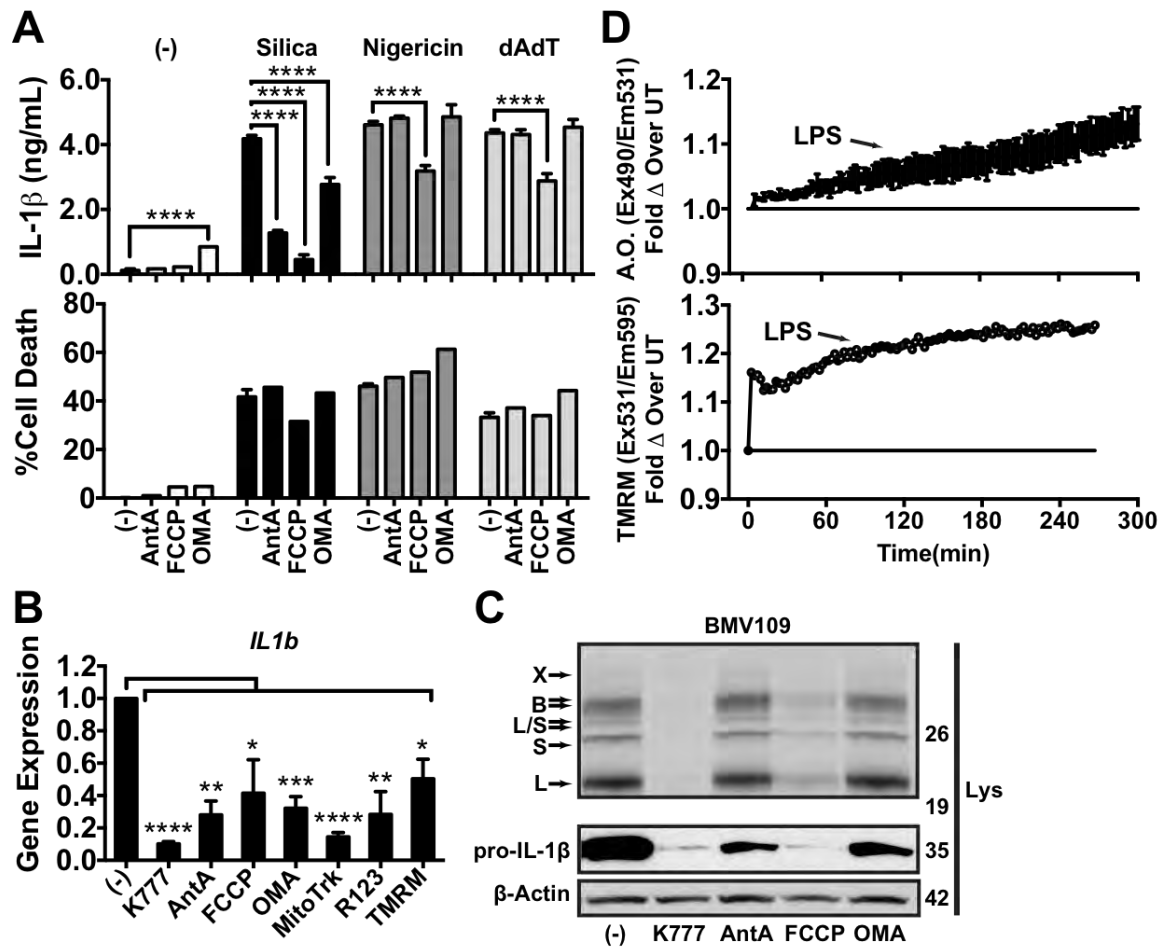
(**Supp. Fig. 4e**). However, subsequent to K777 treatment during priming, the degree of depolarization induced by FCCP was greater than that following LPS priming alone (**Supp. Fig 4i**). This reaffirmed our conclusion that K777 had repolarized the MMP. Moreover, FCCP also inhibited cathepsin activity (as measured in living cells using the activity-based probe BMV109), probably by disrupting the lysosomal pH gradient (**Fig. 6c**). However, AntA and OMA suppressed pro-IL-1 $\beta$  synthesis without affecting cathepsin activity. This indicated that ETC uncouplers, independently of affecting cathepsin activity, can suppress pro-IL-1 $\beta$  synthesis. In summary, inhibition of cathepsins is sufficient, but not necessary, to inhibit pro-IL-1 $\beta$  synthesis, which requires an intact MMP that is uniquely important for particle-induced NLRP3 activation, but not for cathepsin-dependent cell death.



#### **Chapter IV, Figure 6**

**Particle-induced IL-1 $\beta$  secretion and pro-IL-1 $\beta$  synthesis, and not cell death, requires an intact MMP.** In all cases, except part of “D”, PMs were primed with LPS. **(A)** PMs treated with media control (-), AntA (5  $\mu$ M), FCCP (10  $\mu$ M) or OMA (50 nM) and stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). **(B,C)** PMs were primed with LPS for 2h, and treated with media control (-), K777 (15  $\mu$ M), AntA (5  $\mu$ M), FCCP (10  $\mu$ M), OMA (50 nM), MitoTrk (3  $\mu$ M), R123 (25  $\mu$ M), or TMRM (5  $\mu$ M) for an additional 4h and **(B)** IL-1 $\beta$  (*IL1b*) expression analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to media control (-), or **(C)** PMs were probed for cathepsin activity with BMV109; lysates processed and analyzed by SDS-PAGE, phosphor imaging of specific cathepsin activity (arrows on left), and western blotting of pro-IL-1 $\beta$  and  $\beta$ -actin; m.w. markers are on the right in kDa. **(D)** PMs stained with A.O. (top; increasing values = disruption of lysosomes/pH gradient) or TMRM (bottom; increasing values = disruption of mitochondrial membrane potential), then treated with media control (UT) or primed with LPS and fluorescence traces monitored by plate reader at short intervals; traces indicate fold change over UT samples. Error bars represent **(A)** S.D. of technical quadruplicates (IL-1 $\beta$  for silica, nigericin, dAdT), range bars of technical duplicates (IL-1 $\beta$  for AntA, FCCP, OMA; %Cell Death for silica, nigericin, dAdT), or technical singlets (%Cell Death for AntA, FCCP, OMA), **(B)** S.E. of means from three independent experiments or, **(D)** range bars of technical duplicates. Statistical analysis was performed by **(A)** Two-way ANOVA and Dunnett's multiple comparisons test, or **(B)** Two-tailed Student's t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001. Data are representative of **(D, bottom)** two or **(A-C)** at least three independent experiments.

**Figure 6**



## **Particle-induced sterile inflammation is suppressed by cathepsin inhibition *in vivo*.**

The IL-1-dependent sterile inflammatory response to particles has recently been shown to be largely caspase-1-independent *in vivo*(10, 12, 121), despite caspase-1 being absolutely required for IL-1 $\beta$  secretion *in vitro*(10, 12, 144). In fact, a recent study published by our group demonstrates a role for cathepsin C, showing that combined caspase-1 and cathepsin C-deficiency further suppresses this response(121). Moreover, our most recent study demonstrated that a bioavailable cathepsin inhibitor, K777, which has been shown to inhibit cathepsins B, L, S, X, V, K and C, selectively blocks both pro-IL-1 $\beta$  synthesis and NLRP3-activation induced by sterile particles(383, 406). Given our finding that K777 also inhibits caspase-1-independent particle-induced cell death, caspase-1-independent pro-IL-1 $\beta$  synthesis, and NLRP3 activation, it seemed likely that K777 could suppress caspase-1-independent particle-induced inflammation *in vivo*.

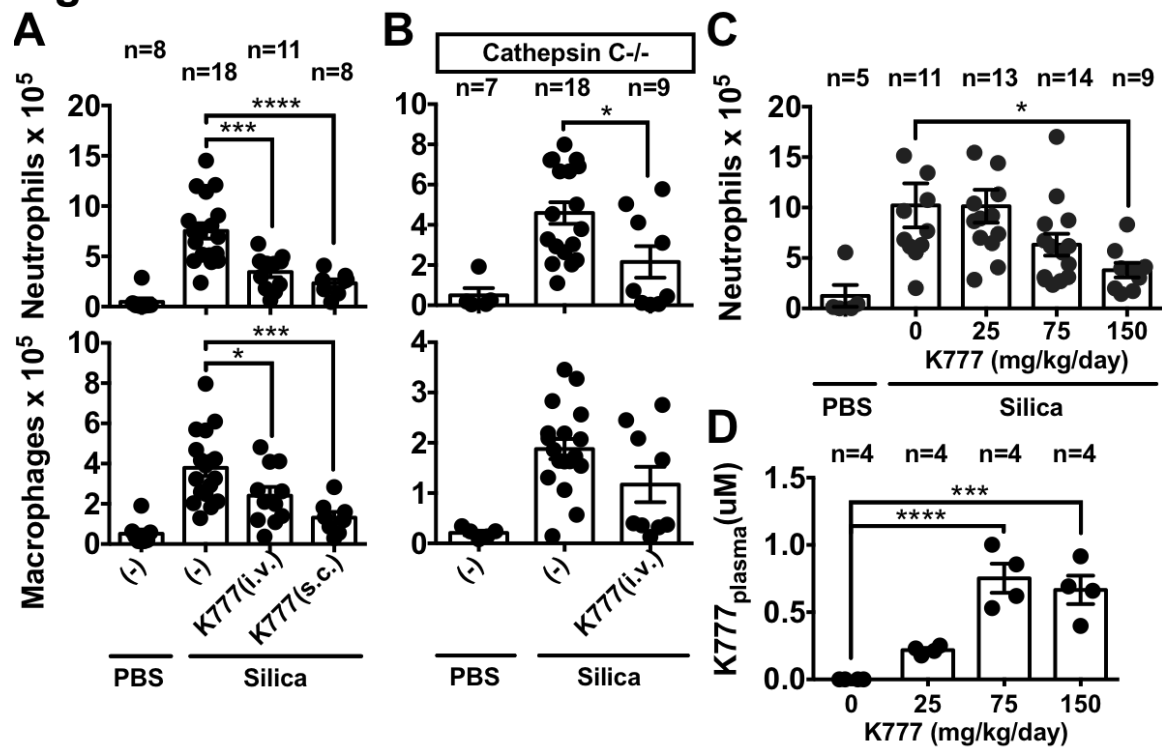
We examined the effect of K777 treatment in a model of IL-1-dependent silica-induced acute peritonitis(121). Indeed, pretreatment with K777, either i.v. or s.c., strongly suppressed both neutrophil and macrophage recruitment in response to silica (**Fig. 7a**). Given the role implicated for cathepsin C in this response(121), we performed the same experiment above with cathepsin C-deficient mice and found again that K777 strongly suppresses this response (**Fig.**

**7b).** This suggests that, even though K777 has been reported to inhibit cathepsin C(383), a substantial proportion of its effect *in vivo* is independent of cathepsin C. We also sought to determine the target concentration of K777 in the plasma necessary to achieve efficacy. To do this, we treated mice via s.c. infusion of K777 at different doses by loading it into osmotic Alzet pumps, which we surgically implanted in the backs of mice for 1 wk. Then, we examined the i.p. silica response as described above. Again, K777 markedly attenuated this response in a dose-dependent manner (up to ~70% reduction) (**Fig. 7c**), and the effective concentration of K777 in the plasma was ~0.75uM (**Fig. 7d**). Together, these data demonstrate in an *in vivo* model that a cathepsin inhibitor, K777, can suppress the IL-1-dependent acute inflammatory response to sterile particles independently of cathepsin C.

#### **Chapter IV, Figure 7**

**Silica-induced acute peritonitis is suppressed by systemic treatment with a cathepsin inhibitor, K777, independently of cathepsin C. (A-C)** Quantification of IL-1-dependent cellular exudates by flow cytometric analysis following i.p. injection of 100 uL PBS or silica (0.2 mg) for 4h. Effect of single-bolus excipient (-) or K777 treatment (62.5 mg/kg i.v., 125 mg/kg s.c.) 1h prior to silica injection is shown in **(A)** WT mice, or **(B)** Cathepsin C<sup>-/-</sup> mice. **(C)** Effect of 1wk s.c. infusion of excipient (0) or different doses of K777 (mg/kg/day) prior to silica injection in WT mice. **(D)** Concentration of K777 in the plasma after 1 wk of treatment with the doses described in “C”. Error bars represent **(A-D)** S.E. of means from the indicated number (n) of mice. Statistical analysis was performed by **(A,B)** Two-tailed Student’s t-test, or **(C,D)** One-way ANOVA and Dunnett’s multiple comparisons test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

**Figure 7**



## **Discussion**

In mouse models, it has been shown that diseases like gout, osteoarthritis, and irritant-induced muscle inflammation are driven by IL-1 $\beta$ , but they do not depend on caspase-1(407-410). Importantly, systemic treatment with the bioavailable cathepsin inhibitor, K777, suppressed the IL-1-dependent *in vivo* inflammatory response to silica. The degree of suppression observed with K777 was more dramatic than that observed in caspase-1-deficient mice examined in most previous studies(10, 12, 121, 130), and this effect was largely independent of cathepsin C. Therefore, it is likely that inhibition of caspase-1-independent pro-IL-1 $\beta$  synthesis and cathepsin C, as well as caspase-1-dependent NLRP3-mediated IL-1 $\beta$  secretion, each contributed to K777's attenuation of this response.

Importantly, independently of caspase-1, pro-IL-1 $\beta$  may be a substrate for an unidentified inflammasome-independent IL-1 $\beta$  activation pathway in intact cells that occurs only *in vivo*. Here, we demonstrated that Ca074Me and K777 can suppress the secretion of IL-1 $\beta$  induced by two different inflammasome-independent stimuli by reducing pro-IL-1 $\beta$  synthesis. Therefore, cathepsin inhibition likely has the potential to be broadly effective against a variety of different inflammasome-dependent and independent IL-1 $\beta$  activating mechanisms that may contribute to *in vivo* responses. Moreover, during an inflammasome-independent form of lytic cell death, pro-IL-1 $\beta$  could be a substrate for cathepsin C-dependent neutrophil proteases upon release into the

extracellular milieu(121, 278-281), along with constitutively active IL-1 $\alpha$  and DAMPs like HMGB1. Indeed, one research group has shown that lysosome disrupting agents, like LLOMe, or alum particles induce inflammasome-independent cell death(155, 163). Although IL-1 $\beta$  generation is not demonstrated in parallel with the findings of these studies, we find similar inflammasome-independence here in parallel with conditions and concentrations of LLOMe or particulate stimuli that induce robust IL-1 $\beta$  secretion. Moreover, in contrast to our study, this group found that a caspase inhibitor, Boc-D-CMK, also suppresses cell death during inflammasome activation. However, it is well known that Boc-D-CMK also inhibits cathepsin B(162), and likely other cathepsins as well. In contrast, we found that only cathepsin inhibitors, and not caspase-1 or pan-caspase inhibitors, suppress particle-induced cell death during NLRP3 activation. Although the impact of this caspase-1-independent cell death response to *in vivo* pathology has not yet been quantified experimentally, we make the case here that cathepsin inhibitors have an advantage over caspase-1 inhibitors in suppressing two key pathological responses to particles.

Many studies have shown that the cathepsin B selective inhibitor Ca074Me suppresses cell death induced by LLOMe, particles and soluble NLRP3 activators(11, 16, 18, 142-144, 155, 160-163, 373, 375-377, 405, 416, 417). However, it is highly likely that these effects have been achieved by inhibiting multiple cathepsins at the concentrations used ((155-159), OUR REF). Here, we found that PMs deficient in the four cathepsins B, L, S and X or in the



five cathepsins B, C, S, L and X showed a large and significant reduction in cell death induced only by silica and not nigericin or dAdT. This phenotype was not evident in single or double (B&L) cathepsin-deficient macrophages. Thus, these data conclude that particle-induced cell death is largely inflammasome-independent, but we also demonstrate a hitherto unrecognized contribution of multiple redundant cathepsins to particle-induced cell death during NLRP3 activation.

Surprisingly, we find that cathepsin X plays a previously unrecognized and non-redundant role in nigericin-induced cell death. As in our previous study, this is consistent with the observation that K777 is a less potent inhibitor of cathepsin X than Ca074Me, which more potently suppresses nigericin-induced cell death and IL-1 $\beta$  activation(406). Indeed, nigericin-induced cell death is entirely dependent on the inflammasome, and our recent study examining cathepsin X in IL-1 $\beta$  responses found that its role in the nigericin response was independent of pro-IL-1 $\beta$  synthesis(406). Therefore, cathepsin X appears to be upstream of nigericin-induced NLRP3 activation and is not essential for priming. Moreover, these data show that K777 is a selective inhibitor of, not only IL-1 $\beta$  secretion, but also, cell death induced by sterile particles during NLRP3 activation.

Our investigation into the mechanism of cathepsin-mediated particle-induced cell death during NLRP3 activation yielded some expected and unexpected results. Our data are consistent with findings of previous studies showing that the Bcl-2 family modulates cell death and IL-1 $\beta$  secretion induced

by a variety of particulate and non-particulate NLRP3 activators(173, 218). However, we found a selective involvement of the Bcl-2 family in these responses to silica and not nigericin or dAdT. The difference in results for non-particulate activators is unclear, but possibly could be due to differences in the cell types analyzed (PM vs bone-marrow-derived macrophages).

The terminal events of NLRP3 activation are thought to require the mitochondria in some way, whether it be the stress-induced evolution of mitochondrial ROS(173), oxidized mitochondrial DNA(174, 218), or cardiolipin(172, 220). Because the Bcl-2 family is known to mediate cathepsin-dependent cell death, which leads to mitochondrial dysfunction and MOMP(172, 200), it would be logical to predict that cathepsin-mediated MOMP causes cell death and NLRP3-dependent IL-1 $\beta$  activation. However, pharmacological activation of MOMP with Bcl-2 inhibitors did not lead to NLRP3 activation. On the contrary, this response triggered a previously unappreciated pathway for concomitant cell death and IL-1 $\beta$  secretion that depends on neither inflammasomes nor RIP3.

Surprisingly, we also found that Bcl-2 inhibition does not enhance particle-induced IL-1 $\beta$  secretion, but instead, strongly enhances cell death. A previous study examining LMD during NLRP3 activation made similar observations for LLOMe, suggesting that LMD actually antagonizes NLRP3-mediated IL-1 $\beta$  secretion(155). Here, we demonstrate that LLOMe and Bcl-2 inhibition both induce early and robust loss of MMP, while silica induces only mild LMD. Indeed,

loss of MMP correlated with high levels of cell death and low levels of IL-1 $\beta$  secretion. Therefore, our data suggest that it is not LMD that antagonizes IL-1 $\beta$  secretion, but rather, it is the loss of MMP. Moreover, this indicates that NLRP3 activation induced by LMD is favored under conditions where MMP is intact.

Together, these data favor a model whereby MMP-dependent processes, like ROS production, contribute to the particle-induced pathway for NLRP3 activation(173). This interpretation contrasts the conclusions of a recent study. That study suggested that mitochondrial ROS are not important for NLRP3 activation since the induction of mitochondrial ROS with ETC uncouplers does not induce IL-1 $\beta$  activation in LPS-primed macrophages(154). While ETC uncoupling can generate mitochondrial ROS, this method for generating mitochondrial ROS also simultaneously destroys the MMP. Here, we show that an intact MMP is necessary for particle-induced NLRP3 activation. Interestingly, we find that this dynamic was less relevant to nigericin-induced NLRP3 activation. We believe that this dichotomy, where particulate NLRP3 activators are more dependent on MMP than non-particulates, actually indicates a disparate dependency of these two classes of NLRP3 activators on cathepsin and MMP-dependent priming.

There is a complex relationship between priming and NLRP3 activation(172). While altering the synthesis of pro-IL-1 $\beta$  can affect any downstream IL-1 $\beta$  activation pathway, the need to prime *de novo* NLRP3 synthesis is a unique feature among inflammasomes(67). In addition, we find

here, and in a previous study, that priming is also more important for particulates compared to a non-particulate NLRP3 stimulus (nigericin)(406). Why this is the case is not clear, however, it could be the result of transcription-independent processes recently implicated in NLRP3 activation(226). For example, one study demonstrated that potassium (K<sup>+</sup>) efflux is a common and essential event for all NLRP3 stimuli, but they also found that LPS priming enhanced K<sup>+</sup> efflux generated by silica, alum, calcium pyrophosphate crystals, and LLOMe, but did not do so for nigericin or ATP(154). Moreover, in that study, Ca074Me and inhibition of lysosomal acidification suppressed K<sup>+</sup> efflux triggered by particulates and not ATP or nigericin. Therefore, particulate stimuli, but not soluble ones, may need a priming step to be able to trigger the prerequisite K<sup>+</sup> efflux.

In summary, this study characterizes a number of potentially important therapeutic characteristics of cathepsin inhibitors relating to the mechanisms of particle-induced inflammatory responses. We show that a bioavailable cathepsin inhibitor is effective at suppressing a largely caspase-1-independent *in vivo* response to sterile particles, and it does this by targeting more than just cathepsin C. Furthermore, we confirm that particles induce inflammasome-independent cell death that depends on multiple cathepsins during NLRP3 activation and identify a novel and non-redundant role for cathepsin X in nigericin-induced cell death and NLRP3 activation. We also implicate the Bcl-2 family specifically in particle-induced cell death and IL-1 $\beta$  secretion, but demonstrate that Bcl-2 inhibition induces a previously undescribed pathway for

concomitant cell death and IL-1 $\beta$  secretion that is independent of and antagonistic toward NLRP3 activation. Indeed, we find that cathepsins are likely involved in priming via a pathway of lysosomal-mitochondrial cross-talk, which requires a stressed but intact MMP, and that this pathway is uniquely important for particle-induced NLRP3 activation. In summary, multiple cathepsins mediate caspase-1-independent particle-driven cell death and pro-IL-1 $\beta$  synthesis, and therefore, they represent tractable and multifaceted drug targets for potential therapeutics aimed at treating IL-1-dependent sterile inflammatory diseases.

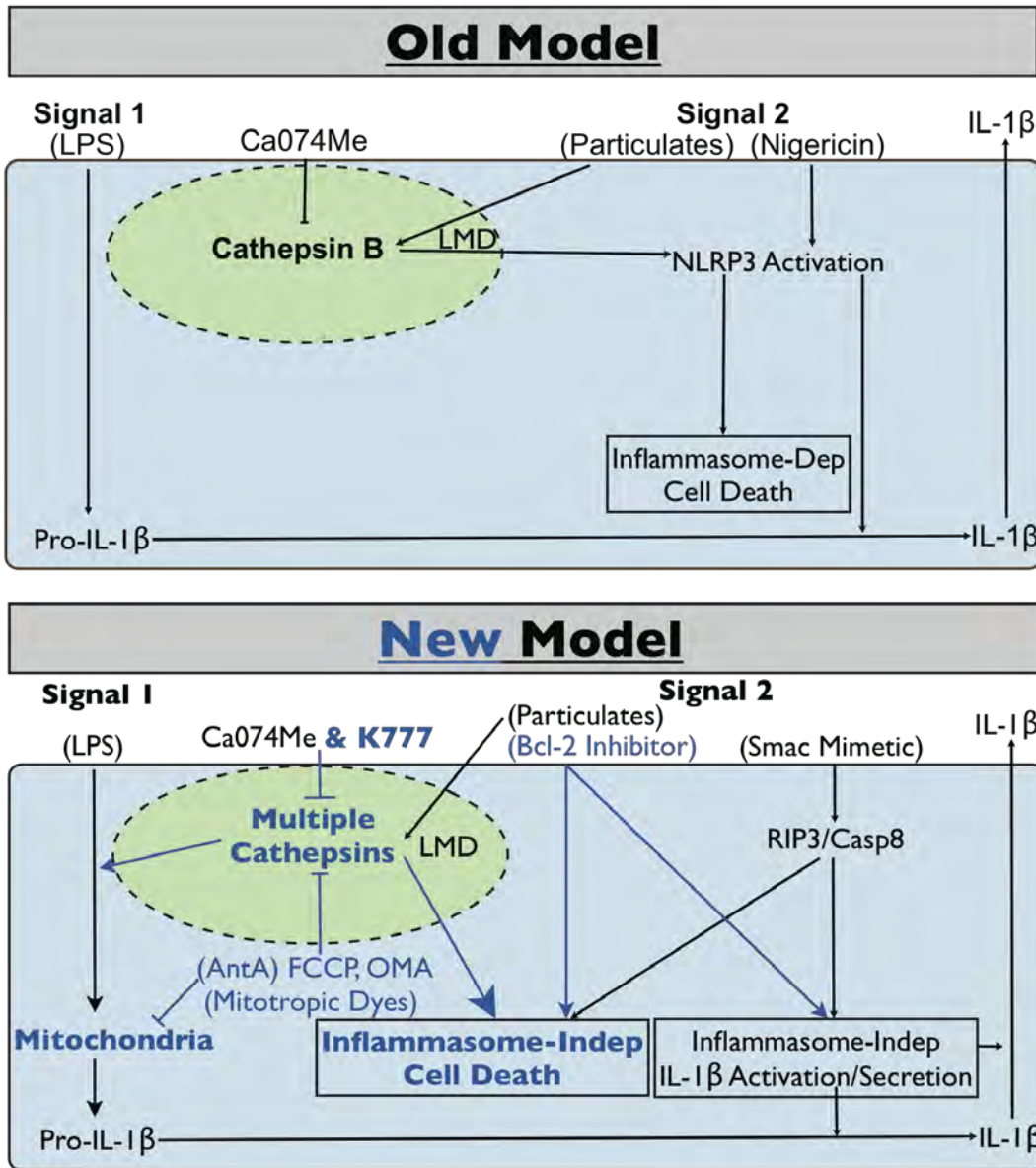
### **Acknowledgments**

We would like to thank John McCullough at  $\mu$ MMS for advice and guidance on the pharmacology and formulation, Alan Wolfe at UCSF for providing the protocol for mass spectrometric analysis of K777 in plasma as well as Scott Shaffer and Andre Kopoyan at the  $\mu$ MMS Proteomics and Mass Spectrometry Facility for standardization and performance of this analysis. Also, Havisha Karnam from the Brown and Khvorova labs as well as Myriam Aouadi from the Czech lab at  $\mu$ MMS for help in the optimization of siRNA knockdowns.

### **Grant Support**

This work was supported by an R01 (Grant# 5R01AI078287-05) to K.L.R., a T32 (Grant# T32AI095213-01), and services from the NHLBI, NIH, DHHS, and the Science Moving Towards Research Translation and Therapy (SMARTT) program via the following contract HHSN268201100015C.

## Summary of Major Contributions in Chapter IV



## **Chapter V: Discussion**



## Chapter V: Discussion

### ***Cathepsins as Therapeutic Targets for the Treatment of Particle-induced Sterile Inflammatory Disease***

I have shown that cathepsin inhibitors are able to suppress two fundamental pathological responses to sterile particles: lytic cell death and IL-1 $\beta$  secretion. Moreover, I recapitulated these effects with more than 25 different cathepsin inhibitors that selectively suppress particle-induced IL-1 $\beta$  secretion and cell death **(see Appendix 6)**. Therefore, it is unlikely that all of these inhibitors have the same non-cathepsin off-targets. Indeed, my genetic data suggests that multiple cathepsins, not just cathepsin B, play redundant roles in both particle-induced lytic cell death and IL-1 $\beta$  secretion. In fact, this redundancy is a hallmark of cathepsin biology, which reflects the major function of cathepsins as non-specific digestive proteases(260, 379, 380). For a researcher, this redundancy presents a formidable challenge. As I have shown, genetic knockouts of single cathepsins and biochemical techniques can overlook the redundant roles for cathepsins in complex biological processes. Therefore, in order to elucidate a role for cathepsins in particle-induced lytic cell death and IL-1 $\beta$  secretion, I generated multigene knockouts and used state-of-the-art biochemical tools. Since I found concordant results from cathepsin deficiency and inhibition of multiple cathepsins, I believe that the biological effects I observed with cathepsin

inhibitors resulted mostly from on-target effects. However, in my discussion on lysosomotropic drugs, I will explain why there is ample room for caution when interpreting the effects of inhibitors. My inhibitor data never matched up perfectly with my genetic data, so the potential role of off-target drug effects that are unrelated to cathepsins must be carefully considered. For the sake of this discussion on cathepsins as therapeutic targets, I will assume that cathepsin inhibitors suppress lytic cell death and IL-1 $\beta$  secretion by inhibiting cathepsins. If this assumption is true, then my findings have important implications for the roles of cathepsins in particle-induced inflammatory disease.

I believe that cathepsins are unique candidates as therapeutic targets for the treatment of particle-induced sterile inflammatory disease. As stated above, the response of macrophages to sterile particles is fundamentally two-sided. Particles induce pathologic cycles of cell death and inflammation(7, 29). My data suggests that cathepsins play fundamental roles on either side of this response, promoting both particle-induced cell death and pro-IL-1 $\beta$  synthesis/IL-1 $\beta$  secretion during NLRP3 activation. Besides cathepsins, no other tractable targets that play a role in both of these responses to sterile particles (and that do not inhibit more general processes like phagocytosis or lysosomal acidification) have been characterized in primed macrophages. Therefore, cathepsins may promote particle-induced inflammatory disease at multiple levels of its pathogenesis.

My data suggests that cathepsins promote pro-IL-1 $\beta$  synthesis. By inhibiting cathepsins, suppression of pro-IL-1 $\beta$  synthesis could influence particle-

induced inflammatory responses under conditions where inhibition of inflammasome activation may not be effective. As covered in Chapter IV, there is a substantial caspase-1-independent component of the *in vivo* acute sterile inflammatory response to necrotic cells and particles(109, 121). Since pro-IL-1 $\beta$  synthesis is a prerequisite for IL-1 $\beta$  activation/secretion by any stimulus, cathepsin inhibitors can potentially suppress IL-1 $\beta$ -dependent responses *in vivo* that are driven by any activation or secretion pathway. Indeed, I have shown that by blocking pro-IL-1 $\beta$  synthesis just before priming (instead of after several hours of priming), cathepsin inhibitors suppressed IL-1 $\beta$  secretion induced by particulate and soluble NLRP3 activators, as well as IL-1 $\beta$  secretion induced by AIM2 inflammasome activation *in vitro* and *in vivo* (**see Appendices 7j and 8b**). Moreover, this principle extended to inflammasome-independent responses as well. I showed that cathepsin inhibitors suppress IL-1 $\beta$  secretion induced by a previously undocumented IL-1 $\beta$ -activating pathway that is initiated by Bcl-2 inhibitors. Moreover, I have shown that IL-1 $\beta$  secretion can also be induced by a membrane permeant cathepsin D (an aspartic protease) inhibitor and two different DNA-damage promoting chemotherapeutics (doxorubicin and etoposide) (**see Appendix 9**). Interestingly, IL-1 $\beta$  secretion induced by the pro-apoptotic (protein-kinase-inhibiting) chemotherapeutic staurosporine (STS) is resistant to cathepsin inhibitor-mediated suppression (**see Appendix 9**). The reason for this is unknown at this time. Therefore, in order to initiate IL-1 $\beta$ -dependent inflammatory responses, inflammasomes and almost any other

intracellular IL-1 $\beta$  activating pathways rely on cathepsin-dependent pro-IL-1 $\beta$  synthesis.

*In vitro*, I have also shown that cathepsin inhibitors suppress particle-induced cell death. It is likely that this is the same cell death-inducing mechanism that occurs *in vivo* when macrophages ingest inflammatory particles. However, even if I find in the future that cathepsin-independent mechanisms (responsible for the residual amount of cell death that remains even when I treat cells with cathepsin inhibitors) drives the response to sterile particles *in vivo*, cathepsin inhibitors will nonetheless limit the availability of pro-IL-1 $\beta$  to post-lytic extracellular activation mechanisms (ex- neutrophil proteases)(328, 329). An added benefit of inhibitors like K777 is that K777 inhibits cathepsin C, which these neutrophil serine proteases rely on for their activation. But keeping to the hypothetical future scenario where particle-induced cell death cannot be completely blocked by cathepsin inhibitors *in vivo*, the mutual dependence of IL-1 $\alpha$  and IL-1 $\beta$  on NF- $\kappa$ B-mediated transcription would lead one to predict that cathepsin inhibitors can also suppress pro-IL-1 $\alpha$  synthesis. If so, then cathepsin inhibitors may actually suppress both halves of the IL-1-dependent *in vivo* response without having to completely block caspase-1 activation or cell death. However, K777 does not appear to inhibit all NF- $\kappa$ B-dependent cytokine production, as it spares TNF- $\alpha$  and some other cytokines for unknown reasons **(see Appendix 10a,b)**. This disparity in results will be discussed in more detail later. For now, a role for cathepsins in the priming of pro-IL-1 $\alpha$  synthesis remains

to be shown. Regardless, inhibition of cathepsin-dependent pro-IL-1 $\beta$  synthesis will limit the availability of this substrate for almost any extracellular IL-1 $\beta$  activating mechanisms that contribute to IL-1 $\beta$ -dependent inflammatory responses resulting from cathepsin-independent lytic cell death.

Importantly, there are situations in which priming is the most important facet of the IL-1-dependent inflammatory response. Since caspase-1 is constitutively active in circulating monocytes, priming alone can activate IL-1 $\beta$ (233, 234). This is interesting since pyroptosis should occur constitutively in these cells as a result of caspase-1 activation, but this does not appear to be the case. However, *Klebsiella pneumoniae* has been shown to induce NLRP3 and ASC-dependent HMGB1 release and pyroptotic-like cell death without requiring caspase-1 *in vivo*, so caspase-1 activation may not necessarily induce pyroptosis in these cells(418). Whatever may be the case for cell death, IL-1 $\beta$  secretion appears to be regulated primarily at the level of priming in circulating monocytes. Therefore, inhibition of priming may be particularly useful for the treatment of septic shock, in which systemic activation of monocyte-derived IL-1 $\beta$  is known to play a critical role(235). Moreover, patients with hyperactivating inflammasome mutations that have fewer restraints on caspase-1 activation are also likely to benefit from inhibition of pro-IL-1 $\beta$  synthesis(236). Therefore, whether IL-1 $\beta$  is activated by caspase-1-independent intracellular or extracellular mechanisms, or immediately upon priming, priming is an essential aspect of all IL-1 $\beta$ -dependent inflammatory responses that may be targeted therapeutically.

*In vivo*, I expect that cathepsin inhibitors will block particle-induced cell death during NLRP3 activation. Blocking lytic cell death has as multifaceted a therapeutic implication for IL-1-dependent sterile inflammatory disease as blocking IL-1 $\beta$  or IL-1 $\beta$  synthesis. As alluded to in the introduction to this thesis, inhibiting or not inhibiting cell death is a critical crossroad in the sterile inflammatory cascade that, once reached, will lead to either the containment or release of copious pro-inflammatory DAMPs. Given that the acute inflammatory response to both necrotic cells and sterile particles depends almost entirely on signaling through IL-1R1(109, 121), the most relevant DAMPs to consider during cell death-induced inflammatory responses are those with direct IL-1 signaling potential. Just as suppressing pro-IL-1 $\beta$  synthesis will prevent extracellular IL-1 $\beta$  activation by neutrophil proteases, so too will prevention of pro-IL-1 $\beta$  release by suppressing particle-induced cell death. Moreover, without lytic cell death, IL-1 $\alpha$  cannot be secreted(113-115). In fact, pro-IL-1 $\alpha$  becomes more inflammatory as a result of molecular interactions that occur during lytic cell death(119). By inhibiting this type of cell death, the IL-1-dependent inflammatory response will most likely be driven only locally by surface-bound pro-IL-1 $\alpha$ . Moreover, without lytic cell death, there will be no release of HMGB1, IL-33, uric acid, ATP, filamentous actin, mitochondrial DAMPs and many other potent inflammatory mediators(50, 51, 85, 93, 94, 98, 103, 116, 117, 217). Thus, cathepsin inhibitors can block both the production and release of inflammatory mediators known to drive particle-induced sterile inflammatory responses.

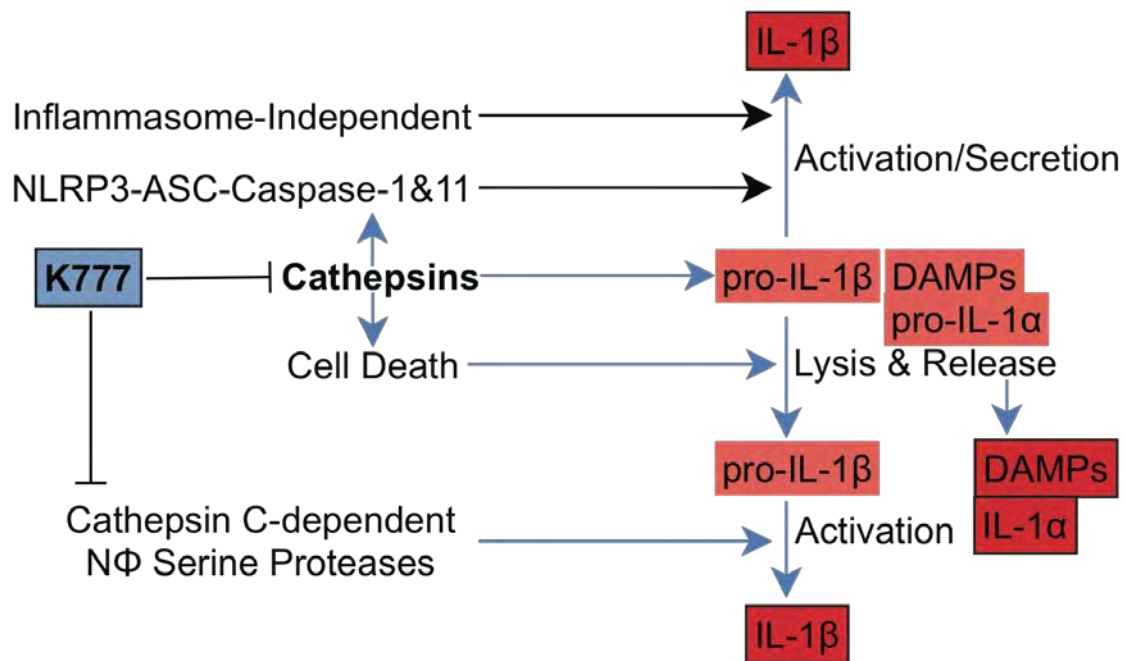
The dualistic quality of cathepsin inhibitors in suppressing both particle-induced IL-1 $\beta$  secretion and cell death stood out among the different inhibitors I examined. Besides cathepsin inhibitors, most other inhibitors that affected IL-1 $\beta$  secretion were unable to suppress particle-induced cell death (again, not counting inhibitors of phagocytosis and lysosome acidification). Importantly, K777 does not affect phagocytosis, which would otherwise account for its selective effects on particulate stimuli (**see Appendix 8c**). Also, as I have shown, caspase inhibitors do not block particle-induced cell death. Instead, ZVAD and YVAD often actually increased cell death. Moreover, inhibition of mitochondrial ROS (with Mitotempo) blocked only IL-1 $\beta$  secretion induced by silica and LLOMe without affecting cell death (**see Appendix 11d**). This suggests that mitochondrial ROS are likely only involved in priming and/or NLRP3 activation, not cell death induced by particles and LMD. Conversely, inhibition of mitochondrial ROS blocked both IL-1 $\beta$  secretion and cell death induced by the Bcl-2 inhibitor, ABT199, suggesting that both of these responses depend entirely on mitochondrial ROS. Although H<sub>2</sub>O<sub>2</sub> has been shown to activate NLRP3 in one study, I found that it does not induce IL-1 $\beta$  secretion, but only causes cell death that cannot be suppressed with either ROS inhibitors, like butylated hydroxyanisole (BHA), or K777 and ZVAD. Over the course of my study, the only non-cathepsin inhibitor that suppressed both particle-induced IL-1 $\beta$  secretion and cell death was the Bax inhibitor, BaxIP5. However, I did not investigate the Bax inhibitor's effect on pro-IL-1 $\beta$  synthesis, so the mechanism its effect on IL-1 $\beta$

secretion is unclear. Later on, I will discuss some lysosomotropic inhibitors that inhibit particle-induced IL-1 $\beta$  secretion and cell death, but it is unclear whether or not they do this by inhibiting cathepsins.



### Chapter V, Figure 1:

**Cathepsins as Therapeutic Targets.** The diagram shows that cathepsins regulate particle-induced IL-1-dependent inflammatory responses by promoting inflammasome activation (NLRP3-ASC-Caspase-1&11), pro-IL-1 $\beta$  synthesis (which provides pro-IL-1 $\beta$  for activation by inflammasome-dependent or independent mechanisms, including pro-IL-1 $\beta$  release during lytic cell death allowing its activation by cathepsin C-dependent neutrophil (N $\phi$ ) serine proteases), and cell death (which can lead to the activation and release of DAMPs and IL-1 $\alpha$ ). Inhibition of cathepsins with drugs, like K777, could therefore suppress multiple components thought to be involved in particle-induced IL-1-dependent inflammatory responses promoted by cathepsins.



## ***K777 as a Therapeutic for Particle-induced Sterile Inflammatory Disease***

I have shown that K777 blocks particle-induced IL-1 $\beta$  secretion and cell death during NLRP3 activation. Moreover, K777 does this selectively; K777 reduced these two responses induced by particulates much more so than those induced by soluble NLRP3 activators, like the K<sup>+</sup> ionophore nigericin, or the AIM2 activator dAdT. Selectivity is important. As discussed in the introduction, sterile inflammatory mechanisms driven by IL-1 serve a physiologic function in response to both cellular injury (eliciting responses for cleanup and repair) and pathogenic infections (killing pathogens induced by sterile particles)(22-24, 84). Therefore, the selectivity of K777 (or new cathepsin inhibitors designed with this application in mind) may provide a therapeutic window in its dose-range where it suppresses pathological responses to sterile particles without unacceptably prohibiting protective responses to microbial pathogens. Of course, cathepsins have basal physiologic roles as well, which must be considered.

Complete abrogation of normal/basal cathepsin activity could be detrimental. Cathepsins play critical roles in antigen presentation, cell-mediated immune defense, and general digestion of proteinaceous extracellular/intracellular products(275-277). Therefore, for K777 to be safe and effective, there must be a therapeutic window where partial inhibition of the cathepsin repertoire permits suppression of overactivity in pathologic

circumstances whilst providing enough basal activity to carry out normal cathepsin functions. The considerable functional redundancy of the mammalian cathepsin repertoire may be consistent with this goal. Since cathepsins generally play a role in digesting proteins in the phagosomes, one might expect that cathepsin deficiencies would result in lysosomal storage diseases. However, there are a minority of lysosomal storage diseases that actually result from cathepsin deficiency, including cathepsin K (Pycnodysostosis) or the aspartic protease cathepsins D and E(419-421). Nonetheless, as discussed below, a cathepsin K inhibitor has already successfully gone through clinical trials for the treatment of osteoporosis(422-424). In fact, cathepsin inhibitors are effective at killing parasites because, unlike mammalian cysteine cathepsins, parasite cathepsin orthologs lack this redundancy. Instead, parasites often depend entirely on only one or a few cathepsins for proteolysis during their life cycle(425). This is one of the major reasons why treatment of parasitic diseases with cathepsin inhibitors, including K777, has been achieved in mammalian models without significant toxicity that might result from inadequate lysosomal function(386-391). On a side note, host-parasite interactions have provided other important insights relevant to cathepsins and inflammation. It has been shown that a variety of parasites encode their own cathepsin inhibitors that suppress inflammatory host-defense mechanisms(426). For example, taeniaestatin from *Taenia taeniaeformis* (tape worms) has been shown to suppress IL-2 production and IL-1-dependent T cell proliferation in mice(427). Whether or not parasite-

derived cathepsin inhibitors implicate a direct role for cathepsins in IL-1 $\beta$  production has not yet been determined, but this represents an interesting correlation nonetheless. Thus, there is a reasonable basis to suggest that there is a therapeutic window in which K777 may be used as an anti-inflammatory therapeutic.

I demonstrated K777's anti-inflammatory efficacy *in vivo*, without observing any obvious systemic intolerance in mice. However, efficacy and systemic tolerability are only two aspects of K777's pharmacology that need to be considered regarding its future as a clinically useful drug. To that effect, a great deal of research investigating K777 as an anti-parasitic or anti-metastatic drug has provided some invaluable foresight into K777's future as an anti-inflammatory therapeutic. K777 has been through extensive preclinical evaluation for the therapeutic targeting of cruzain (the CatL ortholog of *Trypanosoma cruzi*), other parasite cathepsin orthologs, cathepsins B and L for their roles in the progression of certain cancers, and it has been shown to suppress inflammation during pancreatitis by inhibiting cathepsins B, L and S(385-391, 428). Through *in vitro* and *in vivo* testing, many early studies have shown that K777 has suitable oral bioavailability, pharmacokinetics and –dynamics, and an acceptable toxicity profile in rodents, canines and apes(389). Unfortunately, given the results of some more recent (unpublished) studies, K777's disposition as an oral anti-inflammatory drug is not encouraging.

Although phase I clinical trials were expected to begin in late 2013 for the treatment of Chagas' disease (*Trypanosoma cruzi*) with K777, the project was halted due to tolerability issues discovered during comprehensive preclinical tolerability testing (personal communication with the project director at UCSF, Stephanie Robertson, Ph.D.)(390). While mice were highly tolerant of doses as high as 125 mg/kg/day, canines experienced severe dose-related nausea, which, according to Dr. Robertson, is highly predictive for human nausea. Moreover, since K777 irreversibly inhibits the metabolic liver enzyme CYP3A4, which can lead to drug interactions, it caused some liver toxicity at doses >100 mg/kg/day. At 150 mg/kg/day, canines also began to show noticeable weight loss. Importantly, early pharmacokinetic studies also found that the dose-response to K777 was non-linear as a result of CYP3A4 inhibition and inhibition of the endogenous drug-efflux transporter P-glycoprotein (PgP), making dose-related exposure of the targeted peripheral tissues difficult to control(385, 429). At low oral doses, K777 does not reach peripheral tissues well; but at higher doses, inhibition of PgP can result in over exposure. However, this was not the case for i.v. administration, which bypasses first-pass metabolism in the liver that occurs via oral routes<sup>5</sup>. Nonetheless, the FDA expressed concerns to Dr. Robertson's team that K777 is sequestered in lysosomes (see discussion below on

---

<sup>5</sup> **First-pass metabolism** is a phenomenon that occurs when drugs are absorbed through the digestive tract. These drugs go directly to the liver, where a large fraction of the drug is processed, metabolized, and excreted prior to delivery to the rest of the body. I.V. or S.C. administration of drugs largely bypasses first-pass metabolism, leading to greater (~10x) peripheral drug exposure at lower doses with lower liver-associated toxicity.

lysosomotropic drugs) and that it inhibits so many cathepsins. In fact, the first cathepsin inhibitor to make it through clinical trials, Odanacatib (a cathepsin K inhibitor for the treatment of osteoporosis), was specifically designed to be non-basic so that it does not accumulate in lysosomes(422-424). Despite this barrier to moving into clinical trials, K777 has a proven track record for tolerability in many preclinical animal models(389). Thus, K777 can still be investigated in animal models of inflammatory disease as proof-of-concept studies. However, I recognized some properties of K777 that, according to Dr. Robertson, have not been considered in these latest tolerability studies. These properties of K777, which I am about to discuss, may have confounded their results.

K777 has challenging properties for pharmaceutical formulation that may have contributed to its failure in canine tolerability studies. Firstly, K777 is poorly soluble, making it difficult to keep in solution at higher concentrations necessary to deliver adequate doses to mice via an Alzet pump (**see Appendix 12**). Moreover, when I injected K777 too rapidly i.v., mice died almost instantly, probably as a result of K777's precipitation leading to embolic strokes. The HCl salt of K777, which I used in all experiments, was designed to enhance its aqueous solubility by reducing the pH of aqueous solutions and ionizing the molecule. However, this results in acidic formulations that, when delivered s.c. via Alzet pumps, led to K777's inevitable precipitation after neutralization by interstitial fluids. For the purpose of demonstrating its efficacy *in vivo*, I was able to implement a formulation for poorly soluble drugs that caused K777 to

precipitate in a monodispersed (uniform) micellar suspension that facilitated its absorption(371). However, the acidity of K777's HCl salt in solution (pH range from 2-6) likely resulted in the severe local inflammatory responses that I observed in my s.c. Alzet pump study (see discussion on acidity and inflammation below). Therefore, s.c. and i.v. formulations of K777 have been complicated by either poor solubility or unacceptable acidity. From my communications with Dr. Robertson, this phenomenon of precipitation was not fully appreciated in the latest toxicity studies. These latest tolerability studies for K777 were done using oral dosing. Since oral drugs are ideal for eventual prescription, alternative routes were not evaluated for K777 at this stage of development. Moreover, during oral dosing of K777, solubility/pH is probably not such an obvious concern; absorption is likely facilitated by the low pH environment of the stomach. In these studies, K777 was probably absorbed well enough by the stomach not to raise any red flags, so there was nothing prompting an examination of K777's actual solubility in the stomach or of its precipitation in the basic pH environment of the duodenum downstream. Therefore, it may be that the tolerability issues relating to nausea in these studies were related to blockage/irritation of the intestines due to K777's precipitation. Moreover, the non-linear pharmacokinetics of K777, resulting from first-pass metabolism and its inhibition of clearance mechanisms, likely made it difficult to achieve effective systemic exposure and simultaneously prevent liver toxicity. Therefore, between the nausea in canines, the elevation of liver enzymes, and

the unpredictable oral pharmacokinetics, it is not surprising that oral toxicity testing failed. However, the causes of this toxicity/intolerance may not have been accurately attributed since precipitation and alternative routes/formulations, which have linear dose-response kinetics, were not examined.

I provided strong evidence that K777 suppresses what I presume to be fundamental aspects of particle-induced inflammatory pathology. Therefore, there is a reasonable incentive for the continued investigation of K777. Based on my observations and the recent tolerability studies, I believe that the clinical application of K777 as an anti-inflammatory therapeutic may still be possible through its reformulation for non-oral routes with the free base (instead of the HCl salt). To that end, Dr. Rock and I had the free base form of K777 synthesized for us by Julian Adams and John Lee at Infinity Pharmaceuticals in Cambridge. However, future large-scale investigations of K777 in animal models should be conducted along side the development of new/alternative cathepsin inhibitors that possess more manageable pharmaceutical characteristics.

There are other cathepsin inhibitors, like Ca074Me, which have shown similar promise in some *in vivo* models of inflammatory disease. Ca074, the cathepsin B-specific counterpart to Ca074Me, has been shown to inhibit the activation of caspase-1 and caspase-11 in the brain using a mouse model of focal cerebral ischemia(430). Also, beta-amyloid levels and memory have been improved by intrathecal administration of Ca074Me, or the pan-cathepsin inhibitor E64d, in guinea pig models of Alzheimer's(431, 432). Moreover, in



models of polymyositis, Ca074Me has also been used to suppress lung interstitial inflammation and fibrosis in rats, as well as muscle inflammation and apoptosis in guinea pigs(433, 434). In a rat model of adjuvant-induced arthritis, in which cathepsin activity was elevated in inflamed ankle joints, systemic administration of only 2 mg/kg/day (incorporated into the diet) of a vinyl-sulfone (like K777) cathepsin inhibitor reduced inflammation(435). Finally, deficiency of single cathepsins (Cat K, L & S), or treatment with a cathepsin S inhibitor, has been shown to be protective in mouse models of atherosclerosis (*LDL-R*<sup>-/-</sup> or *ApoE*<sup>-/-</sup>)(436-438). However, I found no role for cathepsin B or both cathepsins B & L in hematopoietic cells in an *LDL-R*<sup>-/-</sup> mouse model of atherosclerosis (**see Appendix 1**). On the other hand, caspase inhibitors have a more ambiguous and perhaps less impressive track record.

Caspase inhibitors have a well known propensity for inhibiting cathepsins, suggesting that efficacy of these inhibitors in models of inflammatory disease may be a direct result of these off-target effects. For instance, caspase inhibitors, including BocD-CMK, Z-VAD-FMK, Ac-YVAD-CMK, and Z-WEHD-FMK, have all been shown to inhibit cathepsin B with IC<sub>50</sub>s ranging from 1-10  $\mu$ M(162). This has been observed in multiple other studies as well(142, 342, 343, 439, 440). Moreover, caspase inhibitors have toxicity issues of their own. ZVAD's metabolic byproduct, fluoroacetate, has been shown to be highly hepatotoxic(441). Toxicity aside, caspase inhibition with minocycline failed Phase III trials for the treatment of ALS (Amyotrophic Lateral Sclerosis) due to inefficacy, and it actually caused

more rapid disease progression(442, 443). Therefore, there is a significant portfolio of studies showing cathepsin inhibitors are effective in the treatment of inflammatory diseases in animal models, while caspase inhibitors seem to have all the same pharmacological drawbacks as cathepsin inhibitors without the ability to inhibit pro-IL-1 $\beta$  synthesis or particle-induced cell death during NLRP3 activation.

### ***Importance of Cell Death during NLRP3 Activation***

Lytic cell death and IL-1 $\beta$  activation are intimately connected to one another. One of my key findings was that cell death induced by severe mitochondrial disruption limits IL-1 $\beta$  activation. While cell death may seem to be an obvious impediment to IL-1 $\beta$  secretion, the influence of cell death on IL-1 $\beta$  activation at the cellular level is not that straightforward. Moreover, the influence of cell death on IL-1 $\beta$  activation has been neglected by most studies. To holistically evaluate the influence of cell death and other critical variables over the course of my study, I developed a technique of monitoring relative cell numbers, pro-IL-1 $\beta$  production, IL-1 $\beta$  secretion, TNF- $\alpha$  secretion, LDH release (quantifying cell death) and MTS metabolism (quantifying cell survival) all from the same set of samples in a 96-well format (see methods section). In doing so, I gained an appreciation for numerous otherwise neglected phenomena that influence various measurements of cell death and NLRP3-mediated IL-1 $\beta$  secretion. Moreover, I found that the

important factor for how cell death influences IL-1 $\beta$  production is not simply how much cell death occurs. Instead, it is how cell death occurs, which is paramount to its influence on IL-1 $\beta$  activation.

When IL-1 $\beta$  activation and cell death occur strictly by pyroptosis, both events are completely dependent on caspase-1 and/or caspase-11(24). Although pyroptotic cell death will eventually destroy the synthetic, metabolic, and biochemical machinery necessary to produce, process and secrete IL-1 $\beta$ , IL-1 $\beta$  will be secreted as a natural consequence of caspase-1/11 activation prior to this destructive phenomenon nonetheless. In fact, there should be a relatively high standard of efficiency for IL-1 $\beta$  secretion as function of concomitant pyroptotic cell death that can be derived mathematically from experimental data:

$$\text{IL-1}\beta \text{ Secretion Efficiency} = \text{IL-1}\beta \text{ secreted} / \% \text{ Cell Death}$$

Pyroptotic stimuli, like nigericin or dAdT, induce high levels of IL-1 $\beta$  secretion compared to the amount of concurrent cell death. Perhaps, this equation could be improved by incorporating the fractional activation of synthesized pro-IL-1 $\beta$  into the above equation:

$$\text{Fractional IL-1}\beta \text{ Activation} = \text{IL-1}\beta / (\text{IL-1}\beta + \text{pro-IL-1}\beta)$$

$$\text{IL-1}\beta \text{ Activation Efficiency} = [\text{IL-1}\beta / (\text{IL-1}\beta + \text{pro-IL-1}\beta)] / \% \text{ Cell Death}$$

Whichever equation is used, this can indicate whether non-pyroptotic mechanisms of cell death are occurring during IL-1 $\beta$  activation. Moreover, this can provide information as to the extent of non-pyroptotic death and how this concomitant type of death is influencing IL-1 $\beta$  secretion. Well-characterized (their molecular targets are known) stimuli that induce responses tangential to NLRP3 activation, which interfere with or promote IL-1 $\beta$  activation, can be mechanistically informative using the above concepts (or actual calculations). For example, I have shown that LLOMe and the Bcl-2 inhibitors (ABT199 & ABT263) induce very little IL-1 $\beta$  secretion compared to dAdT, which depends completely on the inflammasome for cell death. At the same time, LLOMe and Bcl-2 inhibitors induce much more cell death than dAdT. Taking the ratio of IL-1 $\beta$  secretion per % cell death augmented (and highlighted) the differences measured between these two sets of stimuli (there is a greater difference between IL-1 $\beta$  secretion per % cell death upon stimulation with dAdT and LLOMe compared to the differences in IL-1 $\beta$  secretion or cell death examined between these two stimuli in isolation). This informed my interpretation in two ways. First, it told me that these stimuli induce an inflammasome-independent mechanism of cell death; had these stimuli, instead, induced cell death through caspase-1, each unit of cell death would have occurred with a concomitant unit of IL-1 $\beta$  secretion. Second, it told me that this mechanism of cell death antagonized IL-1 $\beta$  activation, since the ratio of cell death to IL-1 $\beta$  secretion was so high that 100% cell death would be reached before IL-1 $\beta$  secretion reached the levels induced by

dAdT. Conversely, the concentrations of silica I used induced similar ratios of IL-1 $\beta$  secretion to cell death as dAdT. Therefore, even though I found that silica-induced cell death was inflammasome-independent, this told me that it was occurring in parallel with IL-1 $\beta$  activation rather than antagonizing IL-1 $\beta$  activation. Moreover, as discussed later, in inflammasome-sufficient macrophages, silica-induced cell death may occur entirely by pyroptosis despite the fact that cathepsin-dependent cell death takes over in inflammasome-deficient cells. Although the above equations would predict this is the case, this is a matter for future investigation. In making this connection, that LLOMe and Bcl-2 inhibition induce disproportionate amounts cell death for a given amount of IL-1 $\beta$  secretion, my subsequent observation that LLOMe and Bcl-2 inhibitors cause early and robust mitochondrial depolarization, while silica and dAdT did not, suggested that severe mitochondrial disruption antagonizes IL-1 $\beta$  activation. Indeed, on further examination, I found that an intact MMP is critical for priming of pro-IL-1 $\beta$  synthesis and probably particle-induced NLRP3 activation as well. This helped me explain my finding that cathepsin C-deficient macrophages, which are less able to induce LMD with LLOMe, die less and actually secrete more IL-1 $\beta$  in response to high concentrations of LLOMe (**see Appendix 21**). This finding was corroborated by another study, and suggested that LMD antagonizes IL-1 $\beta$  activation and secretion(163). But, as I just discussed, it does not seem to be direct LMD that is responsible for this, but rather it is mitochondrial disruption downstream of severe LMD. Thus, having information

about concurrent cell death to complement my other measurements of IL-1 $\beta$  synthesis and secretion gave me critical insight into specific phenomena that I may otherwise overlooked. Moreover, without this insight, I may have even misinterpreted my data.

A lack of attention to the influence of cell death is pervasive in the field of inflammasomes. Indeed, this has led to the misinterpretation of published results. For example, the landmark study, which showed that K<sup>+</sup> efflux is a common requirement for NLRP3 activation by all stimuli, is a good example(154). I will refer to this study as “the K<sup>+</sup> study” from now on. The K<sup>+</sup> study found that LPS enhances K<sup>+</sup> efflux by particulates and LLOMe, and not by ATP or nigericin. Their interpretation of this result included the following assumptions:

- 1) Particles and LLOMe induce K<sup>+</sup> efflux, but not as a result of cell death, since this experiment was done in NLRP3<sup>-/-</sup> macrophages.
- 2) Enhancement of particle and LLOMe-induced K<sup>+</sup> efflux by LPS was not a result of LPS enhancing cell death, since this experiment was done in NLRP3<sup>-/-</sup> macrophages.

If this study had actually examined cell death, they would have realized that particle and LLOMe-induced cell death in LPS-primed macrophages is not NLRP3-dependent. Having shown in my study that particle and LLOMe-induced cell death are actually inflammasome-independent, this merits some reinterpretation of these results.

In the K<sup>+</sup> study, K<sup>+</sup> efflux most likely occurred as a result particle and LLOMe-induced cell death, rather than as part of a mechanism leading to the activation of pyroptosis. Considering this, it is interesting that I have not found any inhibitors that reduce particle-induced cell death without causing an equivalent reduction in IL-1 $\beta$  secretion. Perhaps, by causing this drop of intracellular K<sup>+</sup> concentration, cathepsin-dependent lytic cell death fulfills the requirement for lowering intracellular K<sup>+</sup> to facilitate NLRP3 activation. However, my pentuple cathepsin-deficient macrophages did show a more significant reduction in cell death than IL-1 $\beta$  secretion. Therefore, my data suggests particle-induced IL-1 $\beta$  secretion is not dependent on cathepsin-mediated cell death. In fact, as I will discuss later, LPS may also induce K<sup>+</sup> efflux during priming, prior to Signal 2. In fact, LPS may have enhanced particle and LLOMe-induced K<sup>+</sup> efflux by enhancing cell death. Indeed, I found that particle-induced lytic cell death is partially dependent on MyD88 and TRIF (**see Appendix 10d**).

Finally, ignorance of cell death may have led to another misinterpretation in the K<sup>+</sup> study. The K<sup>+</sup> study reported that Ca074Me suppressed K<sup>+</sup> efflux induced by particles and LLOMe. Their interpretation was that cathepsin B is critical for facilitating K<sup>+</sup> efflux induced by these stimuli. Once again, if they had examined cell death, they would have realized that Ca074Me suppresses particle-induced cell death and that this is most likely the reason that Ca074Me suppressed K<sup>+</sup> efflux in their study. Therefore, examination of cell death along side IL-1 $\beta$  secretion is especially critical to understanding and accurately

interpreting such a biological system in which these two variables are so intimately connected.

Cathepsin-dependent cell death in LPS-primed macrophages deserves further characterization. Particle-induced cell death during NLRP3 activation in LPS-primed macrophages may not be equivalent to particle or LMD-induced cell death in other cell types. Macrophages are exceptional in their sensitivity to particle-induced cell death. A single particle of silica is sufficient to kill a macrophage, and prior to the realization that it causes intense inflammatory responses, silica was a commonly used tool for selectively depleting macrophages *in vivo*(165, 444-446). Conversely, silica is relatively non-toxic to most other cell types (immature DCs are less sensitive than macrophages, but are also more sensitive than most other cell types)(165). Thus, macrophages are likely the first and possibly the only cells to die during the initial phases of the particle-induced inflammatory response. It is also possible that their sensitivity to particles indicates mechanistically unique facets of their cell death response, which is probably further distinguished under conditions of LPS-priming.

As discussed briefly above, priming enhances the cell death phenotype of macrophages in response to sterile particles. Even though it has been shown in other cell types that mild LMD leads to apoptosis, while severe LMD can lead to necrosis, this may not be true in LPS-primed macrophages(320). Even though the MTS cell death assay quantifies a loss of mitochondrial metabolism/function during apoptosis and necrosis, I found that particle-induced cell death measured



by the LDH assay (which typically measures lytic/necrotic cell death) was often equivalent to that measured by MTS. Therefore, in LPS-primed macrophages, a more pro-inflammatory necrosis-like phenotype seemed to predominate (even with apoptotic stimuli like STS, doxorubicin and etoposide). Of course, more direct techniques are likely required to examine whether there is actually a mixture of apoptosis, necrosis secondary to apoptosis, necrosis, and pyroptosis. Nonetheless, particle/LMD-induced cathepsin-dependent cell death in LPS-primed macrophages seems to represent a predominantly necrosis-like phenotype. The dependence of this cell death pathway on cathepsins and not inflammasomes suggests that it is a distinct and dominant pathway concomitant with pyroptotic cell death. The fact that particle-induced lytic cell death occurs in the absence of inflammasomes in LPS-primed macrophages does not necessarily mean that pyroptosis does not normally occur as the dominant cell death response to sterile particles when inflammasomes are present. If particles are inducing NLRP3 and caspase-1-dependent IL-1 $\beta$  activation, then they are probably also inducing pyroptosis. However, in the absence of inflammasomes, cathepsin-dependent cell death mechanisms may occur in lieu of pyroptotic cell death mechanisms. While this is just speculation, determining how these two pathways for lytic cell death interact with one another in LPS-primed macrophages is a matter for future investigation. Since it seems that this phenomenon has not been given adequate attention in the inflammasome literature, I propose that, in LPS-primed macrophages, cathepsin-dependent

necrosis-like cell death should be given a name, such as “cathosis” (and cathepsin-dependent apoptosis-like cell death could be referred to as “catheptosis”). This terminology may help distinguish this important and distinct pathway for inflammatory cell death and facilitate a more rigorous scientific discourse into the relationship between cathosis and disease-associated inflammatory processes.

Finally, I should mention some work that has preceded my study on the subject of cathepsin-dependent particle-induced cell death in LPS-primed macrophages. There are other reports that provide genetic evidence for cathepsins in cell death during NLRP3 activation, but I do not believe my work is simply a reproduction of these reports. One of these studies found only minor reductions in cell death using cathepsin B siRNA in LPS-primed microglia stimulated with chromogranulin A for 36h(161). Therefore, the cell type, phenotype, time-point and stimulus are distinct from those I examined. Another study reported that alum-induced cell death was completely dependent on either cathepsin S or cathepsin B(155). They offered no explanation for this finding. Indeed, my work suggests that neither cathepsin B nor S depend on one another for their own activity and that cathepsin B or S alone are not essential for particle-induced cell death. However, this may be a special case for alum that does not apply to silica. This latter study also found no role for cathepsins X, H and C in alum-induced cell death and no role for cathepsins S or B in LLOMe-induced cell death. Instead, they did find that cell death induced by LLOMe was completely

dependent on cathepsin C. In fact, this same group has reported this same finding in three separate studies(155, 163, 405). In their most recent report (and in the other two as well), their interpretation of this result is such that they believe cathepsin C mediates every process downstream of LMD. However, despite them citing papers describing how LLOMe works, they do not seem to either acknowledge or realize that LLOMe relies predominantly on cathepsin C inside lysosomes to become active and initiate LMD in the first place(328, 329). Consistent with my own observations, this same group has also reported that LMD induced by LLOMe and alum in LPS-primed macrophages leads to inflammasome-independent cell death, showing this data in two separate reports(155, 163). However, they did not show this data side-by-side with a productive IL-1 $\beta$  response (the data they did show with LLOMe and alum in one of these papers show that these reagents induced almost no IL-1 $\beta$  secretion), so it is difficult to verify whether these stimuli were activating the inflammasome in any of their experiments. In fact, their conclusion was that cathepsins are not involved in inflammasome activation at all, but instead, that they are involved in necrosis. Therefore, although I am not the first to report this cathepsin-dependent, inflammasome-independent cell death phenomenon in LPS-primed macrophages, I would argue that my data provides a single, accurate and thorough examination of this issue that did not exist prior to my investigation.

## ***Importance of Priming for NLRP3 Activation***

The importance of Signal 1/priming for NLRP3 activation is often unappreciated in the literature. I found a number of reagents that reduce priming, including cathepsin inhibitors, ETC uncouplers and KCl, which have been repeatedly cited by various studies as having discrete effects on Signal 2 of NLRP3 activation **(see Appendices 13 & 14)**. The effects of these reagents on Signal 1 have not been appreciated by such studies. There are many more inhibitors that also reduce priming and/or NF- $\kappa$ B activation. I will not go into all of them here, but it is worth noting the few that have been clearly recognized in studies that have examined mechanisms of NLRP3 activation. ROS inhibitors, IL-10 and Type I IFNs (acting via IL-10) can all block transcriptional priming(184, 239). Moreover, BRCC3 deubiquitinase inhibitors can block transcription-independent priming(226, 229, 230). However, careful attention to Signal 1 in studies examining mechanisms of NLRP3 activation is the exception, not the rule.

It is not surprising that the distinction between Signal 1 and Signal 2 is not fully understood. This relationship is complex. For instance, some stimuli provide Signal 1 and Signal 2. The clearest example of this occurs during stimulation with some pathogens that can both prime and activate NLRP3, like bacteria or viruses(74, 447). Alternatively, other stimuli induce Signal 2 responses that have a unique requirement for a particular facet of Signal 1 in order to activate NLRP3. For example, gram-negative bacteria not only induce priming on their own via LPS, but they also require TRIF downstream of LPS priming to induce Type I

IFN-dependent caspase-11 production in order to activate NLRP3(74). In this example, gram-negative bacteria are uniquely dependent on TRIF signaling, which is largely independent of MyD88-dependent pro-IL-1 $\beta$  synthesis. Therefore, examining pro-IL-1 $\beta$  synthesis or NF- $\kappa$ B activation are not the only transcriptional facets of Signal 1 that are important for Signal 2 of NLRP3 activation for certain stimuli. In fact, overlooking other facets of Signal 1 that are particular to NLRP3 activation in general, like NLRP3 synthesis, have led to much confusion in the field.

Generally, studies examining whether an inhibitor or variable is specifically affecting the mechanism for Signal 2 of NLRP3 activation have relied on NLRP3-independent stimuli as negative controls. This method makes two key assumptions, which are as follows:

- 1) Anything that affects priming will affect all inflammasomes equally.
- 2) Any variable that affects NLRP3-dependent IL-1 $\beta$  activation without affecting NLRP3-independent IL-1 $\beta$  activation must play a specific role in Signal 2 of NLRP3 activation.

Since a requirement for *de novo* NLRP3 transcription is a facet of priming unique to NLRP3 inflammasomes, the first assumption is false(67). Moreover, this principle can be extended to non-transcriptional priming mechanisms that are also unique to NLRP3(226). Because the first assumption is false, the second assumption is also false. Given a differential dependence on Signal 1, a lesser

degree of Signal 1 suppression is likely necessary to reduce NLRP3 activation before Signal 1 also becomes limiting for other inflammasomes. Therefore, there is a window of suppression where one may find that NLRP3 activation is reduced, while activation of another inflammasome is not. Unless a wide range of conditions is tested for any variable, one cannot conclude that Signal 1 is not being affected just because a single condition shows that the variable only affects NLRP3-dependent IL-1 $\beta$  secretion. For example, the K<sup>+</sup> study (discussed earlier) made the two above assumptions(154). They show that elevated extracellular KCl (45 mM) does not affect dAdT induced IL-1 $\beta$  activation. Indeed, I also find this to be the case using 40 mM of KCl (**see Appendix 14a**). However, I found that higher concentrations (80 mM) actually affect dAdT as well (**see Appendix 14b**). While the K<sup>+</sup> study states that higher concentrations of KCl are toxic, I did not find any increase in cell death caused by 80 mM of KCl. I did not see any reduction in cell death either, once again reinforcing my conclusion that particle-induced cell death does not depend on NLRP3; this is assuming that KCl does, in fact, block Signal 2 of NLRP3 activation. Therefore, KCl suppresses NLRP3-mediated IL-1 $\beta$  secretion more than that mediated by AIM2 inflammasomes (activated by dAdT), but its effects are not exclusive to NLRP3. Indeed, other studies have shown that high extracellular KCl also inhibits the NLRP1 inflammasome(133). Therefore, I believe KCl actually blocks Signal 1, as will be discussed in more detail shortly. Indeed, any variable affecting Signal 1 is likely to have effects that are exclusive to NLRP3 activating stimuli under certain

conditions, leading some studies to reach the conclusion that the variable they are examining is specific to the mechanism for Signal 2 of NLRP3 activation. The reality of the situation appears to be much different.

The available data indicate that Signal 1 and Signal 2 do not play discrete roles in NLRP3 activation. Instead, Signal 1 and Signal 2 appear to be continuous events. This continuity is evident at the functional level (Signal 2 for NLRP3 activation depending more on Signal 1 than other inflammasomes), as just described above, but it is also evident temporally. Priming seems to continue for much longer than just a few hours. Even six hours after priming, I found that inhibition of protein synthesis with CHX for three additional hours significantly reduced pro-IL-1 $\beta$  synthesis, as well as IL-1 $\beta$  and TNF- $\alpha$  secretion. Indeed, in standardizing this assay, I found that pro-IL-1 $\beta$  levels inside cells continue to increase for almost 9h after priming with LPS (data not shown). Moreover, a continuous replenishment of the intracellular pool of IL-1 $\beta$  is evident when examining IL-1 $\beta$  activation by immunoblotting (**see Chapter III, Fig. 6**). After 3h of priming and 6h of IL-1 $\beta$  secretion, the pool of intracellular pro-IL-1 $\beta$  is not necessarily drained by IL-1 $\beta$  secretion compared to LPS priming alone. For IL-1 $\beta$  secretion to occur without pro-IL-1 $\beta$  levels dropping inside cells, intracellular pro-IL-1 $\beta$  stores must be continuously replenished as IL-1 $\beta$  is secreted. Since it has been shown that expression of hyperactive NLRP3 mutants in HEK293 cells also exhibit elevated NF- $\kappa$ B activity, inflammasomes might somehow provide positive feedback that primes pro-IL-1 $\beta$  synthesis(416). Surprisingly, this does

not seem to be an autocrine effect of IL-1 $\beta$  or TNF- $\alpha$ , as one might expect. Instead, I have shown that IL-1R1 $^{-/-}$  and TNF-R1&II $^{-/-}$  macrophages exhibit no defect in IL-1 $\beta$  secretion (**see Appendix 15b,c**). Moreover, I have shown that the various inhibitors I mentioned above suppress pro-IL-1 $\beta$  synthesis in macrophages treated only with LPS, so these effects are not a result of suppressing inflammasome-driven positive feedback. Therefore, not only is Signal 1 continuous on a functional level with NLRP3 activation, but Signal 1 (or downstream effects of Signal 1) also persists at some level throughout inflammasome activation for at least nine hours after LPS treatment.

Rather than being a discrete event, Signal 1 overlaps or is continuous with Signal 2, making it difficult to examine Signal 2 in isolation. However, by blocking protein translation during priming with CHX, I was able to stop transcription-dependent priming prior to providing Signal 2 (assuming mRNA does not itself play a role in these responses without being translated into protein). After stopping transcription-dependent priming, cathepsin inhibitors were still able to reduce silica and nigericin-induced IL-1 $\beta$  secretion. This suggested a role for cathepsins in NLRP3 activation. However, despite inhibiting pro-IL-1 $\beta$  synthesis, K777 suppressed silica-induced IL-1 $\beta$  secretion more than that induced by nigericin. Conversely, Ca074Me reduced IL-1 $\beta$  secretion induced by silica and nigericin to a similar degree. The reason for this seems to relate to different dependencies on cathepsin X (nigericin-induced IL-1 $\beta$  secretion depends more on cathepsin X, which is inhibited more effectively by Ca074Me than by K777). Although



cathepsin inhibitors reduce IL-1 $\beta$  secretion induced by these NLRP3-activating stimuli in the absence of transcriptional priming, this does not prove that they are inhibiting Signal2 for NLRP3 activation. Since I know that non-transcriptional priming plays a role in NLRP3 activation, I must consider the possibility that cathepsin inhibitors suppress NLRP3 activation by inhibiting non-transcriptional priming(226, 229, 230). Indeed, my data seems to suggest that this is the case since I observed that the role of cathepsins in Signal 1 was specific to IL-1 $\beta$  without affecting other NF- $\kappa$ B-dependent cytokines.

Although I already emphasized the special requirement of NLRP3 transcription for NLRP3 activation, it should be noted that LPS-induced transcriptional and post-transcriptional priming is not a singular event. To the contrary, LPS-priming appears to be diverse and multifaceted. For example, one of my more perplexing findings was that cathepsin inhibitors suppress pro-IL-1 $\beta$  (and to some extent IL-6 and MCP-1) production induced by LPS, but they do not suppress TNF- $\alpha$  or RANTES synthesis (**see Appendix 10a,b**). Presumably, my observation that the steady-state pools of *IL1b* and *Nlrp3* transcript are affected by cathepsin inhibitors and ETC uncouplers suggests that NF- $\kappa$ B activation was affected. This is also suggested, and even demonstrated, by the findings of another study examining the effects of the cathepsin inhibitor Z-FA-fmk(399). However, the fact that cathepsin inhibition did not affect all NF- $\kappa$ B-dependent cytokines indicates additional layers of complexity to these pre- and post-transcriptional priming events. In recent years, the complexity of NF- $\kappa$ B-mediated

gene regulation and crosstalk with other signaling pathways and transcription factors has been gaining appreciation(448). In fact, there are five members of the NF- $\kappa$ B family that play different roles in transcriptional regulation dependent on prevailing signaling pathways that can lead to a canonical, non-canonical and atypical pathway of NF- $\kappa$ B gene transcription. Perhaps, cathepsin inhibitors affect only one part of this pathway and not others. Alternatively, cathepsin inhibitors may differentially affect post-transcriptional regulatory mechanisms governing the stability or degradation of these cytokines. Before considering this possibility, I would like to propose a possible upstream pathway to provide some context.

Based on my data, it seems that lysosomal-mitochondrial crosstalk may contribute to the priming of pro-IL-1 $\beta$  and NLRP3 synthesis. LPS has been shown to induce mild LMD, the production of mitochondrial ROS(123, 124, 181-183), K<sup>+</sup> efflux(188-192), and metabolic changes that cause cytosolic acidification(449). I have shown that LPS also causes mild LMD and mild mitochondrial depolarization, which is blocked by cathepsin inhibition with K777. Indeed, ROS inhibitors have been shown to block priming, and mitochondrial ROS have been suggested by multiple reports to be important for NF- $\kappa$ B activation(184, 450). Moreover, I found that inhibition of cathepsins, the ETC, or K<sup>+</sup> efflux also blocks priming. Since I also found that ETC uncoupling can inhibit priming without affecting cathepsin activity, the preponderance of existing data suggests that mitochondria are downstream of cathepsin-dependent priming events. Therefore, K777 may inhibit priming by inhibiting cathepsin-dependent

pro-death Bcl-2 family member activation necessary to induce mitochondrial ROS-mediated NF- $\kappa$ B activation. How K<sup>+</sup> efflux fits into all of this may need to be resolved by further experimentation, but K<sup>+</sup>/H<sup>+</sup> exchange at the plasma membrane could potentially facilitate cathepsin activity in the cytosol through cytosolic acidification(202). Indeed, cytosolic acidification is another well-documented effect of LPS priming (via the Warburg Effect) and a phenomenon closely associated with inflammatory cell types(449). Given the apparent involvement of LMD, cathepsins, the ETC, ROS, and K<sup>+</sup> efflux in priming, claiming these processes as events exclusive to Signal 2 of NLRP3 activation should require extensive efforts to differentiate Signal 1 from Signal 2.

Having proposed the lysosomal-mitochondrial LPS-priming pathway above, the role of cathepsins in regulating pro-IL-1 $\beta$  synthesis, and not the synthesis or TNF- $\alpha$ , may be explained by the following. Besides cytosolic acidification, the LPS-induced Warburg Effect causes another metabolic disturbance that directly relates to pro-IL-1 $\beta$  synthesis. By increasing glutamine metabolism via the mitochondrial TCA cycle, LPS induces an elevation in the glutamine metabolite succinate in the cytosol via the mitochondrial transporter VDAC(451). While mitochondrial ROS can promote IL-1 $\beta$  transcription by inducing NF- $\kappa$ B-dependent HIF-1 $\alpha$  synthesis, succinate is necessary to stabilize the intracellular pool of HIF-1 $\alpha$ (451-453). Under these conditions, stabilized HIF-1 $\alpha$  can act as a transcription factor to directly initiate late/sustained pro-IL-1 $\beta$  transcription without affecting the production of TNF- $\alpha$ (451). Therefore, during

LPS-mediated mild lysosome disruption, it is possible that cathepsins initiate mitochondrial stress via the Bcl-2 family, leading to ROS production, HIF-1 $\alpha$  synthesis and stabilization, and the selective promotion of pro-IL-1 $\beta$  synthesis. Presumably, severe mitochondrial disruption prevents further ROS production and succinate-mediated stabilization of HIF-1 $\alpha$ , thereby abbreviating pro-IL-1 $\beta$  synthesis. It is also possible that, via the above lysosome→cathepsin→mitochondria pathway, cathepsins can somehow specifically enhance the stability of *IL1b* mRNA. Indeed, it is well-known that the steady-state pool of *IL1b* mRNA is stabilized by LPS-signaling(454). Additionally, it may also be the case that other variables, which have been previously implicated in Signal 2 of NLRP3 activation, actually roles related to the above IL-1 $\beta$ -specific LPS-priming pathways that have been overlooked.

While it may not be fully understood, the distinction between Signal 1 and Signal 2 is often not given appropriate consideration in experimental approaches. Many tools used to examine Signal 2 of NLRP3 activation, like fluorescent indicator dyes, have been used in such a way as to mistake Signal 1-related phenomena as being components of Signal 2. These tools depend on measuring signal changes that are relative to a given baseline, and this baseline is often chosen after priming. For instance, the K<sup>+</sup> study examines K<sup>+</sup> efflux and ROS production relative to a baseline generated in LPS-primed macrophages(154). If it were true that priming is completed within a few hours after LPS stimulation, and no longer playing any role in NLRP3 activation, choosing an LPS-primed

baseline instead of an untreated baseline is a reasonable approach to examining Signal 2 of NLRP3 activation. However, as I have shown, LPS-priming causes changes in both lysosomal integrity and MMP that are observed only when data are plotted relative to untreated (unprimed) controls. As a result, in the K<sup>+</sup> study, the effects of priming on K<sup>+</sup> efflux, and of K<sup>+</sup> efflux on priming, were not measured. Even though K<sup>+</sup> efflux is a known effect of LPS-signaling in macrophages and a variety of other cell types(188-192), the K<sup>+</sup> study did not measure LPS-induced K<sup>+</sup> efflux or consider its role in the priming of NLRP3 activation. Conversely, I examined this and found that high extracellular KCl does indeed affect IL-1 $\beta$  synthesis and that KCl can non-specifically inhibit both NLRP3 and AIM2-mediated IL-1 $\beta$  secretion (**see Appendix 14**). Another recent study claims that K<sup>+</sup> efflux is induced by the interaction of crystals with plasma membrane, and that IL-1 $\beta$  activation induced by this process can be inhibited with high extracellular KCl or ion channel blockers(455). However, while they do not examine pro-IL-1 $\beta$  levels for the ion channel blockers, in the only western blot in which they show pro-IL-1 $\beta$  levels and KCl treatment, pro-IL-1 $\beta$  is dramatically reduced. Therefore, when investigating any cellular dynamic for its role in NLRP3 activation, unless already established, the baseline measurement should be untreated, unprimed controls. Alternatively, pro-IL-1 $\beta$ , intracellular IL-1 $\beta$  or some other indicator of priming should be measured in all experiments.

Given all of the issues I just examined, it is also possible that Signal 1 and Signal 2 are fundamentally inseparable events. Perhaps, LPS-signaling alone is

insufficient to provide a strong enough signal to activate NLRP3, and thus, Signal 2 may augment Signal 1 enough to reach the threshold for NLRP3 activation. This has been suggested previously by studies showing that overexpression of NLRP3 causes spontaneous activation of the NLRP3 inflammasome(185, 456). In this sense, activators of NLRP3 may only be differentiated from one another based on what complementary components of Signal 2 they provide to augment Signal 1 and/or the upstream mechanisms of how they induce these cellular changes.

### ***Silica Vs. Nigericin-induced NLRP3 Activation***

Particles require phagocytosis and lysosomal acidification to activate NLRP3(144). This indicates a role for lysosomal proteases or lysosomal acidity itself in NLRP3 activation. Indeed, as I have shown, inhibiting lysosomal acidification reduces silica-induced IL-1 $\beta$  secretion more than it affects that induced by nigericin (**see Appendix 13**). However, if lysosomal acidity were equally important for both stimuli, they would both be affected equally. Since they are not affected equally, lysosomal acidity is not equally important for both stimuli. Here, I hypothesized that cytosolic acidification following LMD contributes to NLRP3 activation, which might explain why lysosomal acidity may more important for particle-induced NLRP3 activation (269). Nigericin, on the other hand, induces K<sup>+</sup> efflux, which is known to induce cytosolic acidification via H<sup>+</sup>/K<sup>+</sup> exchange at the cell membrane(201, 202). Therefore, the reason why

inhibition of lysosomal acidification affects silica-induced IL-1 $\beta$  activation more than that induced by nigericin may be linked to their different methods of cytosolic acidification.

While K<sup>+</sup> efflux and the disruption of lysosomes or mitochondria have all been implicated in NLRP3 activation, they are also known mechanisms for acidifying the cytosol(269, 457, 458). Moreover, the cytosol acidifies during apoptosis in response to a variety of stimuli, and nigericin is often used in these studies as a positive control for cytosolic acidification(457). Furthermore, it has been shown that apoptotic stimuli induce cytosolic acidification via Bax-induced mitochondrial alkalinization, which is inhibited by OMA(458). In agreement with that study, a subsequent study showed that Bcl-2 overexpression in transgenic mice, or OMA treatment, prevents cytosolic acidification and protects against ischemia-reperfusion injury(459). This same study found that Bcl-2 directly associated with and inhibited VDAC activity, which is interesting since it has also been shown that cytosolic acidification dramatically increases VDAC activity and the MMP(460). The fact that all of the variable that affect cytosolic acidification also seem to influence NLRP3 activation may not be coincidental.

In contrast to inhibition of lysosomal acidification, KCl affects nigericin-induced IL-1 $\beta$  secretion more than it affects that induced by silica (**see Appendix 13**). As expected for a K<sup>+</sup> ionophore, this suggests that nigericin is more dependent on K<sup>+</sup> efflux than silica, which likely acidifies the cytosol via LMD independently of K<sup>+</sup> efflux mechanisms. I also found that nigericin and ATP

(which also induces K<sup>+</sup> efflux) induce rapid loss of lysosomal pH gradients (likely by cytosolic acidification) and loss of lysosomal cathepsin activity (**see Appendix 16**). This suggests that cathepsin activity is not essential for NLRP3 activation by non-particulate stimuli, but that cytosolic acidification is common to both particulate and non-particulate stimuli. Furthermore, when I incubated macrophages in basic medium, which alkalinizes the cytosol, silica and nigericin-induced IL-1 $\beta$  secretion were reduced equally (**see Appendix 17a**). When I incubated macrophages in acidic medium (RPMI lacking bicarbonate), silica and nigericin-induced IL-1 $\beta$  secretion are enhanced equally (**see Appendix 17b**). Although this may relate to Signal 2 in some way, this is all more likely due to effects on Signal 1, since IL-1 $\beta$  production is clearly reduced in basic medium. Moreover, dAdT is affected similarly in both of the conditions of altered pH described above. Cytosolic acidification will be discussed in more detail again below. For now, I would like to emphasize that Signal 1 and Signal 2 are difficult to differentiate from one another, while the upstream mechanisms of NLRP3 activation (direct LMD or direct K<sup>+</sup> efflux) can be distinguished for different stimuli.

Incidentally, I observed that in RPMI without bicarbonate CaCl<sub>2</sub> is unable to activate NLRP3. The reason for this seemed to be that without bicarbonate, CaCl<sub>2</sub> does not precipitate with as a particulate carbonate salt, a finding that was corroborated by a subsequent study and raised questions about whether the



release of intracellular calcium stores in certain mediums (like RPMI) can cause precipitation and NLRP3 activation(154).

Although the upstream mechanisms described are recognizably distinct from one another, I found a more downstream variable important of NLRP3 activation that distinguishes silica from nigericin (also, see discussion on cathepsin X below). IL-1 $\beta$  secretion induced by silica seems more dependent on the ETC than nigericin or dAdT. I know this because, like cathepsin inhibitors, ETC uncouplers reduce pro-IL-1 $\beta$  synthesis despite suppressing silica-induced IL-1 $\beta$  secretion more than that induced by nigericin or dAdT. On one hand, it makes sense that these inhibitors reduce silica-induced IL-1 $\beta$  secretion more than they reduce dAdT-induced IL-1 $\beta$  secretion, since NLRP3 must be synthesized during priming prior to NLRP3 inflammasome activation. On the other hand, it is surprising that these inhibitors do NOT reduce nigericin-induced IL-1 $\beta$  secretion more than they reduce that induced by dAdT, since nigericin also depends on NLRP3 synthesis during priming in order to induce IL-1 $\beta$  secretion. While K777 also preferentially suppressed silica-induced IL-1 $\beta$  secretion compared to that induced by nigericin, the special non-redundant involvement of cathepsin X in nigericin-induced NLRP3 activation seemed to explain this difference to some degree (K777 is not a great inhibitor of cathepsin X). The situation appears to be different for ETC uncouplers. OMA and AntA uncoupled the ETC and reduced silica-induced IL-1 $\beta$  secretion without affecting cathepsin activity or nigericin-induced IL-1 $\beta$  secretion. Therefore, regarding the particulate

bias of ETC uncouplers, cathepsins do not seem to explain this difference. Whatever the reason may be, there is something about the ETC that is especially important for particle-induced IL-1 $\beta$  secretion and this could relate to either effects on Signal 1 or 2. A recent study reported cathepsin-dependent mitochondrial dysfunction and ROS production follows virus-induced LMD(339) and another study using RNA viruses found, as I have, that complete loss of MMP antagonizes NLRP3 activation(224). Therefore, it seems that mitochondrial ROS may be the factor generated by an intact, but not completely destroyed, ETC/MMP that is especially important for particle-induced NLRP3 activation. Indeed, I found that ROS inhibitors, especially the mitochondrial ROS inhibitor Mitotempo, preferentially reduce silica and LLOMe-induced IL-1 $\beta$  secretion (**see Appendix 11**). And, just like ETC uncouplers, Mitotempo did not reduce cell death induced by LLOMe or silica, indicating the ROS and ETC-dependent mechanism of particle-induced IL-1 $\beta$  secretion is downstream of cathepsins. However, like anything else, although I observed a preferential affect on silica and not nigericin, this does not necessarily mean that silica and nigericin do not depend more or less on different facets of Signal 1 that are being mediated by mitochondrial ROS.

Despite the inherent ambiguity of this system, a mechanistic role for ROS in NLRP3 activation is worth serious contemplation. I noticed that the LRR region of NLRP3 seems to have an unusual repeating motif of evenly spaced cysteines that is not present to the same degree in other LRRs, and this pattern has not

been recognized in the literature (**see Appendix 18**). Cysteines are easily oxidized by ROS, and those present on NLRP3 may become oxidized by mitochondrial ROS. Subsequently, they may also be reduced again by cytosolic acidification (via processes discussed below) and form disulfide bonds intra or inter-molecularly. Either by oxidation or oxidation followed by reduction of these cysteine may thereby cause conformational changes in NLRP3 that are important its NLRP3 activation, such as changes that permit cleavage of its LRR by cathepsins (as has been suggested to occur for the LRRs of TLRs 3,7 & 9)(400, 401). This is an intriguing hypothesis that warrants further investigation.

### ***Lysosomal tropism, Lysosomal pH & Cytosolic Acidification in IL-1 $\beta$ Activation***

Over the course of my investigation, I was forced to ask whether cathepsin inhibitors were acting via off-target effects. Off-targets of drugs are usually targets closely related or similar in function to on-targets, which might include other cathepsins, other cysteine proteases or proteases in general. In fact, while my collaborators at UCSF have used a cell-free system to screen a diverse panel of potential cysteine and non-cysteine protease targets for K777(personal communication), their screen failed to identify cathepsin X as a target of K777. This indicates that cell-free inhibitor screens do not always agree with *in situ* biochemistry. Therefore, it is difficult to know how many other closely related off-targets there might be for K777.

Besides active-site-based off-targets, another possibility for off-target drug effects is when the chemical moieties of a drug that are not part of the on-target binding interface (parts of the chemical not designed for a particular active site) bind in some way to other unrelated enzymes or molecules. While there are constraints limiting the variability of any small molecule inhibitor's on-target moiety, there can be much more variability in other parts of a small molecule. Therefore, it is unlikely for different inhibitors in the same class to have share off-targets unrelated to their intended targets, since their only common structural feature is their on-target moiety. And since I have shown that the effects of K777 and Ca074Me on particle-induced IL-1 $\beta$  secretion and cell death are reproduced by most of the 25 or more different cathepsin inhibitors I examined, K777 and Ca074Me most likely affect these responses via their on-target moieties. **(see Appendix 6)**. Conversely, these drugs may still have similar effects on particle-induced IL-1 $\beta$  secretion and cell death because they share a more general biochemical property. Indeed, one characteristic shared by most of these cathepsin inhibitors is that that they are amphiphilic lysosomotropic weak bases with secondary and tertiary amines.

It is well established that weak amphiphilic bases can cause dose-dependent increases in lysosomal pH(461-463). As mentioned in the introduction, when discussing lysosomotropic detergents, lysosomotropic agents are amphiphilic so they can pass through membranes(464). They are also weakly basic, causing them to become ionized and trapped in acidic

compartments(464). This ionization occurs via their acquisition of a hydrogen ion ( $H^+$ ) from hydronium ( $H_3O^+$ ), leaving one less  $H^+$  to contribute to the acidity of the solution. Thus, these agents increase lysosomal pH. While the molecule-specific chemistries influencing the neutralizing property of weakly basic drugs are diverse, causing wide variations in the dose-to-pH relationship, the concentration of weak base necessary to increase lysosomal pH by 1 generally occurs within a range from  $\sim 1 - 1000 \mu M$ (461). Therefore, despite my genetic evidence for the roles of cathepsins in NLRP3 activation and priming, it seems that some additional off-target effects of these inhibitors may be achieved by their influence on lysosomal pH. To investigate this, I made a short-list of amphiphilic drugs that are weak bases and began to randomly probe the literature for reports these drugs have anti-inflammatory properties. What I found was surprisingly in line with my suspicions.

A landmark paper for the IL-1 field came out in 1990, discovering that IL-1 $\alpha$  and IL-1 $\beta$  are secreted via an unconventional route (Cytoplasm $\rightarrow$ Secreted instead of ER $\rightarrow$ Golgi $\rightarrow$ Exosome $\rightarrow$ Secreted)(465). This same study also noted that a classic lysosomotropic weak base, methylamine ( $CH_3NH_2$ ), inhibited IL-1 $\beta$  secretion. Another classic agent known to raise lysosomal pH, chloroquine, has also been shown in numerous studies to reduce IL-1 $\beta$  and IL-6 expression(466). Moreover, another study found that a whole class of newly-synthesized diaminic carbonates, which are diprotic weak bases, protected mice from LPS-induced shock(467). Though this study noted a striking similarity between the effects of

these diaminic carbonates and those they observed for chloroquine, they were unable to figure out exactly why these both types of compounds were reducing inflammatory responses.

This theme of lysosomotropic weak bases having unexplained anti-inflammatory effects is pervasive. Time and again, various studies have attempted to connect the on-target effects of weakly basic drugs with their anti-inflammatory properties. In contrast, I recognized through meta-analyses that anti-inflammatory properties are common features of weakly basic drugs. For example, Imatinib (Gleevec) is a treatment for chronic myelogenous leukemia, which targets several kinases (such as c-kit and c-abl) involved in driving malignant proliferation(468). Moreover, studies with imatinib have noticed that it has profound anti-inflammatory properties. One study found that it prevents TNF- $\alpha$  production and NF- $\kappa$ B activation by inhibiting I $\kappa$ B phosphorylation and that it also protects mice from LPS-induced liver failure(469). This study attributes the kinase inhibitory activity of imatinib to this effect, and this may be accurate. However, no specific target has since been attributed to this effect of imatinib. As another example, the anti-depressant imipramine (also a weak amphiphilic base) was shown as early as 1966 to have potent anti-inflammatory effects in a rat model of arthritis(470). Moreover, various macrolide antibiotics, like azithromycin, have been shown to possess anti-inflammatory properties, such as reducing neutrophil/macrophage chemotaxis and the expression of IL-1, IL-6 and TNF- $\alpha$ (471). The list goes on. Again, the anti-inflammatory mechanism of action for

these drugs is unknown, but they are all known to be amphiphilic weak bases (usually amines)(463). Here, I propose that their effects are at least partly due to increasing lysosomal pH and that this should be considered in future drug and inhibitor-based studies.

Intriguingly, prior to finding an association between these weakly basic drugs and their anti-inflammatory properties in the literature, I had already begun testing this hypothesis using several of these lysosomotropic drugs. Out of a random sample of lysosomotropic drugs, I found that they all had the ability to inhibit pro-IL-1 $\beta$  synthesis as well as particle-induced IL-1 $\beta$  secretion and cell death (**see Appendix 13a**). Moreover, I found similar effects using lysosomotropic dyes, indicating that the use of these dyes to study lysosomal function in biological processes must be done carefully and in parallel with other confirmatory techniques. Whether a study examines a lysosomotropic dye or drug, the resulting data should be interpreted with due consideration for the effects of these chemicals on lysosomal pH.

Our genetic evidence for the role of cathepsins in pro-IL-1 $\beta$  synthesis and cell death suggests that neutralization of pH by lysosomotropic weak bases affects these processes by inhibiting the pH-dependent activity of cathepsins. However, there are ~60 different lysosomal hydrolases and 25 known lysosomal membrane proteins, most of which rely on acidic lysosomal pH(472). Therefore, it seems likely that some additional off-target effects of these inhibitors may be achieved by their influence on lysosomal pH and these other lysosomal proteins.

However, as I already touched upon, several big questions still remains to be answered: Why does lysosomal pH not matter for non-particulate NLRP3 activators like ATP and Nigericin, or AIM2 activation by dAdT, as much as it does for particulate NLRP3 stimuli? Is the acidity of the lysosomal fluid itself important for pro-IL-1 $\beta$  synthesis or particle-induced IL-1 $\beta$  secretion and cell death?

A handful of studies have made a connection between cytosolic acidification and NLRP3 activation (see also the section on silica and nigericin above). One study showed that extracellular acidosis activates NLRP3(473). In this study, acidic medium caused rapid cytosolic acidification and NLRP3 activation, which depended on K<sup>+</sup> efflux (which is likely due to H<sup>+</sup>/K<sup>+</sup> exchange at the cell membrane(202)), while basic medium suppressed this response. Surprisingly, they also found that dissipation of lysosomal pH using BafA caused cytosolic acidification and activated IL-1 $\beta$ . Additionally, it has also been shown that the influenza ion channel protein M2 activates NLRP3 by causing cytosolic acidification(474). The M2 channel was shown to translocate to the golgi in order to achieve this effect on cytosolic pH. Given this evidence, it seems that cytosolic acidification may be an important mechanism of NLRP3 activation. Indeed, the relationship between cytosolic acidification and inflammation is pervasive.

Cytosolic acidification occurs under conditions known to favor inflammatory states. A phenomenon called the Warburg Effect takes place in inflammatory macrophages and DCs, and is characterized by a switch from oxidative phosphorylation to glycolysis(449). The opposite is true for M2 (anti-



inflammatory) macrophages and regulatory T cells. This change in cellular metabolism (oxidative phosphorylation → glycolysis) can be induced by stimuli like LPS via TLR4 (via NF-κB) and is suppressed by anti-inflammatory cytokines like IL-10. Moreover, enhanced glycolysis leads to more mitochondrial ROS production (more NADH to complex I and less oxidative phosphorylation leaves more mitochondrial respiratory chain components in the reduced state that react with O<sub>2</sub>) and a shunting of pyruvate into lactic acid, thereby acidifying the cytosol. Interestingly, the Warburg Effect also occurs in cancer cells. As a result of their disruption of normal tissue architecture, tumors tend not to be well vascularized. This leads to hypoxia, so cancer cells switch to glycolysis as their primary mechanism for ATP generation. Given that cathepsin inhibitors, like K777, have been under investigation as anti-cancer therapeutics(385), and here as anti-inflammatory therapeutics, this connection may be relevant to my study.

We observed in earlier experiments with immortalized (cancerous) bone-marrow-derived macrophages (IMMPs) that cathepsin B&L double deficiency resulted in complete loss of particle and nigericin-induced IL-1β secretion without affecting that induced by dAdT or TNF-α secretion (**Appendix 19**). Whether this is due to metabolic changes, altered cell death mechanisms, or reduced redundancy of cathepsins in these cancer cells is unknown. Therefore, this remains an intriguing phenomenon for future investigations.

## ***Cathepsin X in Nigericin-mediated NLRP3 Activation***

Although it used to be thought that cathepsin X was ubiquitously expressed, its expression has recently been shown to be restricted mostly to cells of the immune system(475). Moreover, its expression was also shown to be elevated in various malignancies and in the glial cells of patients with Alzheimer's disease(475). Importantly, cathepsin X contains integrin-binding motifs (RGD), and it is known to bind to heparin sulfate proteoglycans that regulate integrin signaling(476, 477). Integrins are important for forming focal adhesions between cells or with the extracellular matrix (ECM), which induces actin cytoskeletal rearrangements important for facilitating cellular signaling during migration, proliferation and differentiation(478). Cathepsin X interacts with and regulates  $\beta$ 3-integrins on monocytes and macrophages as well as  $\beta$ 2-integrins involved in adhesion and phagocytosis on T cells (LFA-1/CD11a) and monocytes (Mac-1/CD11b)(274). Therefore, cathepsin X has important roles in regulating integrin-mediated signaling in immune cells.

Integrins are critical players in inflammatory responses. Mac-1 (CD11b) is known to regulate a variety of macrophage processes like phagocytosis and the oxidative burst, but it has also been shown to regulate TLR-induced NF- $\kappa$ B activation(479). Moreover, fibrinogen binding by Mac-1 can stimulate chemokine production by macrophages through TLR4(480). Integrins have also been shown to modulate caspase activation in neutrophils, and they play a critical role in host inflammatory responses carried out by leukocytes *in vivo*(481, 482). For

example, Mac-1 (and LFA-1 & VLA-4) is critical for neutrophil and monocyte extravasation during systemic inflammatory responses via its interaction with ICAM-1 on the endothelium(483, 484). Therefore, integrins play multiple roles in regulating cellular and *in vivo* inflammatory responses.

Recently, integrins have been directly implicated in NLRP3 activation. A recent study demonstrated that  $\beta$ 1-integrins could mediate Signal 1 of inflammasome activation in intestinal epithelial cells by binding to pathogenic bacteria, like *Yersinia enterocolitica*(447). Moreover, it has also been shown that  $\alpha$ 5 $\beta$ 1 integrin binds to the surface protein (Td92) of a periodontopathogen called *Treponema denticola*, which leads to the simultaneous priming and activation of NLRP3(485). Interestingly, cathepsins B and L have been shown necessary for ebolavirus to invade host cells and their ability to regulate this process requires  $\alpha$ 5 $\beta$ 1 integrin(486). Although cathepsin X may play a role in regulating  $\beta$ 1-integrin activity to induce NLRP3 activation, this has never been demonstrated.

Given the existing literature, and my finding that cathepsin X plays a non-redundant role specifically in nigericin-mediated NLRP3 activation, it seems that its integrin-related functions may mediate this role. As I just described, cathepsin X can regulate  $\beta$ 3 and  $\beta$ 2 integrins, which are known to play roles in inflammatory responses by regulating adhesion, migration and even TLR-mediated NF- $\kappa$ B activation in monocytes, macrophages, and neutrophils. Moreover,  $\beta$ 1 integrins have been suggested to play a direct role in NLRP3 activation and can also modulate cathepsin B and L activity. Whether cathepsins X, B & L play redundant

or cooperative roles in these processes is unknown. However, there seems to be a strong link between cathepsins, integrins and inflammation. Indeed, I found that cathepsin X activity was mostly extracellular, and that the impermeant extracellular cathepsin inhibitor JPM-565 was much more effective at reducing IL-1 $\beta$  secretion induced by nigericin than that induced by silica (**see Appendix 20b**). Moreover, when I examined macrophage responses on cellulose ester membranes instead of cell culture treated plastic, IL-1 $\beta$  secretion and cell death induced by silica and dAdT were normal (**see Appendix 20a**). Conversely, these responses to nigericin were completely absent. This suggests that cathepsin X regulates integrin-mediating signaling, which may be critical for the nigericin-induced pathway for NLRP3 activation. Moreover, because of cathepsin X's putative role in integrin-mediated signaling, inhibition of cathepsin X by K777 *in vivo* may have reduced IL-1-dependent neutrophil and monocyte recruitment into the peritoneum via interference with cell migration, adhesion, or extravasation. Therefore, the role of cathepsin X in nigericin-induced (and other) inflammatory responses remains an intriguing area of future research.

### ***An Evolutionary Perspective on LMD-Mediated NLRP3 Activation***

It is well known that a number of different pathogens (bacteria, viruses, parasites etc.) use the phagosome as a means of cell invasion(487). Usually, acidification of the phagosome upon fusion with the lysosome activates the pathogenic

invasion mechanisms. Alternatively, some pathogens prevent lysosomal fusion altogether in order to survive within the phagosome. Interestingly, cathepsins B and L have been shown to be critical for entry of corona-, filo- and paramyxoviruses into host cells, since these viruses require activation of viral glycoproteins required for membrane fusion(488). In fact, *in vitro* studies have shown that K777 can completely block Ebolavirus infection of host cells, or SARS (Severe Acute Respiratory Syndrome) coronavirus infection in combination with serine protease inhibitors(488). Thus, K777 is now a lead candidate for the treatment of Ebola hemorrhagic fever. There are several bacteria that escape phagosomes, including *Listeria monocytogenes*, *Shigella flexnari*, *Mycobacterium tuberculosis*, and *Streptococcus pyogenes*(487). Importantly, all of these bacteria have been shown to activate NLRP3 through LMD. *Listeria* does so via listeriolysin O (LLO) (375), *Streptococcus pyogenes* via streptolysin O (SLO) (489), and *Mycobacterium* via the pore-forming protein ESAT6 (490, 491). Despite activating NLCR4 inflammasomes, *Shigella flexnari* also activates NLRP3 via its type III secretion system(492). Although the innate immune system has PRRs designed to recognize these pathogens, many have evolved ways to disguise themselves(493). Moreover, after invasion, these pathogens have devised ways to co-opt or suppress the host's cell death and immune defense mechanisms(493-495). Therefore, it seems that LMD is the primary event that should incite a response from a macrophage being invaded by such a pathogen. The moment LMD occurs seems like the most advantageous time for secretion of

anti-inflammatory cytokines and lytic cell death, so that the invaded cells can call for help from other immune cells and destroy the intracellular niche of the pathogen. Conversely, waiting until a pathogen is well inside the cytoplasm, at which point it may or may not cause mitochondrial disruption, may be too late. By this time, pathogenicity factors may have already silenced the host cell's defense mechanisms, allowing the microbe to establish itself as a long-term resident. In line with this reasoning, I have shown that even mild LMD induced by silica, without significant MOMP, can cause a profound NLRP3 activation. However, as covered in the introduction, this seems to be a pathological response to silica that was meant for a pathogen.

The purpose of mitochondrial disruptive cell death seems to be much different than LMD-mediated cell death. It is well known that mitochondrial disruption is the primary organelle orchestrating the initiation of apoptosis, often employed by both the intrinsic and extrinsic pathways(294). Apoptosis is classically known to be non-lytic and non-inflammatory, as would be expected given its physiologic roles in cell turnover during development and tissue renewal in adult organisms(496). Apoptosis is therefore a process that cells have “decided” to initiate, rather than a reaction to an imminent threat. Indeed, I found that IL-1 $\beta$  activation was antagonized by early mitochondrial disruption. In fact, severe LMD induced by LLOMe caused robust mitochondrial disruption, which is probably the reason why LLOMe induced lots of cell death with little concomitant IL-1 $\beta$  secretion. Consistent with this observation, I also found that cathepsin C-

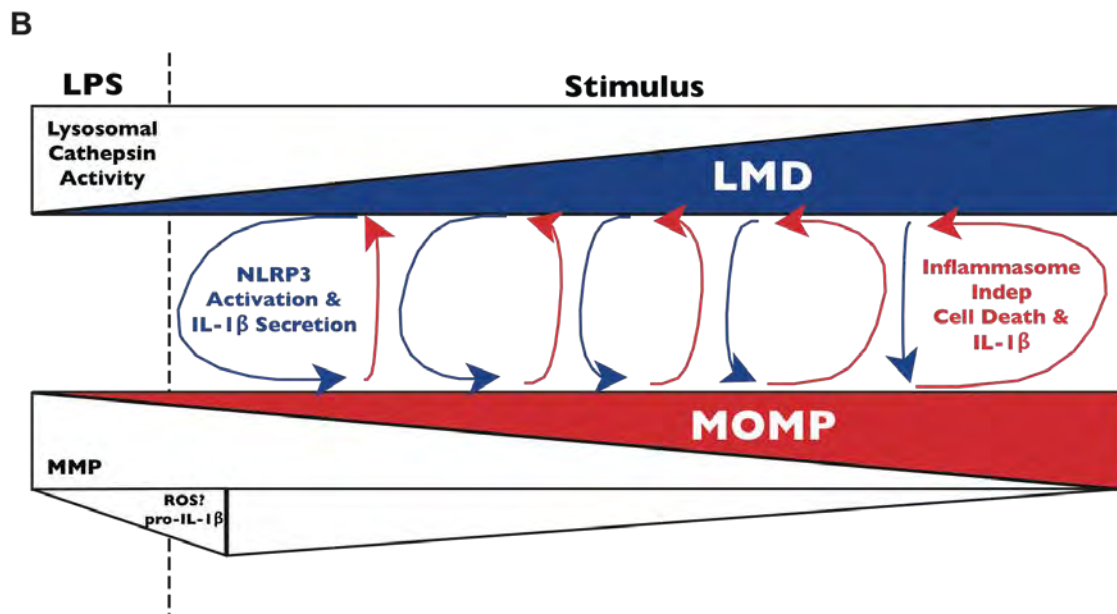
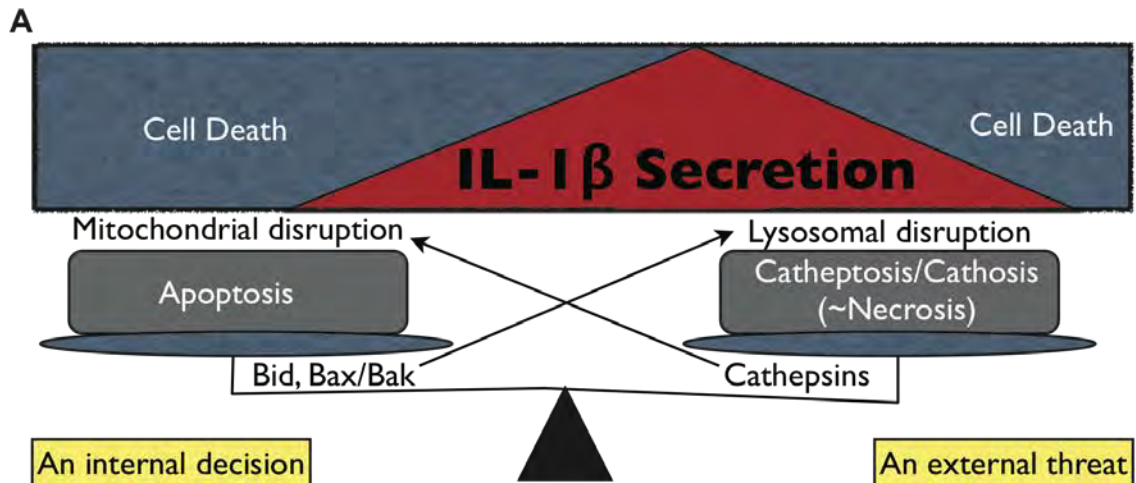
deficient macrophages, which less efficiently activate LLOMe-mediated lysosome disruption, actually secreted more IL-1 $\beta$  and experienced less cell death when they were stimulated with high concentrations of LLOMe (**see Appendix 21a**). This has been corroborated by another study as well(163). Similarly, it has been shown that completely inactive SLO mutants could not activate NLRP3, while partially active SLO mutants induced even better IL-1 $\beta$  secretion than WT SLO(497). Whether or not this is because WT SLO caused more mitochondrial disruption than partially active SLO mutants remains to be elucidated. Nonetheless, as a fundamental mechanism of apoptosis, mitochondrial disruption seems to antagonize inflammatory responses. If this is true, it has important implications for controlling inflammation in clinical contexts where cell death is desirable. For example, apoptosis is the desired response from chemotherapeutics in the treatment of cancer, since tumor lysis syndrome is the inflammatory and potentially deadly lytic alternative(498). Therefore, in formulating therapeutic strategies for cancer, especially hematologic malignancies like acute or chronic myelogenous leukemia, it may be appropriate to consider ways to disrupt mitochondria as directly as possible to prevent a robust inflammatory response.

**Chapter V, Figure 2:**

**A lysosomally dominant crosstalk involving mitochondria is optimal for particulate-mediated IL-1 $\beta$  secretion. (A)** The diagram depicts the mutual involvement of lysosomes and mitochondria in particle-induced IL-1 $\beta$  activation. Mitochondrial disruption (left) generally leads to apoptotic death. Although mild mitochondrial disruption subsequent to lysosomal disruption (right) occurs during NLRP3 activation, severe disruption of either the mitochondria or the lysosome leads primarily to cell death without IL-1 $\beta$  activation. Lysosomal disruption generally leads to both apoptotic (“Catheptosis”) and necrotic (“Cathosis”) phenotypes, though both types of cell death appear to be dependent on cathepsins. Cathepsins are known to cause mitochondrial disruption via activation of the Bcl-2 family (Bid, Bax/Bak), and the Bcl-2 family is known to cause or enhance lysosomal disruption either directly or through induction of mitochondrial ROS production leading to peroxidation of the lysosomal membranes via the Fenton Reaction. As apoptosis is an active process generally initiated through cell signaling and not severe organelle damage, this process represents an “internal decision” by cells to die rather than a defensive pro-inflammatory mechanism initiated by an “external threat,” as is the case with lysosome disruption. **(B)** The diagram shows a more direct relationship between the lysosomal and mitochondrial disruption. Mild LMD and MOMP occur during priming to increase pro-IL-1 $\beta$  synthesis and ROS production. When a stimulus is provided, LMD upstream of MOMP promotes NLRP3 activation and IL-1 $\beta$  secretion. But as LMD becomes more severe, an inefficient MOMP-dominant pathway of inflammasome-independent cell death and IL-1 $\beta$  activation occurs that antagonizes lysosomal cathepsin activity, NLRP3 activation, ROS production and pro-IL-1 $\beta$  synthesis. If this pathway begins with MOMP and not LMD, then NLRP3 is not activated at all by the time LMD is induced and lots of cell death occurs with concomitant low levels of IL-1 $\beta$  activation.



A lysosomally dominant cross-talk involving mitochondria is optimal for particulate-mediated IL-1 $\beta$  secretion



## ***Model of Particle-Induced NLRP3-mediated IL-1 $\beta$ Secretion and Cell Death***

### **Chapter V, Figure 3:**

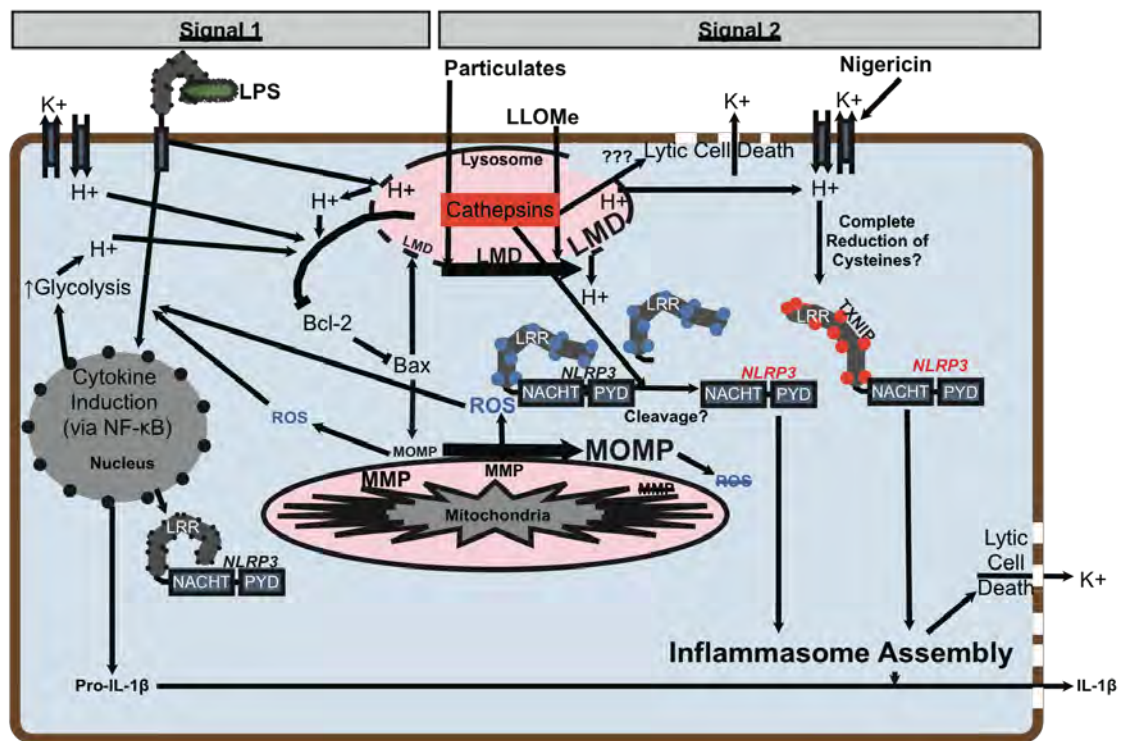
When a patrolling tissue macrophage encounters an inflammatory particle, it ingests the particle. Unable to digest the particle at first, it starts producing NADPH oxidase-dependent ROS and ATP required to increase its efforts to digest the particle. However, the particle's charged/oxidized surfaces can also generate ROS leading to further lipid peroxidation and LMD. LMD initiates cathepsin-dependent lytic cell death by both unknown mechanisms and through activation of pro-death Bcl-2 family members, releasing HMGB1 and other DAMPs into the environment that prime other macrophages and epithelial cells that contribute to priming via surface expressed pro-IL-1 $\alpha$  in the local environment.

Priming in these macrophages is principally driven by NF- $\kappa$ B, but it is also promoted by cathepsins, K<sup>+</sup> efflux and mitochondrial ROS production; mild LMD releases cathepsins into the cytosol causing mild mitochondrial depolarization via the Bcl-2 family, which generates mitochondrial ROS that activates redox-sensitive transcription factors like HIF-1 $\alpha$ . At the same time, NF- $\kappa$ B activation causes a switch from oxidative phosphorylation to glycolysis, mildly acidifying the cytosol with lactic acid (in concert with cytosolic acidification from mild LMD and K<sup>+</sup> efflux) to facilitate cathepsin activity and generating ATP as the mitochondria become progressively more compromised. During priming, pro-IL-1 $\beta$  is synthesized along with pro-IL-1 $\alpha$  and more NLRP3, which is modified by transcription-independent priming mechanisms and recruited to the outer mitochondrial and ER membranes. Mitochondrial ROS oxidizes the numerous cysteines in the LRR domain of NLRP3, causing conformational changes, but not enough for activation.

Next, one of these primed macrophages encounters a sterile particle released by another dying macrophage and ingests it. Again, the particle causes some LMD, leading to further cathepsin release and cytosolic acidification to that facilitates cathepsin activity. The oxidized NLRP3 LRR is cleaved off of the NLRP3 protein by cathepsins, causing its activation. Alternatively, nigericin causes rapid cytosolic acidification via cathepsin X-dependent K<sup>+</sup> efflux channels. This reduces some of the oxidized cysteines in the LRR of NLRP3 that facilitates binding to oxidized TXNIP (liberated from thioredoxin by mitochondrial ROS) via disulfide bonding. Cleavage or binding of TXNIP moves the LRR away from the PYD of NLRP3 allowing the recruitment of ASC and caspase-1 into inflammasome complexes. At the same time, more cathepsin-Bcl-2 family-mediated mitochondrial stress and ROS production continues to drive pro-IL-1 $\beta$  synthesis to replenish intracellular stores as IL-1 $\beta$  is secreted via pyroptotic cell

death. Pyroptosis or cathosis occurs slowly at first, but facilitates  $K^+$  efflux to balance the  $Ca^{2+}$  influx necessary to activate calpain for pro-IL-1 $\alpha$  processing. Finally, the mitochondria can no longer maintain membrane potential and they disintegrate, leading to the cessation of further pro-IL-1 $\beta$  synthesis. Having lost the ATP required to power the  $Na^+/K^+$  pump, plasma membrane potential is completely lost and the cell bursts, releasing mitochondrial components, other DAMPs, including IL-1 $\alpha$ , and the particles into the environment.

### Comprehensive Model of Particle-Induced NLRP3 Activation and Cell Death



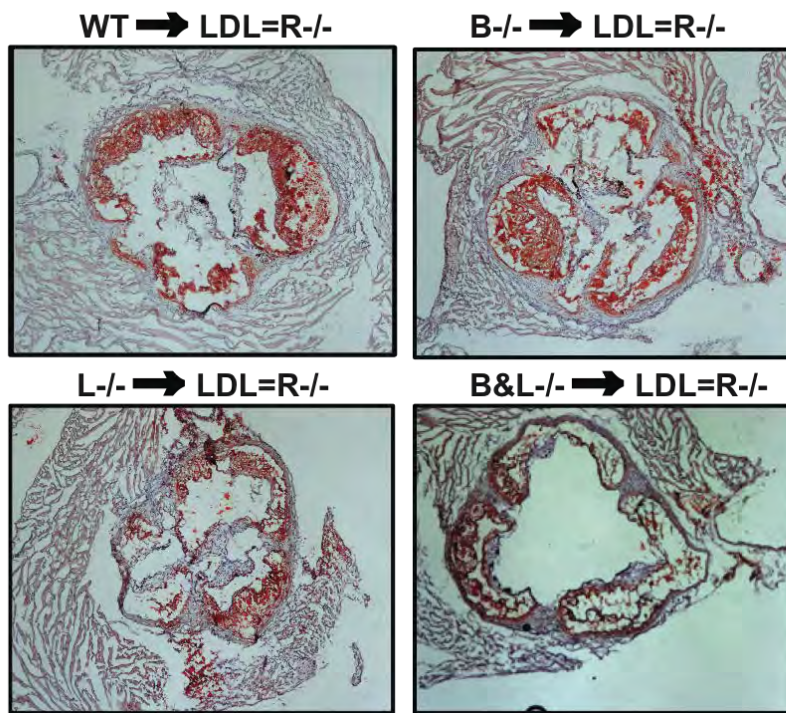
# Appendices

## Appendices

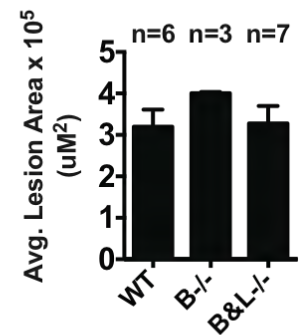
### **Appendix 1:** *Cathepsins B, L or B&L in bone-marrow-derived cells are not essential for atherogenesis.*

*LDL-R*<sup>-/-</sup> mice were lethally irradiated and reconstituted with bone marrow from WT mice or mice lacking cathepsins B, L or B&L. After 8 wks of reconstitution, these mice were given a high fat atherogenic diet for an additional 8 wks, prior to sacrifice. Hearts and aortic trunk were frozen in OTC (Optimal Cutting Temperature) medium and serially sectioned by cryostat. Sections the aortic sinus were stained with Oil-Red-O before plaque area was quantified and averaged. **(A)** Representative sections of aortic sinus from the indicated chimeric mice. **(B)** Quantification of average (avg.) lesion area from *LDL-R*<sup>-/-</sup> chimeric mice reconstituted with bone marrow from the indicated donors. Error bars represent S.E. of means from the indicated number (n) of mice.

**A**



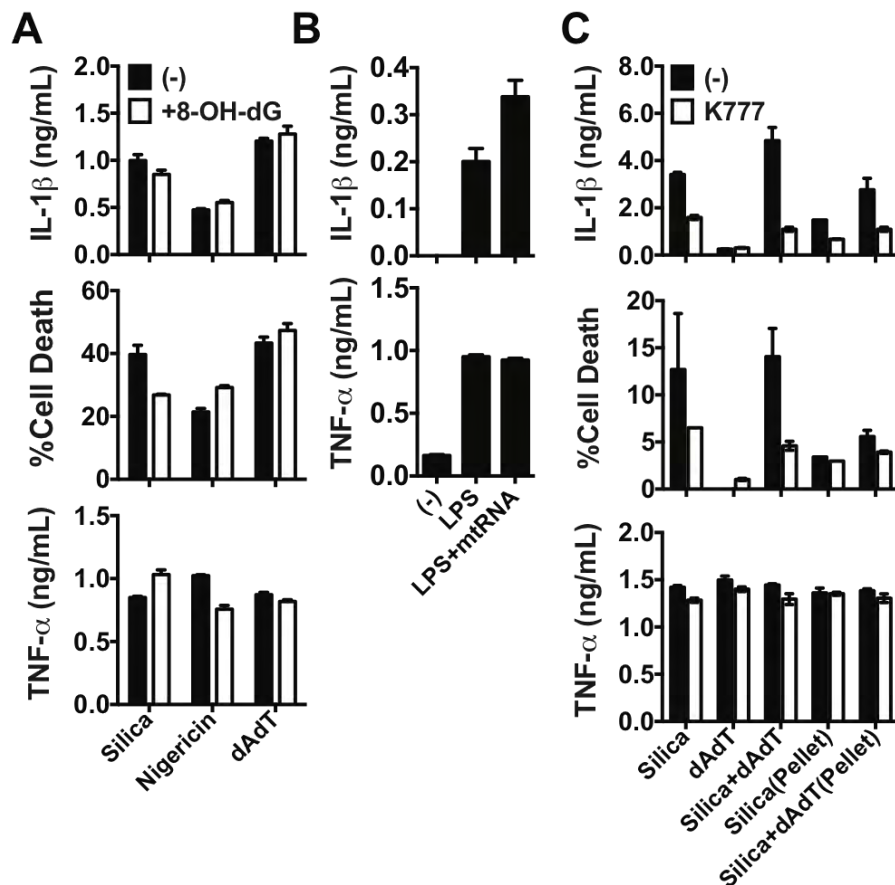
**B**



Ca

**Appendix 2:** 8-OH-dG does not inhibit NLRP3 activation, mitochondrial RNA induces some IL-1 $\beta$  secretion, and silica-induced IL-1 $\beta$  secretion can be enhanced by binding to dAdT.

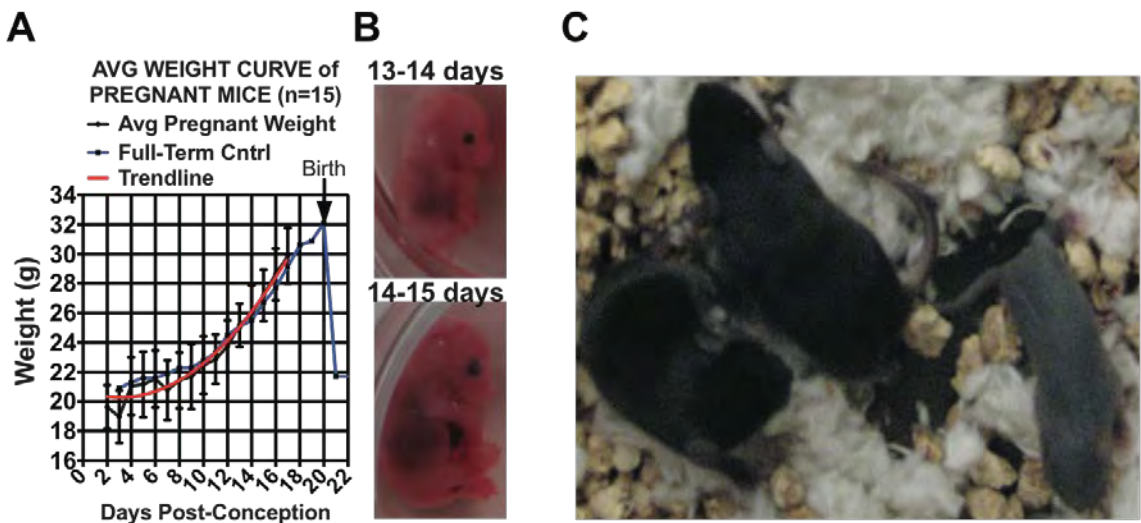
**(A)** LPS-primed PMs were treated with media control (-) or 8-OH-dG (200  $\mu$ M) and then stimulated with silica (50  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). **(B)** PMs were primed with media control (-) or LPS, then stimulated with 1.5  $\mu$ g of mitochondrial RNA isolated from mouse hepatocytes that was complexed with Lipofectamine 2000 using the same protocol as that used for dAdT. **(C)** LPS-primed PMs were stimulated with silica (50  $\mu$ g/mL), dAdT (0.5  $\mu$ g/mL) without complexation to Lipofectamine 2000, silica pelleted alone and resuspended in fresh RPMI, or silica mixed with dAdT prior to being pelleted and resuspended in fresh RPMI. Error bars represent **(A)** S.D. of technical triplicates or, **(B)** range bars of technical duplicates. Data are representative of **(A,C)** one or, **(B)** two independent experiments.





**Appendix 3: Novel Protocol: Timing the harvest of fetal livers for hematopoietic stem cell collection by predicting date of conception based on weight curve of pregnant females.**

**(A)** After setting up mating pairs, female mice in each pair were weighed daily to establish a general weight curve during pregnancy to aid in the prediction of the day of conception. Black line represents the average of weight trends from 15 pregnant female mice. Blue line represents the curve of a control mouse whose date of conception was known based on the identification of a vaginal plug, and this mouse was followed until she gave birth. The red line represents the trendline for the data from the 15 mice. **(B)** Fetal mice from 13-14 days post-conception (top) or 14-15 days post-conception. **(C)** 14 do cathepsin B-deficient (left; black and furry) mice and their cathepsin B&L double-deficient sibling (right; pale and hairless).

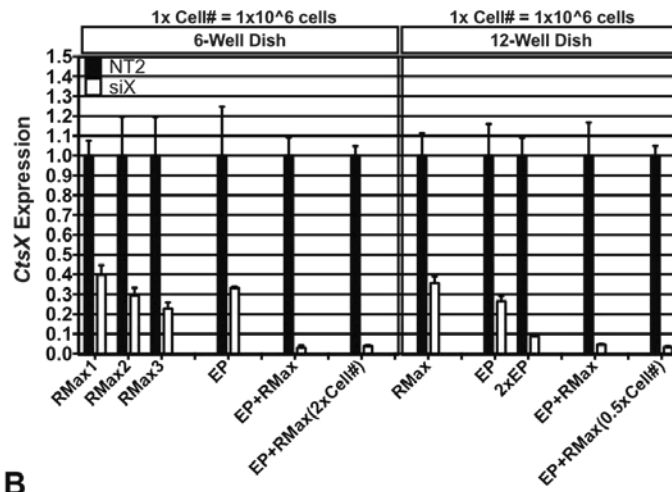


***Appendix 4: Novel Protocol: Optimal siRNA knockdown is achieved in PMs with minimal toxicity by combining Endoportor and RNAiMax.***

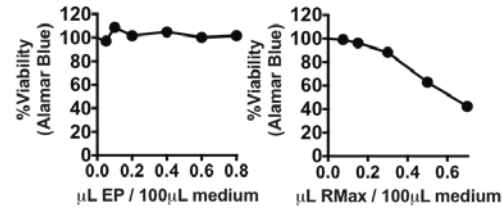
**(A)** PMs were plated in the indicated cell culture dishes at  $1 \times 10^6$  cells per well (in one case 2x this number of cells was used and in another 0.5x this number of cells were used as indicated) and treated with non-targeting siRNA (NT2) or siRNA targeting cathepsin X (siX) that was complexed with either 0.1, 0.15, or 0.2 uL of RNAiMax (RMax 1,2 ro 3, respectively) per 100 uL of final medium volume, 0.11 uL of Endoportor (EP) or 0.22 uL of Endoportor (2xEP) per 100uL of final medium volume, or 0.15 uL of RNAiMax together with 0.11 uL of Endoportor (EP+RMax) per 100 uL of final medium volume. After 48h, cathepsin X (*CtsX*) expression was analyzed by qPCR, normalized to GAPDH expression and plotted relative to non-targeting controls. **(B)** PMs were treated with titrations of either Endoportor (EP) (left) or RNAiMax (RMax) (right) for 48h and cell viability was analyzed by Alamar Blue. **(C)** LPS-primed PMs were treated with media control or K777 (20  $\mu$ M) prior to stimulation with Endoportor (EP; 10  $\mu$ M; note that 0.11 uL of EP in 100 uL is 1.1  $\mu$ M); solid bars indicated analysis of supernatants, hashed bars indicated analysis of lysates. Error bars represent **(A)** S.D. of technical triplicates or, **(C)** range bars of technical duplicates. Data are representative of one independent experiment. **(see next page for detailed protocol)**



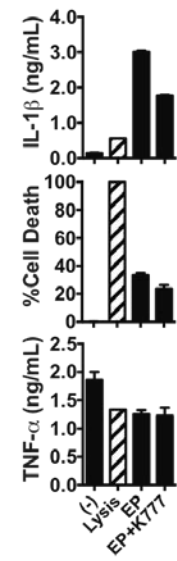
**A**



**B**



**C**



## **Endoport & RNAiMax Transfection Solution for Macrophages**

**By Gregory M. Orlowski (2014)**

**Note:** These calculations are for exact volumes. The actual calculations should account for volumes lost during pipetting and on the sides of tubes (it is critical that the scaling of the calculations is done correctly).

***Overestimate the Theoretical Final Volume of siRNAMix and carry this down through all calculations, but then mix the solutions (beginning with both MiniMixes) according to the exact volumes needed to arrive at the Actual Final Volume of siRNAMix.***

**For 50nM siRNA** (increase or decrease Endoport, not RNAiMax, if changing siRNA concentration)

Endoport (EP) → use 0.11uL per 0.1mL of 10%Media(Final Volume)

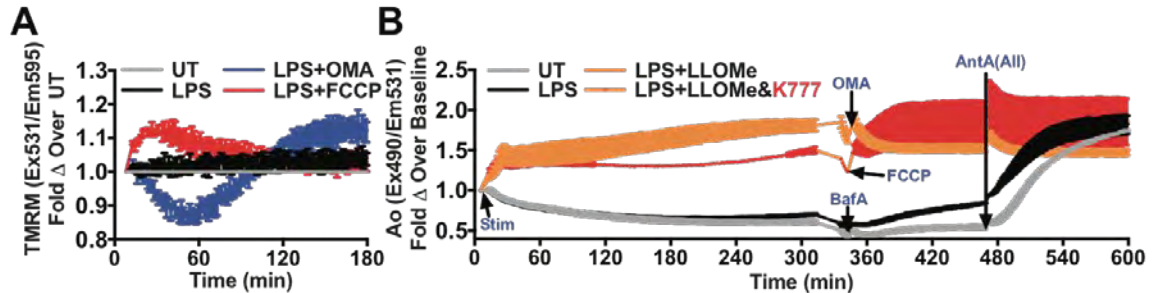
RNAiMax (RMax)→ use 0.15uL per 0.1mL of 10%Media(Final Volume)

### **Protocol:**

- 1) Calculate volume of **MasterMix** (RMax + EP + siRNA):
  - a.  $0.2 \times \text{Final Volume of siRNAMix in 10\% Media} = \text{MasterMix Volume}$
- 2) Calculate volume of both **MiniMixes** (RNAiMax in OptiMem & siRNA + EP in OptiMem)
  - a.  $0.5 \times \text{MasterMix Volume} = \text{MiniMix Volumes}$
- 3) Calculate how much RNAiMax is needed for the **RMax MiniMix**:
  - a.  $[(\text{Final Volume of siRNAMix})/0.1\text{mL}] \times 0.15 = \text{RNAiMax Volume}$
- 4) Calculate how much EP and siRNA you will need for your **EP/siRNA MiniMix**:
  - a.  $[(\text{Final Volume of siRNAMix})/0.1\text{mL}] \times 0.11 = \text{EP Volume}$
  - b.  $[(50\text{nM})(\text{Final Volume of siRNAMix})]/(\text{Stock siRNA Conc.}) = \text{siRNA Volume}$
- 5) Add the **EP & siRNA** to the **EP/siRNA MiniMix** in OptiMem
- 6) Add the **RMax** to the **RMax MiniMix** in OptiMem
- 7) Wait 5 Minutes Exactly
- 8) Combine both **MiniMixes**(1:1), which is now the **MasterMix**, vortex briefly just to mix, incubate 30min RT
- 9) Add 10%Media to **MasterMix**
  - a.  $0.8 \times \text{Final Volume of siRNAMix} = \text{Volume of 10\%Media}$
- 10) Add **Final siRNAMix** to cells in appropriate final volumes:
  - a. 96-well = 70uL/well
  - b. 12-well = 0.8mL/well
  - c. 6-well = 2mL/well
- 11) Do not change the media until the desired knockdown period is over (96h is recommended; add fresh media on top if old media yellows)

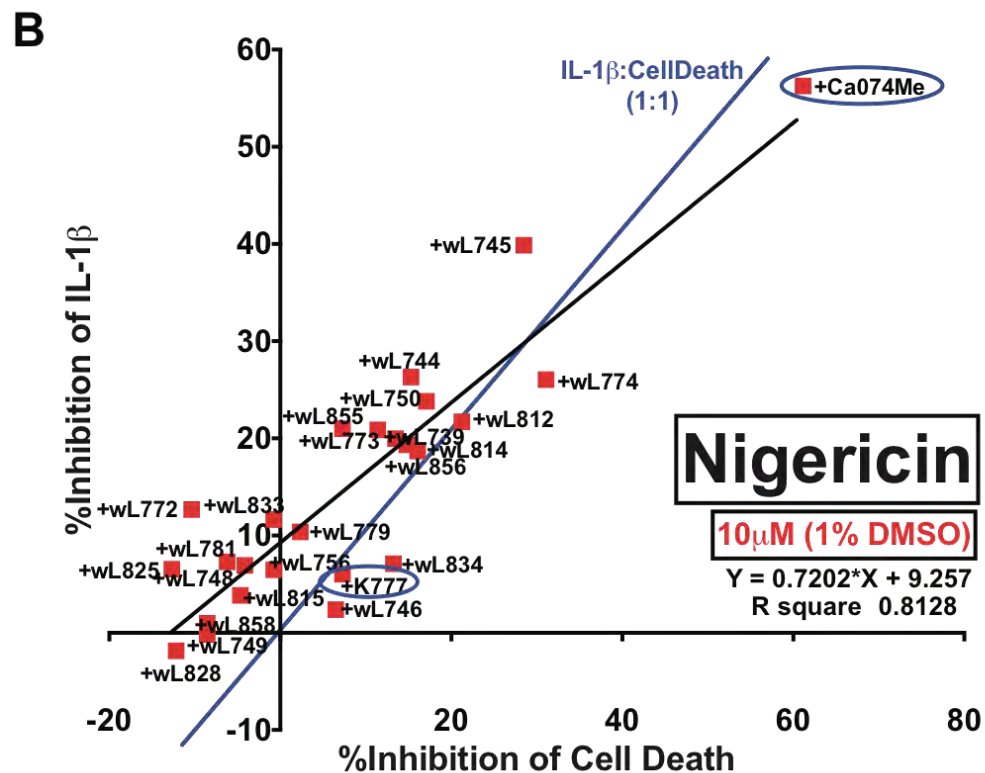
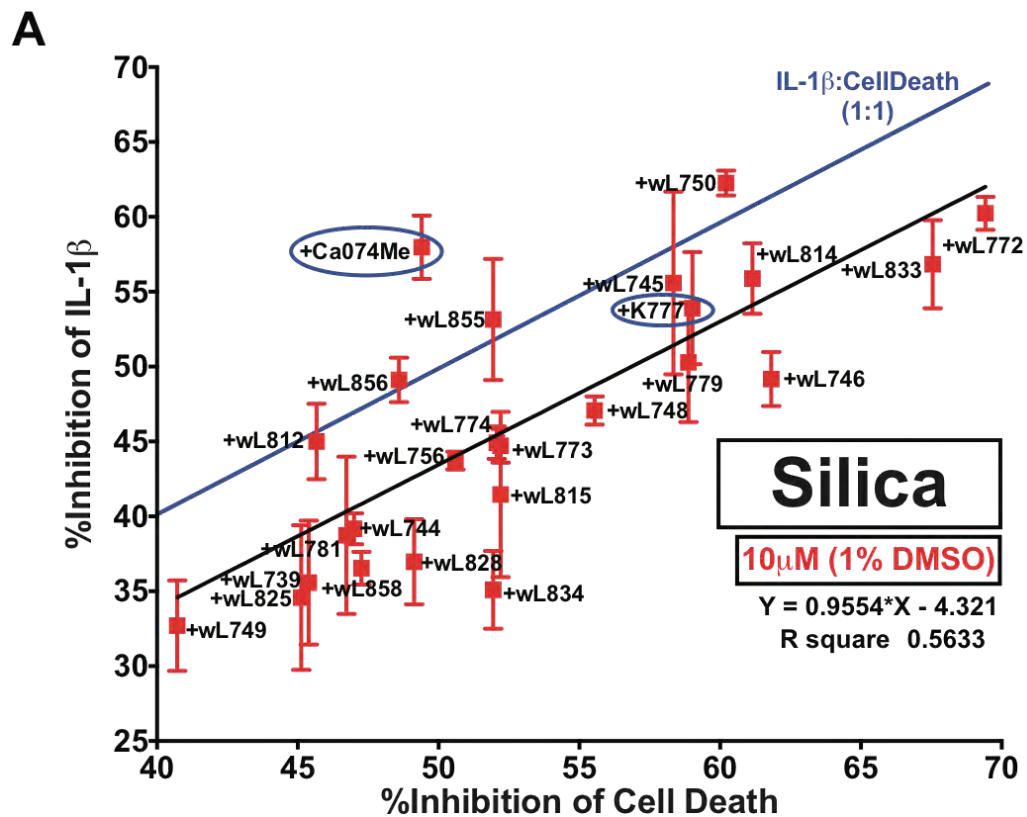
**Appendix 5: Novel Protocol: Acridine Orange and TMRM 96-well format for the real-time monitoring of lysosomal and mitochondrial disruption.**

**(A)** PMs stained with TMRM (increasing values = disruption of mitochondrial membrane potential) were primed with LPS or left untreated (UT), then treated with media control (UT or LPS), FCCP (2  $\mu$ M) or OMA (5  $\mu$ g/mL); fluorescence monitored at short intervals and plotted as fold change over untreated. **(B)** PMs stained with A.O. (increasing values = disruption of lysosomes/pH gradient) were treated with media control (untreated = UT) or primed with LPS before treatment with media control or K777 (15  $\mu$ M) prior to stimulation with media control or LLOMe (1 mM); fluorescence traces monitored by plate reader at short intervals and plotted as fold change over baseline; after ~6h, samples from the indicated traces were treated with media control (UT), Bafilomycin A (BafA; 50 nM), FCCP (2  $\mu$ M) or OMA (5  $\mu$ g/mL); and after ~8h, samples from all traces treated with AntA (All; 5  $\mu$ M). Error bars represent range bars of technical duplicates. Data are representative of three independent experiments.



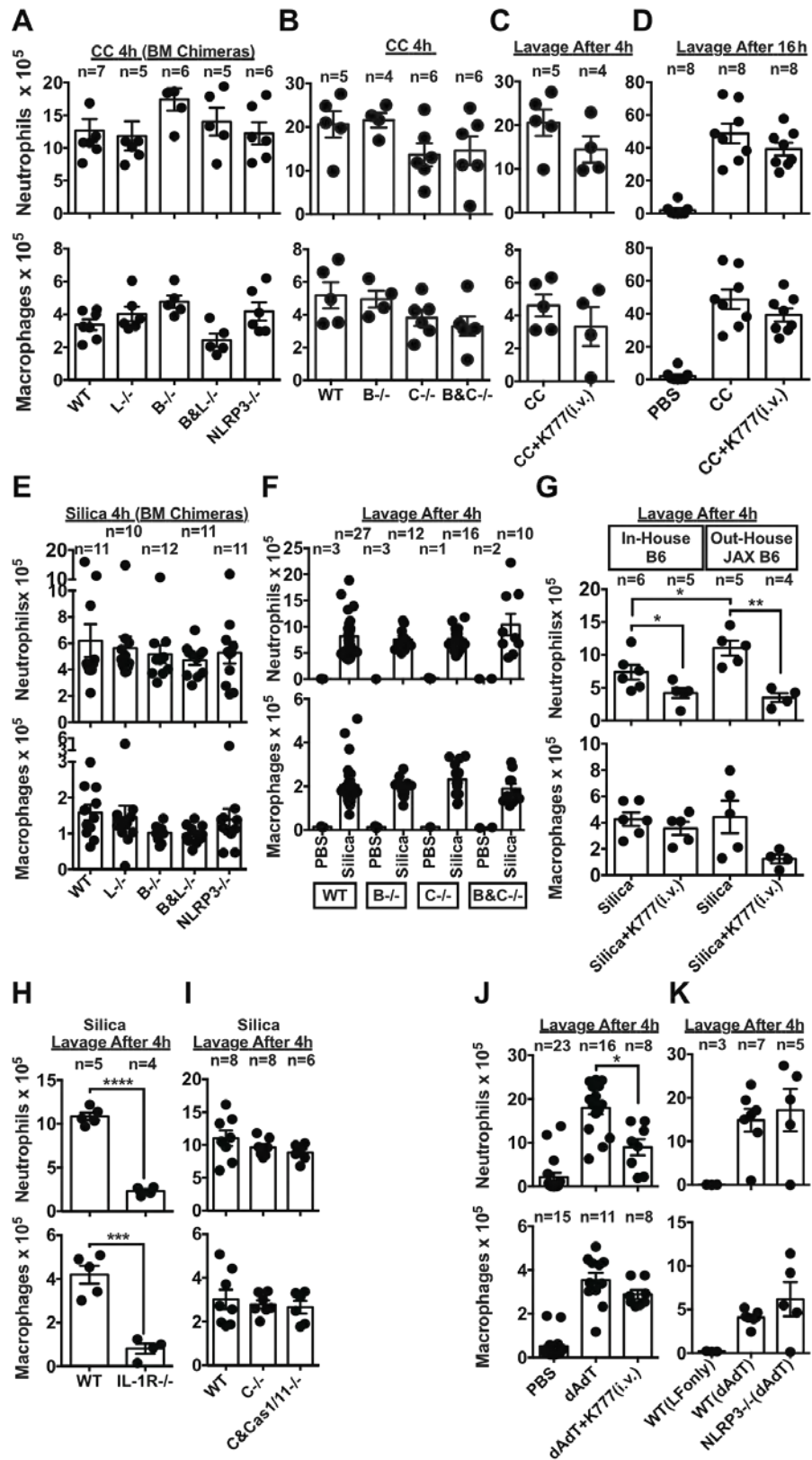
***Appendix 6: Twenty-five different cathepsin inhibitors all exhibit similar efficacy for inhibiting silica and nigericin-induced IL-1 $\beta$  secretion and cell death in LPS-primed macrophages.***

In all experiments, PMs were primed with LPS. **(A,B)** PMs were treated with 10  $\mu$ M of each cathepsin inhibitor in a final concentration of 1% DMSO (wLXXX inhibitors were synthesized by Matt Bogyo's lab at Stanford and exhibit similar pan-cathepsin inhibition profiles) and stimulated with either **(A)** silica (40  $\mu$ g/mL) or **(B)** nigericin (2  $\mu$ M). Data are plotted at percent inhibition of IL-1 $\beta$  secreted or cell death relative to treatment with 1% DMSO control and stimulation. Blue circles highlight K777 and Ca074Me. Blue lines indicate the theoretical trendline for a 1:1 relationship between inhibition of IL-1 $\beta$  secretion and cell death. Black lines are the trendlines for the experimental data and the accompanying equations and R-squared values are shown on the lower right of each graph. Error bars represent range bars of technical duplicates. Data are representative of two independent experiments.



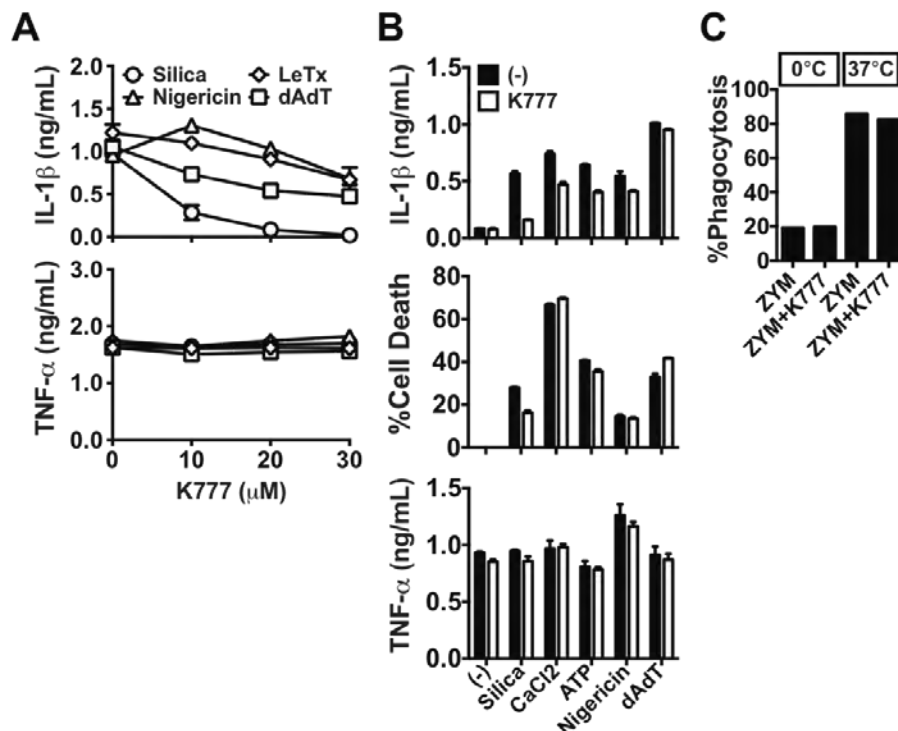
## ***Appendix 7: Compilation of additional in vivo acute peritonitis experiments.***

In all experiments, mice were injected i.p. with the indicated stimuli (or PBS control) for the indicated amount of time, then peritoneal exudates were collected by lavage and analyzed by flow cytometry. **(A-D)** CC-induced acute peritonitis (“A”= 3 mg CC, “B-D” = 0.2 mg CC). **(A)** Lethally irradiated WT mice were reconstituted with bone marrow from the indicated donor mice (WT, cathepsin L, B, B&L, or NLRP3-deficient mice). **(B)** WT mice or cathepsin B, C or B&C-deficient mice. **(C,D)** WT mice pre-treated with excipient control (PBS & CC) or 62.5 mg/kg K777 i.v. (CC+K777) 1h prior to stimulation. **(E-I)** Silica-induced peritonitis (0.2 mg silica). **(E)** Lethally irradiated WT mice were reconstituted with bone marrow from the indicated donor mice (WT, cathepsin L, B, B&L, or NLRP3-deficient mice). **(F)** WT mice or cathepsin B, C or B&C-deficient mice. **(G)** WT mice from either the on-site mouse colony (In-House B6) or ordered <1wk prior from JAX Mice (Out-House B6) were pre-treated with excipient control (PBS & Silica) or 62.5 mg/kg K777 i.v. (Silica+K777) 1h prior to stimulation. **(H)** WT mice or IL-1R1-deficient mice. **(I)** WT, cathepsin C-deficient or cathepsin C/caspase-1&11 triple-deficient mice. **(J,K)** dAdT-induced acute peritonitis (3 µg dAdT). **(J)** WT mice pre-treated with excipient control (PBS & dAdT) or 62.5 mg/kg K777 i.v. (dAdT+K777) 1h prior to stimulation. **(K)** WT or NLRP3-deficient mice stimulated with either Lipofectamine 2000 alone (LFOonly) or dAdT complexed with Lipofectamine 2000 (dAdT). Error bars represent S.E. of means from the indicated number (n) of mice. Statistical analyses were performed by Two-tailed Student’s t-test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .



**Appendix 8: K777 selectively suppresses IL-1 $\beta$  secretion and cell death induced by particulate NLRP3 stimuli without affecting general phagocytic machinery.**

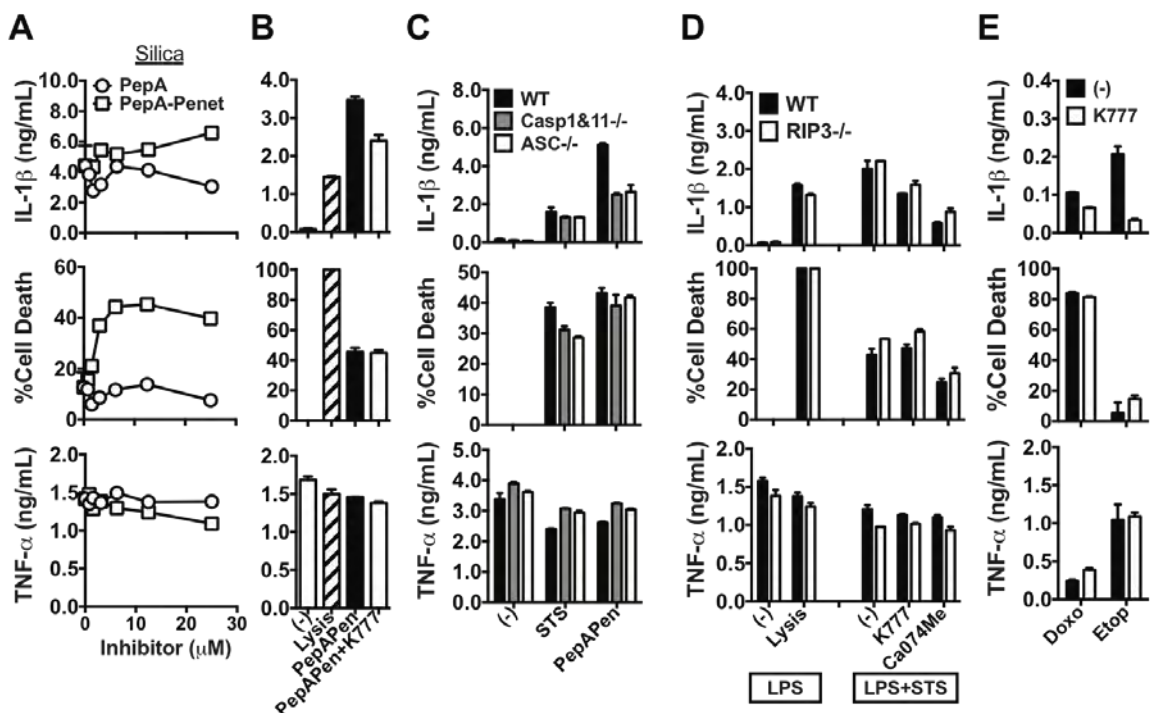
In all experiments, cells were primed with LPS. **(A)** Immortalized bone-marrow-derived macrophages from B6/129Sev hybrid mice were treated with a titration of K777 concentrations and then stimulated with silica (100  $\mu\text{g/mL}$ ), nigericin (2  $\mu\text{M}$ ), anthrax lethal toxin (LeTx; 0.5  $\mu\text{g/mL}$ ), or dAdT (0.5  $\mu\text{g/mL}$ ). **(B)** PMs were treated with media control (-) or K777 (20  $\mu\text{M}$ ) and then stimulated with silica (50  $\mu\text{g/mL}$ ), CaCl<sub>2</sub> (1 mM), ATP (2 mM), nigericin (2  $\mu\text{M}$ ) or dAdT (0.5  $\mu\text{g/mL}$ ). **(C)** PMs were kept either on ice (0°C) or incubated (37°C) throughout the procedure as indicated. After 2h of LPS priming, PMs were treated with media control or K777 (15  $\mu\text{M}$ ) as indicated for 1h. Next, FITC-conjugated zymosan particles (400  $\mu\text{g/mL}$ ) were added to PMs for an additional 45 min and then all samples were placed on ice, labeled with APC-conjugated anti-CD11b antibodies, diluted in 0.2% trypan blue (quenches surface bound/extracellular FITC) and the percent of CD11b<sup>+</sup> cells that were FITC<sup>+</sup> (indicated phagocytosis of FITC-zymosan) was analyzed by flow cytometry. Error bars represent **(A)** S.D. of technical triplicates, **(B)** range bars of technical duplicates. Data are representative of three **(A,B)** or one **(C)** independent experiment(s).





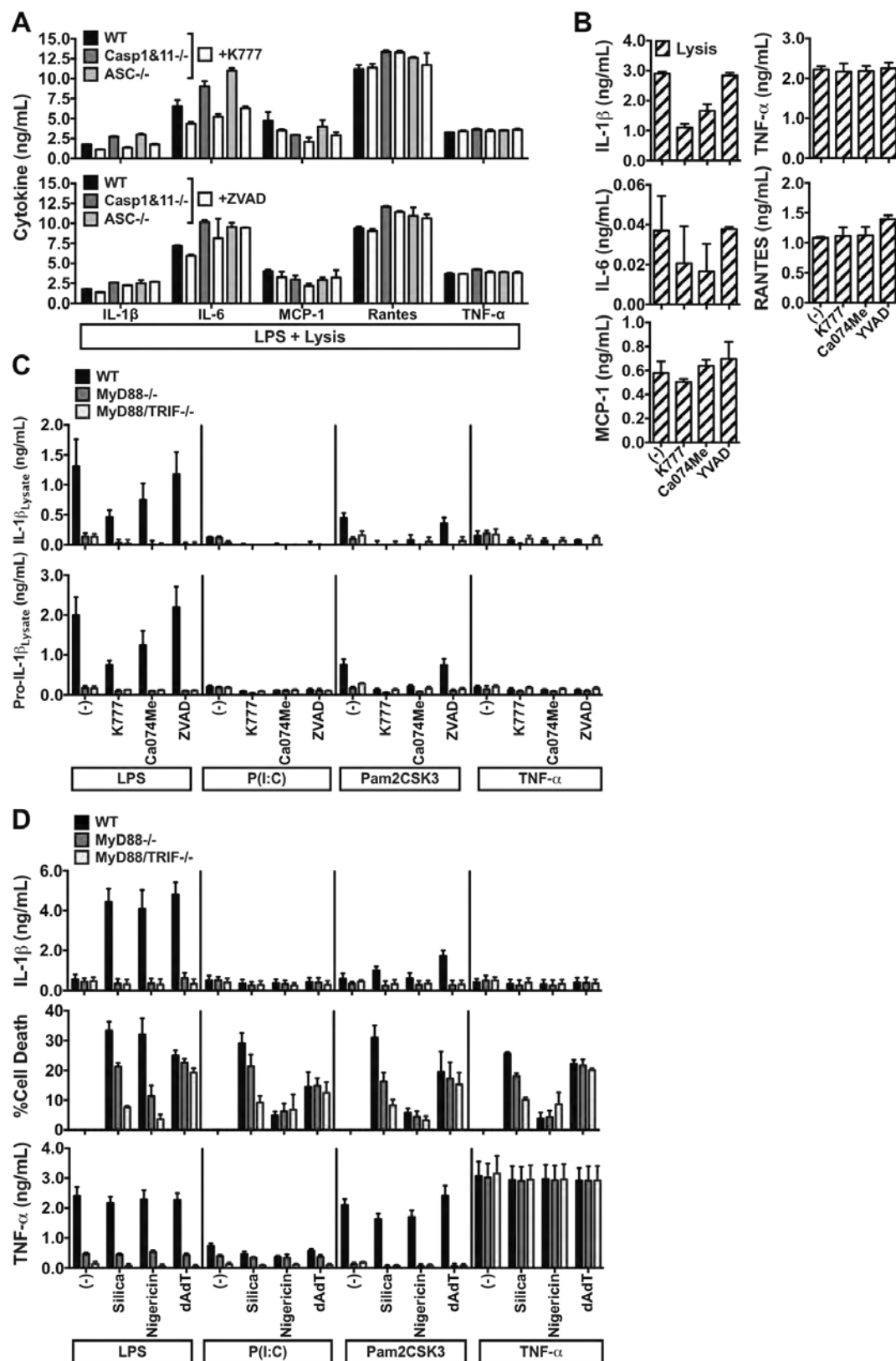
**Appendix 9: A cathepsin D inhibitor (Pepstatin A-penetratin), staurosporine, and chemotherapeutics (doxorubicin and etoposide) induce inflammasome-independent IL-1 $\beta$  secretion with variable sensitivity to suppression with K777.**

In all experiments, PMs were primed with LPS. **(A)** PMs were treated with the indicated concentrations of the poorly membrane permeant cathepsin D inhibitor pepstatin A (PepA) and its cell permeant analog pepstatin A-penetratin (PepA-Penet) and then stimulated with silica (30  $\mu$ g/mL). **(B)** PMs were treated with media control (-), lysis or PepAPen) or K777 (20  $\mu$ M) and then stimulated with pepstatin A-penetratin (PepAPen; 22.5  $\mu$ M). Supernatants (solid bars) or lysates (hatched bars) were analyzed. **(C)** PMs from WT mice or mice lacking caspase-1&11 or ASC were stimulated with media control (-), staurosporine (STS; 5  $\mu$ M) or PepAPen (22.5  $\mu$ M). **(D)** PMs from WT mice or mice lacking RIP3 were treated with media control (-) or lysis, K777 (20  $\mu$ M) or Ca074Me (20  $\mu$ M), and then stimulated with media control (LPS) or STS (LPS+STS; 5  $\mu$ M). **(E)** PMs were treated with media control or K777 (20  $\mu$ M) and then treated for 20h with the chemotherapeutic drugs doxorubicin (10  $\mu$ M) or etoposide (100  $\mu$ M). Cell death was analyzed by MTS assay. Error bars represent **(B-D)** range bars of technical duplicates or, **(E)** S.D. of technical triplicates. Data are representative of **(A,C,D)** one or, **(B,E)** two independent experiments.



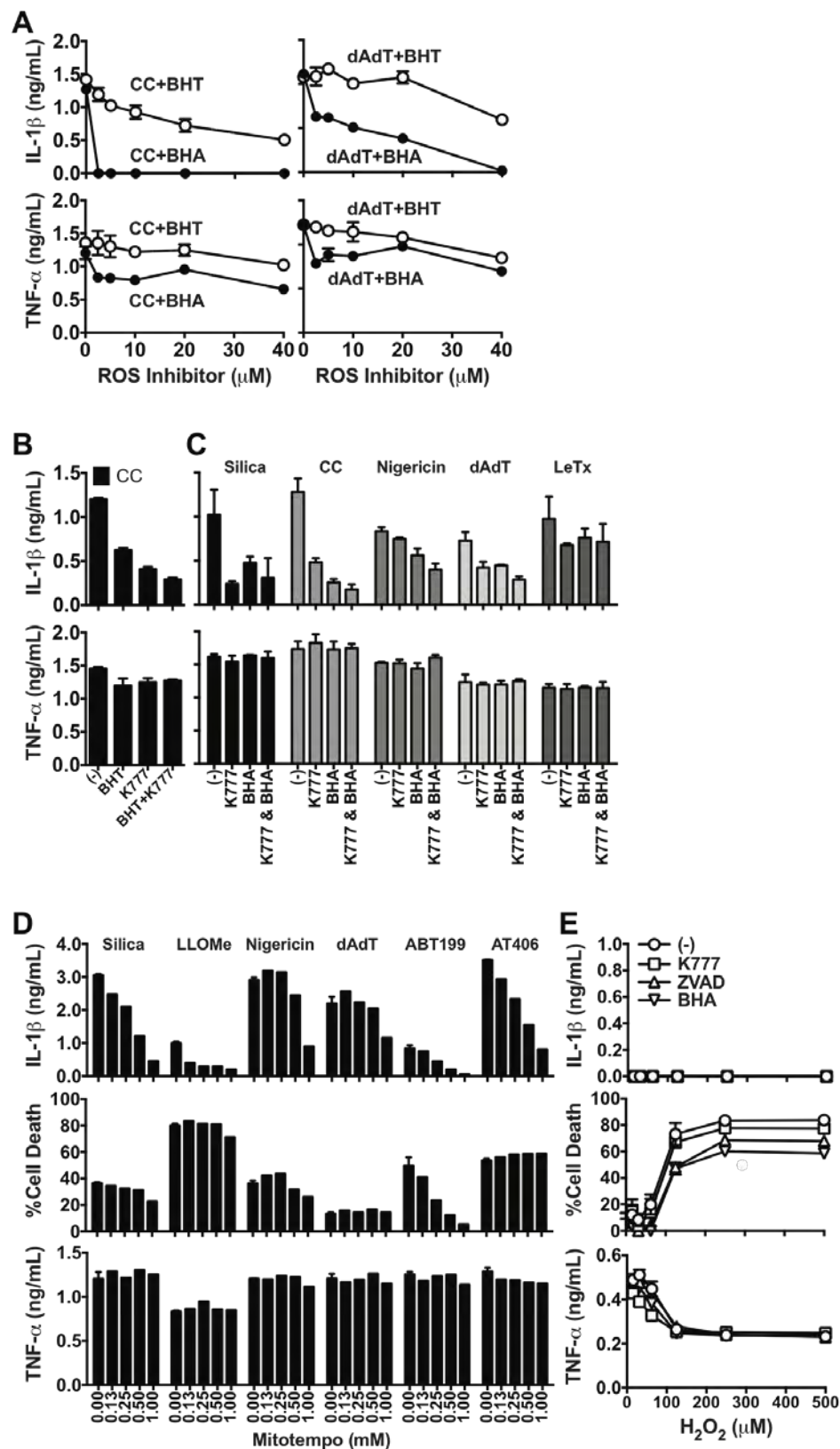
***Appendix 10: Cathepsin inhibitors suppress pro-IL-1 $\beta$  synthesis regardless of priming mechanism and silica/nigericin-induced cell death depends on MyD88 and/or TRIF.***

**(A)** PMs from WT mice or mice lacking caspase-1&11 (Casp1&11<sup>-/-</sup>) or ASC were primed for 2h, then treated with media control (shaded bars), K777 (15  $\mu$ M) or ZVAD (10  $\mu$ M) for an additional 7h and the indicated cytokines were measured in the lysates by ELISA. **(B)** Same as in “A” except were treated with K777 (20  $\mu$ M), Ca074Me (20  $\mu$ M) or YVAD (15  $\mu$ M). **(C,D)** PMs from WT mice or mice lacking MyD88 or MyD88 & TRIF were primed with either LPS (200 ng/mL), poly(I:C) (100  $\mu$ g/mL), Pam2CSK3 (200 ng/mL), or TNF- $\alpha$  (1  $\mu$ g/mL) for 2h and then **(C)** treated with media control (-), K777 (15  $\mu$ M), Ca074Me (15  $\mu$ M) or ZVAD (10  $\mu$ M) for an additional 7h and IL-1 $\beta$  and pro-IL-1 $\beta$  were measured in the lysates by ELISA, or **(D)** treated with media only for 1h and then stimulated with silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), dAdT (0.5  $\mu$ g/mL). Error bars represent range bars of technical duplicates. Data are representative of **(A,B)** two, **(C)** three or, **(D)** one independent experiment(s).



***Appendix 11: ROS inhibitors selectively attenuate particle-induced IL-1 $\beta$  secretion, but not cell death.***

In all experiments, cells were primed with LPS. **(A)** PMs were treated with titrations of butylated hydroxyanisol (BHA) or butylated hydroxytoluene (BHT) and then stimulated with either CC (1 mg/mL) or dAdT (0.25  $\mu$ g/mL). **(B)** PMs were treated with BHT (200  $\mu$ M), K777 (20  $\mu$ M) or BHT & K777 and then stimulated with CC (1 mg/mL). **(C)** Immortalized bone-marrow-derived macrophages from B6-129Sev hybrid mice were treated with BHA (100  $\mu$ M), K777 (20  $\mu$ M) or BHA & K777 and then stimulated with silica (100  $\mu$ g/mL), CC (1 mg/mL), nigericin (2  $\mu$ M), dAdT (0.5  $\mu$ g/mL) or anthrax lethal toxin (LeTx; 0.5  $\mu$ g/mL). **(D)** PMs were treated with a titration of the mitochondrial ROS inhibitor Mitotempo, then stimulated with silica (40  $\mu$ g/mL), LLOMe (0.75 mM), nigericin (2  $\mu$ M), dAdT (0.3  $\mu$ g/mL), ABT199 (5  $\mu$ M) or AT406 (15  $\mu$ M). **(E)** PMs were treated with media control (-), K777 (30  $\mu$ M), ZVAD (5  $\mu$ M) or BHA (10  $\mu$ M) and then stimulated with a titration of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Error bars represent **(A-C,E)** S.D. of technical triplicates or, **(D)** S.D. of technical triplicates for 0 mM Mitotempo and other samples single data points. Data are representative of **(A-C)** two, **(D)** one or, **(E)** three (for H<sub>2</sub>O<sub>2</sub> titration, but only 1 repeat for inhibitors) independent experiment(s).



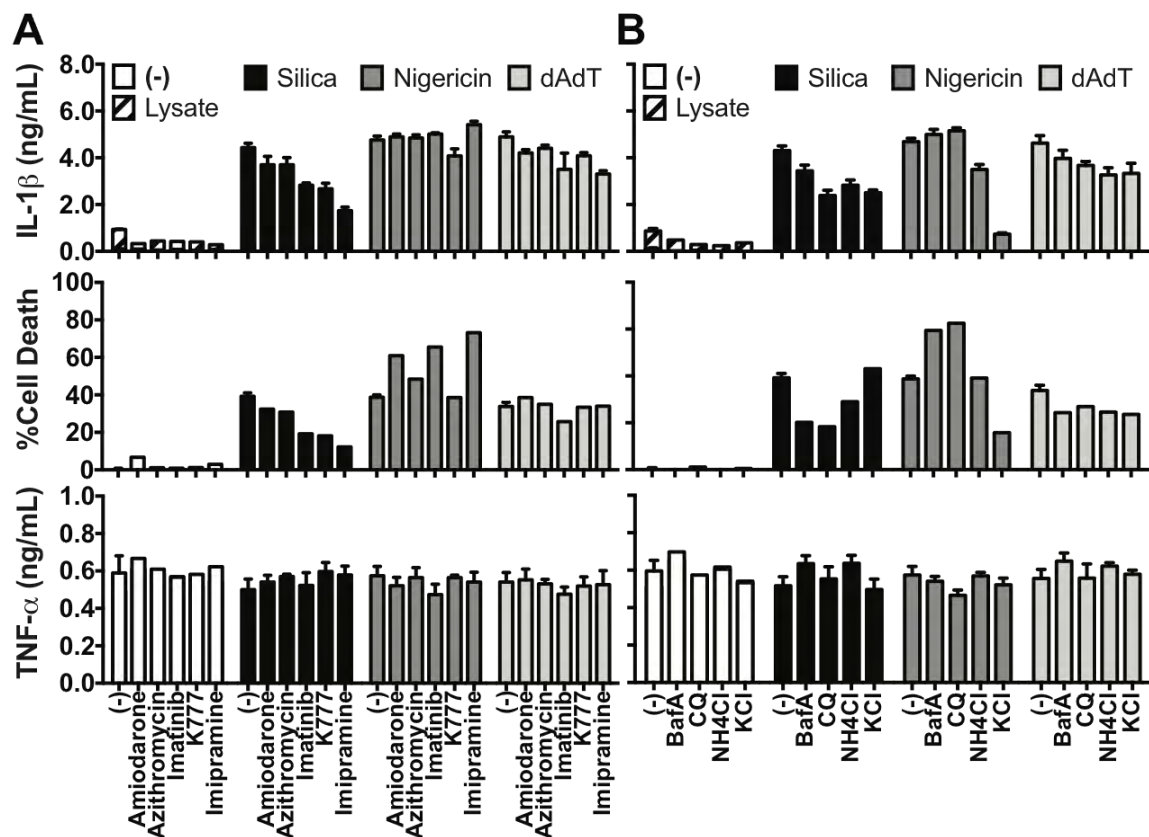
***Appendix 12: Drug Formulation: K777 requires low pH to remain soluble at high concentrations, but the ELP formulation permits absorption of K777 as a monodispersed suspension that causes local inflammation.***

**(A-F)** pH electrode analysis of K777-ELP formulation and solubility (precipitation is indicated by “\*”). **(A)** The indicated concentrations of the K777-ELP formulation (250 mg/mL or 435 mM K777 in 25% PEG-300, 25% glycofurol, 25% Cremophor ELP, 15% ethanol, 10% propylene glycol) were diluted in water. **(B)** Dilutions of the excipient (ELP formulation) alone. **(C,D)** K777-ELP was diluted 1:10 in water (now 25 mg/mL) and the indicated volumes of chemical buffers (top) or proteinaceous buffers (bottom) were added, resulting in the corresponding pH values until precipitation (\*) was observed. **(E)** K777-ELP was diluted 1:10 in the indicated aqueous buffers and pH was titrated with the indicated volumes of 0.1 N NaOH until precipitation (\*) was observed. **(F)** K777-HP $\beta$ CD formulation (250 mg/mL or 435 mM K777 in 43.5% 1-hydroxypropyl  $\beta$ -cyclodextran, 56.5% water) was diluted 1:10 in water (now 25 mg/mL) and pH was titrated with the indicated volumes of 0.1 N NaOH until precipitation (\*) was observed. **(G)** Comparison of DMSO-K777 formulation (300 mM K777 in 50% DMSO, 35% PEG-400, 15% ethanol) and ELP-K777 formulation (300 mM K777; formulation as in “A”) after precipitation by light microscopy (100X) showing a polydispersed (non-uniform) aggregating precipitate for the DMSO formulation (left) and a monodispersed (uniform) micelle suspension for the ELP formulation (right). **(H)** Disassembled osmotic Alzet pump showing a cross-section of the semi-permeable synthetic shell lined with salts that facilitates interstitial fluid influx, thereby compressing the rubber bladder and ejecting the drug out of the regulator port (facing downward). **(I)** Alzet pumps containing 75 mg/kg/day doses of K777 in the DMSO (left) or ELP (right) formulations were surgically implanted in the backs of mice for 1 wk (in the orientation shown in “H”). The DMSO formulation shows the solid aggregating precipitate of K777 in the position where the port of the Alzet pump was located (red circle), while the ELP formulation is completely absorbed into the tissues. **(J-L)** Hematoxylin/eosin-stained transdermal tissue sections (embedded in paraffin) from the backs of mice surgically implanted with Alzet pumps for 1 wk; panels on the left and right represent two separate samples at 10X (top) and 40X (bottom) captured by video light microscopy. **(J)** The excipient ELP formulation (no K777) showing that the formulation itself does not cause local inflammation. **(K)** The K777-ELP formulation dosed at 25 mg/kg/day showing tissue edema and some infiltrating macrophages and neutrophils. **(L)** The K777-ELP formulation dosed at 75 mg/kg/day showing intense neutrophilic infiltrates and caseating necrosis.



**Appendix 13:** *Lysotropic drugs (amphiphilic weak bases) and lysosome/cytosolic acidification inhibitors selectively suppress particle-induced IL-1 $\beta$  secretion and cell death.*

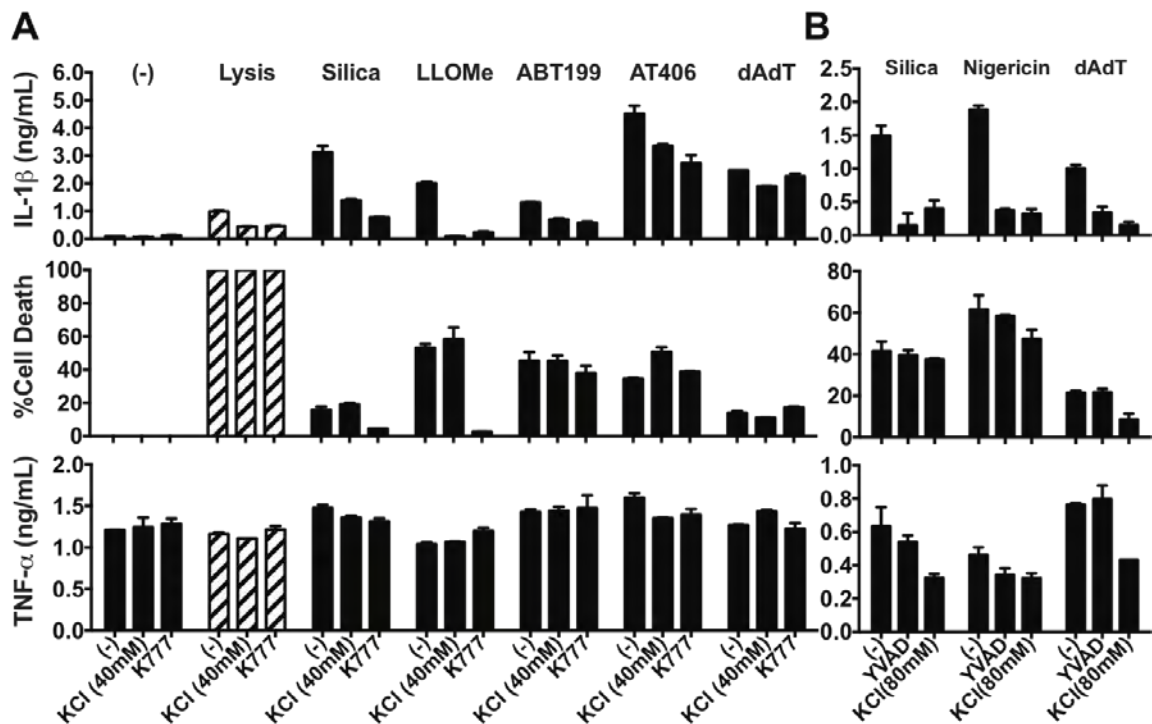
In all experiments, PMs were primed with LPS. PMs were treated with **(A)** amiodarone (5  $\mu$ M), azithromycin (80  $\mu$ M), imatinib (15  $\mu$ M), K777 (15  $\mu$ M), imipramine (60  $\mu$ M) or, **(B)** bafilomycin A (BafA; 200 nM), chloroquine (CQ; 30  $\mu$ M), ammonium chloride (NH<sub>4</sub>Cl; 15 mM) or KCl (50 mM) and then **(A,B)** stimulated with silica (80  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). Error bars represent range bars of technical duplicates. Data are representative of three (K777, KCl, BafA, CQ, NH<sub>4</sub>Cl; all others for lysates) or two (amiodarone, azithromycin, imatinib, imipramine) independent experiments.





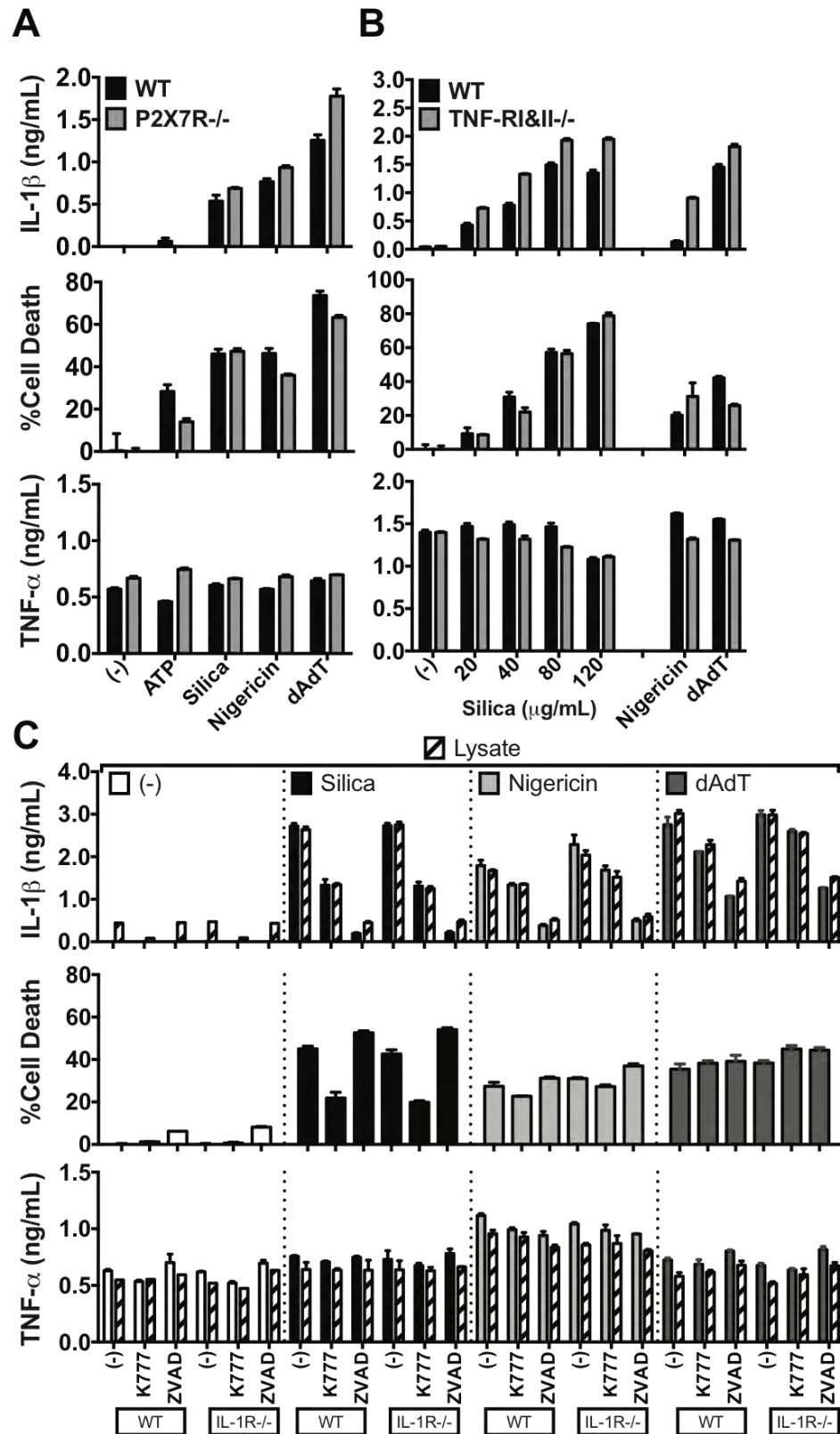
**Appendix 14:** Lower concentrations of KCl selectively suppress IL-1 $\beta$  secretion induced by Silica and LLOMe (& NLRP3-dependent stimuli), but higher concentrations of KCl suppress IL-1 $\beta$  non-selectively.

In all experiments, PMs were primed with LPS. **(A)** PMs were treated with media control (-), KCl (40 mM) or K777 (15  $\mu$ M), and then stimulated with media control (-) or Lysis, silica (40  $\mu$ g/mL), LLOMe (0.75 mM), ABT199 (5  $\mu$ M), AT406 (10  $\mu$ M) or dAdT (0.5  $\mu$ g/mL); supernatants (black bars) or lysates (hatched bars). **(B)** PMs were treated with media control (-), KCl (80 mM) or YVAD (20  $\mu$ M), and then stimulated with media control (-) or Lysis, silica (40  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). Error bars represent range bars of technical duplicates. Data are representative of three (Silica, Nigericin, dAdT) or two (LLOMe, ABT199, AT406) independent experiments.



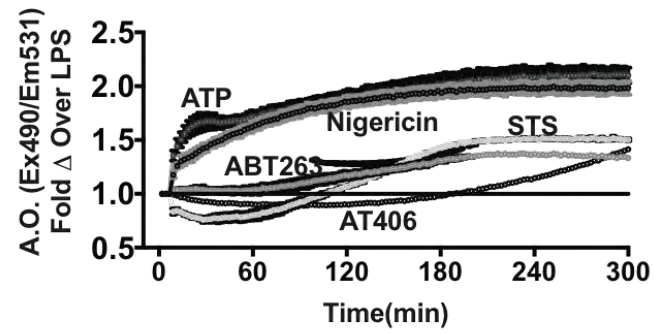
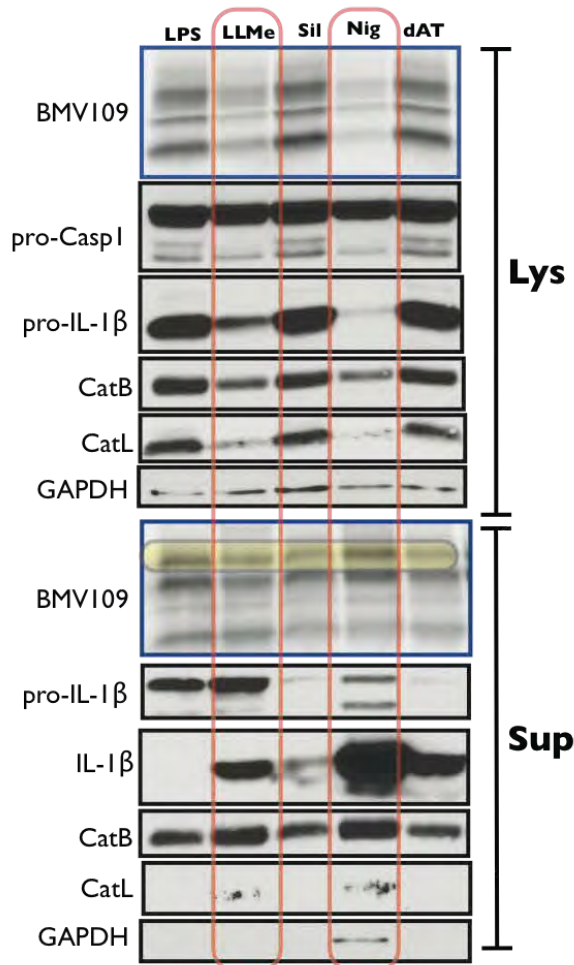
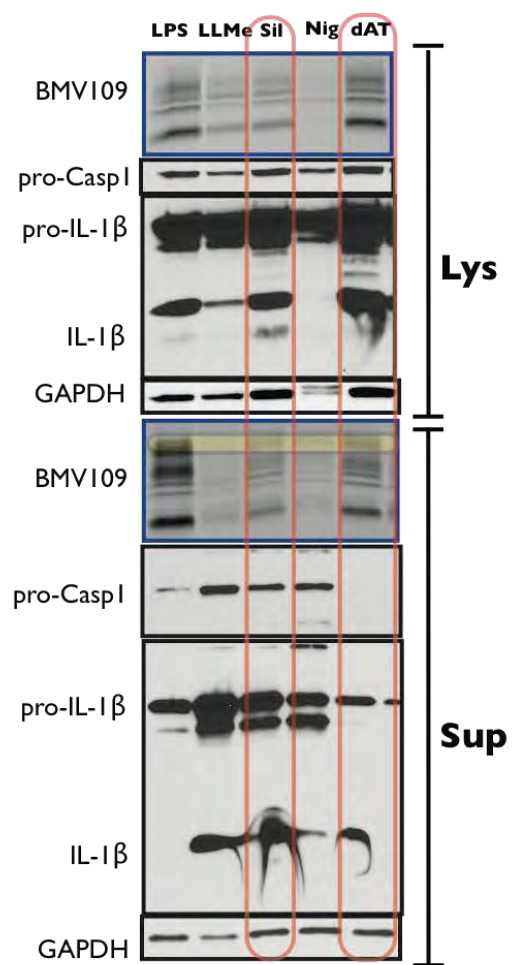
***Appendix 15: P2X7R, IL-1R1, or TNF-RI&II do not contribute to IL-1 $\beta$  synthesis or particle-induced IL-1 $\beta$  activation and cell death in LPS-primed macrophages.***

In all experiments, PMs were primed with LPS. **(A)** PMs from WT mice or mice lacking P2X7R were stimulated with media control (-), ATP (1 mM), silica (40  $\mu$ g/mL), nigericin (2.5  $\mu$ M) or dAdT (0.3  $\mu$ g/mL). **(B)** PMs from WT mice or mice lacking both TNF-RI and TNF-RII were stimulated with media control (-), the indicated concentrations of silica, nigericin (1  $\mu$ M) or dAdT (0.5  $\mu$ g/mL). **(C)** PMs from WT mice or mice lacking IL-1R1 were treated with media control (-), K777 (15  $\mu$ M), or ZVAD (10  $\mu$ M), and then stimulated with media control (-), silica (80  $\mu$ g/mL), nigericin (1.5  $\mu$ M), or dAdT (0.5  $\mu$ g/mL). Supernatants (solid bars) or lysates (hatched bars) were analyzed. Error bars represent **(A,B)** S.D. of technical triplicates or, **(C)** range bars of technical duplicates. Data are representative of individual experiments.



***Appendix 16: ATP and Nigericin rapidly disrupt lysosomal pH gradients and intracellular cathepsin activity.***

In all experiments, PMs were primed with LPS. **(A)** A.O.-stained PMs were primed with LPS and stimulated with ATP (2 mM), nigericin (2  $\mu$ M), STS (5  $\mu$ M), ABT263 (15  $\mu$ M) or AT406 (15  $\mu$ M) and green fluorescence intensity was measured at short intervals by plate reader; data are plotted as fold change of the control treated with LPS only and error bars are range bars of duplicates. **(B,C)** PMs were stimulated with media control (LPS), LLOMe (LLMe; 2 mM), silica (80  $\mu$ g/mL), nigericin (2  $\mu$ M), or dAdT (dAT; 0.5  $\mu$ g/mL) for either **(B)** 1h or **(C)** 6h, prior to probing for cathepsin activity for an additional 1h; lysates and supernatants were processed by SDS-PAGE, cathepsin activity was measured by phosphor-imaging, and pro-caspase-1 (pro-Casp1), pro-IL-1 $\beta$ , IL-1 $\beta$ , cathepsin B (CatB), cathepsin L (CatL), and GAPDH were measured by Immunoblotting (note: active caspase-1 (p10) could not be examined since separating cathepsin bands requires that low m.w. proteins are run off the gel). Yellow ovals in “B and C” highlight that cathepsin X activity is mostly extracellular (compare with corresponding bands in the lysate), and red ovals highlight LLOMe and nigericin both causing a rapid reduction in intracellular cathepsin activity during stimulation in “B”. Conversely, red ovals show that silica causes late reduction in intracellular cathepsin activity during stimulation, while dAdT does not affect intracellular cathepsins activity in “C”. Data are representative of **(A)** three independent experiments, **(B,C)** one experiment.

**A****B****1 hour of Signal 2****C****6 hours of Signal 2**



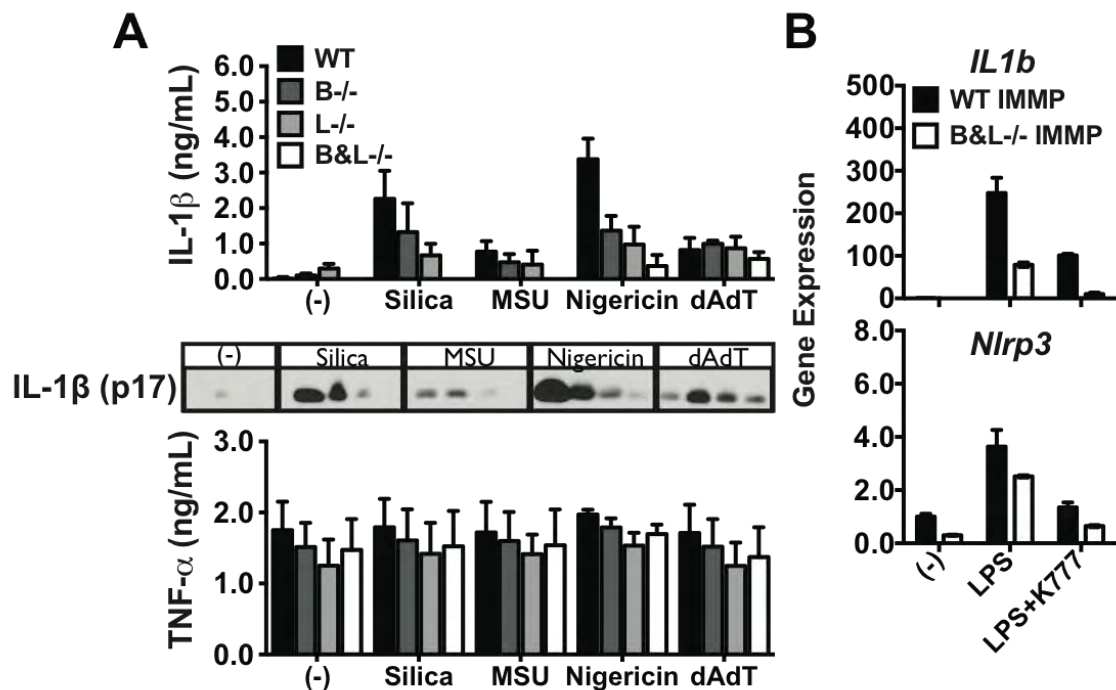
**Appendix 18: Regularly spaced cysteine motifs on the LRR of NLRP3 and NLRP3 cleavage sites.**

	>sp Q8R4B8 NALP3_MOUSE NACHT, LRR and PYD domains-Containing protein 3 OS=Mus musCulus GN=Nlrp3 PE=2 SV=1 MTSVR
PYD	CKLAQYLEDLEDVDLKKFKMHLEDYPPKEG CIPVPRGQMEKADHLDLATLMIDFNGEKAWAMAVWIFAAINRR DLWEKAKKDQPE
misc.	WNDTCTSHSSMVCQEDSLEEEMGLLGYLSRISICKKKKDYCKMYRR HVRSRFYSIKDRNARLGESVDLNSRYTQLQLVKEHPSKQERE HELLTIGRTKMRDSPMSSSLKLELLFEPEDGHSEPV
NACHT	HTVVFQGAAGIGKTI LARKIMLDWALGKLFKDKFDYLFYIH CREVSLRTPRSLADLIVSCWPDNPPVCKILRKPSRILFLMDGFDELQGAFFEHIQEV CTDWQKAVRGDILLSSLIRKKLLPKASLLITTRPVALEKLQHLLDHPHVEILGFSEAKRKEYFFKYFSNE LQAREAFRLIQENEVLFTM CFIPLVCWIVCTGLKQQMETGKSLAQTSKTTTAVYVFFLSSLLQSRGGIEEHLFSDYLQGL CSLAADGIWNQKILFEE CDLRKHGLQKTDVSAFLRMNVFQKEVD CERFYSFSHMTFQEFFAAMYILLE
misc.	EEAEGETVRKGP GG CSDLLNRDVKVLENYGKFEKGYLI FVVRFLFGLVNQERTSYLEKKLS CKISQQVRLELLKWIEV KAKAKLQWQPSQLELFY CLYEMQEEDFVQSAMDHFPKIEINLSTRMD HVVSSFCIKNCHRVKTL SLGFFHN SPKEEEEE RRGG RPLDQ
LRR	VQCVFPDTHVACSSRLVNCCLTSSFCRGLFSSSLSTNR SLTELDLSDNTLGDPGMRVL CEALQHPGC NIQRLWLGRCGLSHQCCFDISSVLSSSQ KLVELDLSDNALGDFGIRLL CVGLKHLLC NLQKLWLVSCCLTSACCQDLALVLSNHS LTRLYIGENALGDSGVQVL CEKMKDPQC NLQKLGLVNSGLTSI CCSALTSVLKTNQ NFTHLYLRSNALGDTGLRLL CEGLLHPDC KLQMLELDNCSLTSWSCWNLS TILTHNH SLRKLNLGNNDLGDLCVVTI CEVLKQQGCLLQSLQLGEMYNRET KRALEALQEEKPELTIVFEISW



**Appendix 19: NLRP3-mediated IL-1 $\beta$  secretion is completely dependent on cathepsins B&L in immortalized bone-marrow-derived macrophages (IMMPs).**

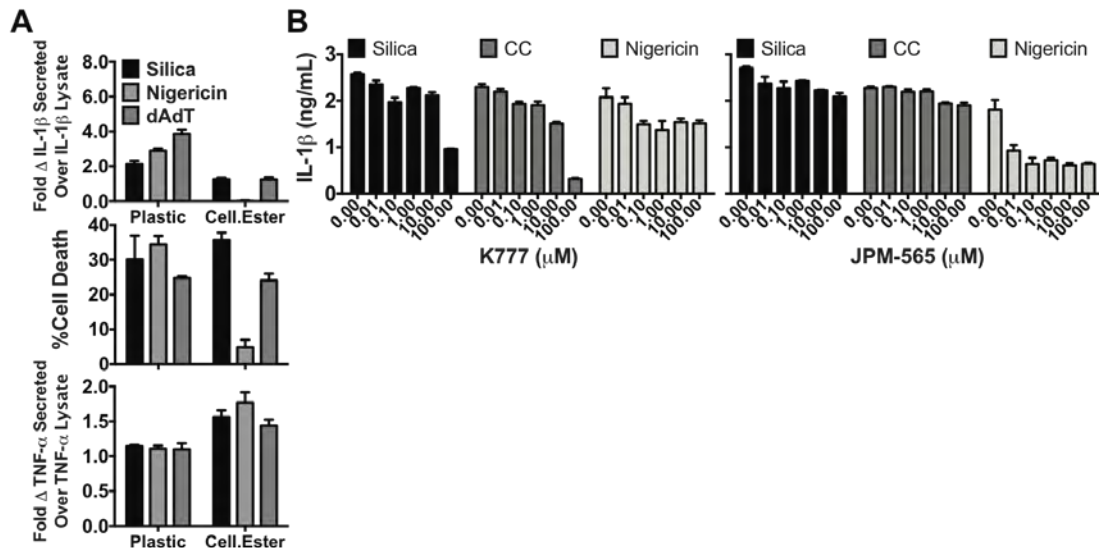
**(A)** LPS-primed IMMPs were stimulated with media control (-), silica (500  $\mu$ g/mL), MSU (300  $\mu$ g/mL), nigericin (2  $\mu$ M) or dAdT (0.65  $\mu$ g/mL) and cytokines analyzed in the supernatants by ELISA (top and bottom) or precipitated and IL-1 $\beta$  secretion analyzed by immunoblotting (middle). **(B)** IMMPs from WT or cathepsin B&L-deficient mice were treated with media control (-) or primed with LPS for 3h prior to analysis of IL-1 $\beta$  (*IL1b*) and NLRP3 (*Nlrp3*) expression by qPCR; data are normalized to GAPDH expression and plotted relative to (-) from WT cells. Error bars represent **(A)** S.E. of means from four (Silica, MSU, dAdT) or three (nigericin) independent experiments or, **(B)** S.D. of technical triplicates from one independent experiment.





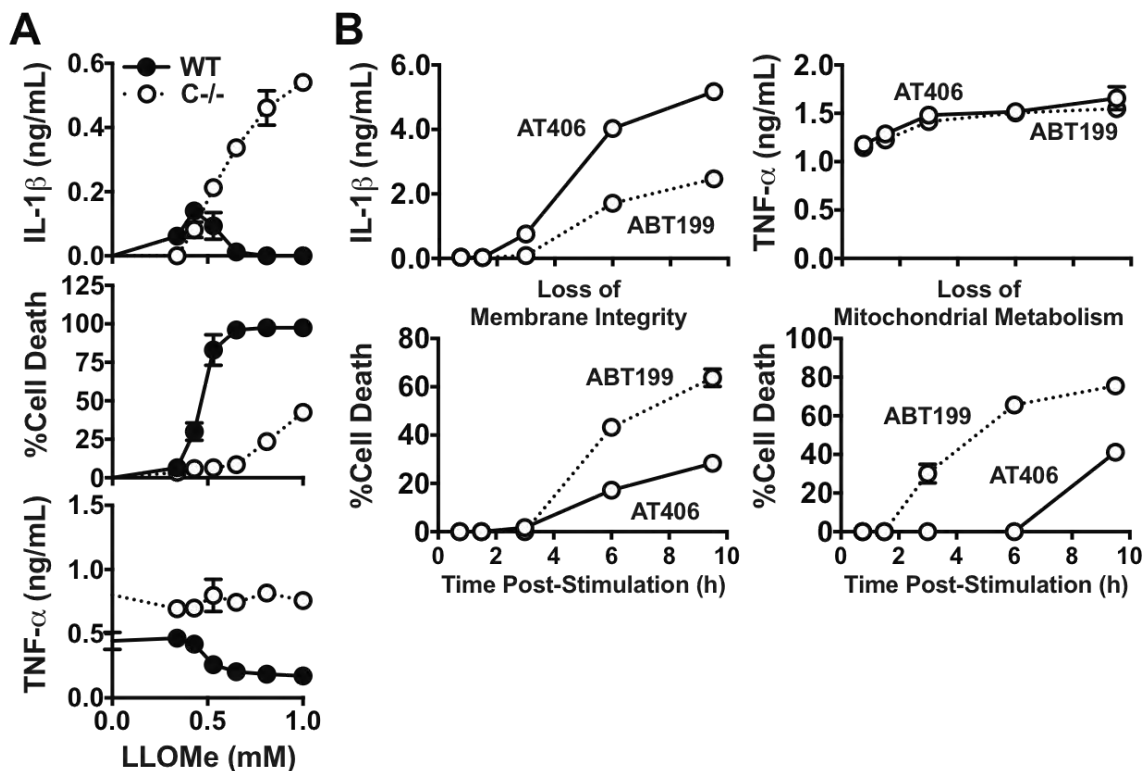
**Appendix 20: Nigericin-induced NLRP3 activation requires strong binding of integrins to a substrate and extracellular cathepsin activity.**

In all experiments, PMs were primed with LPS. **(A)** PMs were plated on either cell culture treated plastic or cellulose ester membranes (Millipore, Cat# MAHA S45 10) prior to stimulation with silica (80  $\mu\text{g/mL}$ ), nigericin (1.5  $\mu\text{M}$ ) or dAdT (0.5  $\mu\text{g/mL}$ ). **(B)** PMs were treated with titrations of either the cell-permeant cathepsin inhibitor K777 or the cell-impermeant cathepsin inhibitor JPM-565 prior to stimulation with silica (300  $\mu\text{g/mL}$ ), CC (500  $\mu\text{g/mL}$ ), or nigericin (2  $\mu\text{M}$ ). Error bars represent **(A)** S.D. of technical quadruplicates or, **(B)** S.D. of technical triplicates. Data are representative of one independent experiment.



**Appendix 21:** Attenuation of robust LMD-mediated cell death enhances IL-1 $\beta$  secretion, while early mitochondrial disruption suppresses IL-1 $\beta$  secretion.

In all experiments, PMs were primed with LPS. **(A)** PMs from WT or cathepsin C-deficient mice were stimulated with a range of LLOMe concentrations. **(B)** PMs were stimulated with either the Bcl-2 inhibitor ABT199 (5  $\mu$ M) or the Smac mimetic AT406 (10  $\mu$ M) and samples were analyzed at the indicated time points; cell death was measured either by LDH assay (Loss of Membrane Integrity) or MTS assay (Loss of Mitochondrial Metabolism) to demonstrate how much earlier ABT199 causes mitochondrial disruption compared to AT406. Error bars represent **(A)** S.D. of technical triplicates or, **(B)** range bars of technical duplicates. Data are representative of **(A)** two independent experiments, **(B)** one experiment.



## Bibliography

1. Kumar, V., A. K. Abbas, N. Fausto, S. L. Robbins, and R. S. Cotran. 2005. *Robbins and Cotran pathologic basis of disease*. Elsevier Saunders, Philadelphia.
2. Shen, H., D. Kreisel, and D. R. Goldstein. 2013. Processes of sterile inflammation. *J Immunol* 191:2857-2863.
3. Otsuki, T., M. Maeda, S. Murakami, H. Hayashi, Y. Miura, M. Kusaka, T. Nakano, K. Fukuoka, T. Kishimoto, F. Hyodoh, A. Ueki, and Y. Nishimura. 2007. Immunological effects of silica and asbestos. *Cell Mol Immunol* 4:261-268.
4. Cullinan, P., and P. Reid. 2013. Pneumoconiosis. *Prim Care Respir J* 22:249-252.
5. Morishige, T., Y. Yoshioka, A. Tanabe, X. Yao, S. Tsunoda, Y. Tsutsumi, Y. Mukai, N. Okada, and S. Nakagawa. 2010. Titanium dioxide induces different levels of IL-1 $\beta$  production dependent on its particle characteristics through caspase-1 activation mediated by reactive oxygen species and cathepsin B. *Biochem Biophys Res Commun* 392:160-165.
6. Skocaj, M., M. Filipic, J. Petkovic, and S. Novak. 2011. Titanium dioxide in our everyday life; is it safe? *Radiol Oncol* 45:227-247.
7. Castranova, V., and V. Vallyathan. 2000. Silicosis and coal workers' pneumoconiosis. *Environ Health Perspect* 108 Suppl 4:675-684.
8. Eltom, S., M. G. Belvisi, C. S. Stevenson, S. A. Maher, E. Dubuis, K. A. Fitzgerald, and M. A. Birrell. 2014. Role of the inflammasome-caspase1/11-IL-1/18 axis in cigarette smoke driven airway inflammation: an insight into the pathogenesis of COPD. *PLoS One* 9:e112829.
9. Nair, P. N., U. Sjogren, and G. Sundqvist. 1998. Cholesterol crystals as an etiological factor in non-resolving chronic inflammation: an experimental study in guinea pigs. *Eur J Oral Sci* 106:644-650.
10. Duewell, P., H. Kono, K. J. Rayner, C. M. Sirois, G. Vladimer, F. G. Bauernfeind, G. S. Abela, L. Franchi, G. Nunez, M. Schnurr, T. Espevik, E. Lien, K. A. Fitzgerald, K. L. Rock, K. J. Moore, S. D. Wright, V. Hornung, and E. Latz. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357-1361.
11. Rajamaki, K., J. Lappalainen, K. Oorni, E. Valimaki, S. Matikainen, P. T. Kovanen, and K. K. Eklund. 2010. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One* 5:e11765.
12. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.
13. Zarins, B., and V. K. McInerney. 1985. Calcium pyrophosphate and pseudogout. *Arthroscopy* 1:8-16.

14. Pantanowitz, L., and K. Balogh. 2004. Charcot-Leyden crystals: pathology and diagnostic utility. *Ear Nose Throat J* 83:489-490.
15. Dostert, C., G. Guarda, J. F. Romero, P. Menu, O. Gross, A. Tardivel, M. L. Suva, J. C. Stehle, M. Kopf, I. Stamenkovic, G. Corradin, and J. Tschopp. 2009. Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PLoS One* 4:e6510.
16. Masters, S. L., A. Dunne, S. L. Subramanian, R. L. Hull, G. M. Tannahill, F. A. Sharp, C. Becker, L. Franchi, E. Yoshihara, Z. Chen, N. Mullooly, L. A. Mielke, J. Harris, R. C. Coll, K. H. Mills, K. H. Mok, P. Newsholme, G. Nunez, J. Yodoi, S. E. Kahn, E. C. Lavelle, and L. A. O'Neill. 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.
17. Halle, A., V. Hornung, G. C. Petzold, C. R. Stewart, B. G. Monks, T. Reinheckel, K. A. Fitzgerald, E. Latz, K. J. Moore, and D. T. Golenbock. 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9:857-865.
18. Niemi, K., L. Teirila, J. Lappalainen, K. Rajamaki, M. H. Baumann, K. Oorni, H. Wolff, P. T. Kovanen, S. Matikainen, and K. K. Eklund. 2011. Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. *J Immunol* 186:6119-6128.
19. Ather, J. L., K. Ckless, R. Martin, K. L. Foley, B. T. Suratt, J. E. Boyson, K. A. Fitzgerald, R. A. Flavell, S. C. Eisenbarth, and M. E. Poynter. 2011. Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J Immunol* 187:64-73.
20. Pinney, J. H., and H. J. Lachmann. 2012. Systemic AA amyloidosis. *Subcell Biochem* 65:541-564.
21. Blancas-Mejia, L. M., and M. Ramirez-Alvarado. 2013. Systemic amyloidoses. *Annu Rev Biochem* 82:745-774.
22. Ashida, H., H. Mimuro, M. Ogawa, T. Kobayashi, T. Sanada, M. Kim, and C. Sasakawa. 2011. Cell death and infection: a double-edged sword for host and pathogen survival. *J Cell Biol* 195:931-942.
23. Miao, E. A., I. A. Leaf, P. M. Treuting, D. P. Mao, M. Dors, A. Sarkar, S. E. Warren, M. D. Wewers, and A. Aderem. 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol* 11:1136-1142.
24. Brodsky, I. E., and R. Medzhitov. 2011. Pyroptosis: macrophage suicide exposes hidden invaders. *Curr Biol* 21:R72-75.
25. Ellson, C. D., R. Dunmore, C. M. Hogaboam, M. A. Sleeman, and L. A. Murray. 2014. Danger-associated molecular patterns and danger signals in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 51:163-168.
26. Kono, H., Rock, K. L. . 2008. How dying cells alert the immune system to danger. *Nature Reviews: Immunology*:10.
27. Galkina, E., and K. Ley. 2009. Immune and inflammatory mechanisms of atherosclerosis (\*). *Annu Rev Immunol* 27:165-197.

28. Mossman, B. T., and A. Churg. 1998. Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med* 157:1666-1680.
29. Dutta, D. M., Brig M. . 2007. Crystalline silica particles mediated lung injury. *KONA: Powder and Particle Journal*:12.
30. Janeway, C. 2005. *Immunobiology : the immune system in health and disease*. Garland Science, New York.
31. Alberts, B. 2002. *Molecular biology of the cell*. Garland Science, New York.
32. Rock, K. L., B. Benacerraf, and A. K. Abbas. 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med* 160:1102-1113.
33. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gelfer. 1979. Properties and applications of monoclonal antibodies directed against determinants of they Thy-1 locus. *J Immunol* 122:2491-2497.
34. Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805-820.
35. Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.
36. Anderson, K. V., and C. Nusslein-Volhard. 1984. Information for the dorsal--ventral pattern of the Drosophila embryo is stored as maternal mRNA. *Nature* 311:223-227.
37. Rosetto, M., Y. Engstrom, C. T. Baldari, J. L. Telford, and D. Hultmark. 1995. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. *Biochem Biophys Res Commun* 209:111-116.
38. Morisato, D., and K. V. Anderson. 1994. The spatzie gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. *Cell* 76:677-688.
39. Dushay, M. S., B. Asling, and D. Hultmark. 1996. Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila. *Proc Natl Acad Sci U S A* 93:10343-10347.
40. Silverman, N., R. Zhou, R. L. Erlich, M. Hunter, E. Bernstein, D. Schneider, and T. Maniatis. 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. *J Biol Chem* 278:48928-48934.
41. Brooks, S. A., J. E. Connolly, and W. F. Rigby. 2004. The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. *J Immunol* 172:7263-7271.
42. Rajasingh, J., E. Bord, C. Luedemann, J. Asai, H. Hamada, T. Thorne, G. Qin, D. Goukassian, Y. Zhu, D. W. Losordo, and R. Kishore. 2006. IL-10-induced TNF-alpha mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *FASEB J* 20:2112-2114.
43. Zhao, W., M. Liu, and K. L. Kirkwood. 2008. p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. *J Biol Chem* 283:1778-1785.
44. Paschoud, S., A. M. Dogar, C. Kuntz, B. Grisoni-Neupert, L. Richman, and L. C. Kuhn. 2006. Destabilization of interleukin-6 mRNA requires a putative RNA

- stem-loop structure, an AU-rich element, and the RNA-binding protein AUF1. *Mol Cell Biol* 26:8228-8241.
45. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, D. McMurray, D. E. Smith, J. E. Sims, T. A. Bird, and L. A. O'Neill. 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413:78-83.
  46. Moore, K. J., L. P. Andersson, R. R. Ingalls, B. G. Monks, R. Li, M. A. Arnaout, D. T. Golenbock, and M. W. Freeman. 2000. Divergent response to LPS and bacteria in CD14-deficient murine macrophages. *J Immunol* 165:4272-4280.
  47. Zanoni, I., R. Ostuni, L. R. Marek, S. Barresi, R. Barbalat, G. M. Barton, F. Granucci, and J. C. Kagan. 2011. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147:868-880.
  48. Kagan, J. C., T. Su, T. Horng, A. Chow, S. Akira, and R. Medzhitov. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 9:361-368.
  49. Yamasaki, S., E. Ishikawa, M. Sakuma, H. Hara, K. Ogata, and T. Saito. 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol* 9:1179-1188.
  50. Zhang, J. G., P. E. Czabotar, A. N. Policheni, I. Caminschi, S. S. Wan, S. Kitsoulis, K. M. Tullett, A. Y. Robin, R. Brammananth, M. F. van Delft, J. Lu, L. A. O'Reilly, E. C. Josefsson, B. T. Kile, W. J. Chin, J. D. Mintern, M. A. Olshina, W. Wong, J. Baum, M. D. Wright, D. C. Huang, N. Mohandas, R. L. Coppel, P. M. Colman, N. A. Nicola, K. Shortman, and M. H. Lahoud. 2012. The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. *Immunity* 36:646-657.
  51. Ahrens, S., S. Zelenay, D. Sancho, P. Hanc, S. Kjaer, C. Feest, G. Fletcher, C. Durkin, A. Postigo, M. Skehel, F. Batista, B. Thompson, M. Way, C. Reis e Sousa, and O. Schulz. 2012. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity* 36:635-645.
  52. Iborra, S., H. M. Izquierdo, M. Martinez-Lopez, N. Blanco-Menendez, C. Reis e Sousa, and D. Sancho. 2012. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. *J Clin Invest* 122:1628-1643.
  53. Zelenay, S., A. M. Keller, P. G. Whitney, B. U. Schraml, S. Deddouche, N. C. Rogers, O. Schulz, D. Sancho, and C. Reis e Sousa. 2012. The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. *J Clin Invest* 122:1615-1627.
  54. Sancho, D., O. P. Joffre, A. M. Keller, N. C. Rogers, D. Martinez, P. Hernanz-Falcon, I. Rosewell, and C. Reis e Sousa. 2009. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458:899-903.
  55. Bhat, N., and K. A. Fitzgerald. 2014. Recognition of cytosolic DNA by cGAS and other STING-dependent sensors. *Eur J Immunol* 44:634-640.

56. Hornung, V., A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D. R. Caffrey, E. Latz, and K. A. Fitzgerald. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458:514-518.
57. Ishii, K. J., T. Kawagoe, S. Koyama, K. Matsui, H. Kumar, T. Kawai, S. Uematsu, O. Takeuchi, F. Takeshita, C. Coban, and S. Akira. 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 451:725-729.
58. Kis-Toth, K., A. Szanto, T. H. Thai, and G. C. Tsokos. 2011. Cytosolic DNA-activated human dendritic cells are potent activators of the adaptive immune response. *J Immunol* 187:1222-1234.
59. Yoshida, H., Y. Okabe, K. Kawane, H. Fukuyama, and S. Nagata. 2005. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat Immunol* 6:49-56.
60. Stetson, D. B., J. S. Ko, T. Heidmann, and R. Medzhitov. 2008. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134:587-598.
61. Ahn, J., D. Gutman, S. Saijo, and G. N. Barber. 2012. STING manifests self DNA-dependent inflammatory disease. *Proc Natl Acad Sci U S A* 109:19386-19391.
62. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.
63. Latz, E., T. S. Xiao, and A. Stutz. 2013. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 13:397-411.
64. Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218.
65. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20:319-325.
66. Faustin, B., L. Lartigue, J. M. Bruey, F. Luciano, E. Sergienko, B. Bailly-Maitre, N. Volkmann, D. Hanein, I. Rouiller, and J. C. Reed. 2007. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell* 25:713-724.
67. Bauernfeind, F. G., G. Horvath, A. Stutz, E. S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B. G. Monks, K. A. Fitzgerald, V. Hornung, and E. Latz. 2009. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183:787-791.
68. Franchi, L., T. Eigenbrod, and G. Nunez. 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.

69. Masters, S. L., A. Simon, I. Aksentijevich, and D. L. Kastner. 2009. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (\*). *Annu Rev Immunol* 27:621-668.
70. Garlanda, C., F. Riva, E. Bonavita, and A. Mantovani. 2013. Negative regulatory receptors of the IL-1 family. *Semin Immunol* 25:408-415.
71. Sutterwala, F. S., and R. A. Flavell. 2009. NLRC4/IPAF: a CARD carrying member of the NLR family. *Clin Immunol* 130:2-6.
72. Vigano, E., and A. Mortellaro. 2013. Caspase-11: the driving factor for noncanonical inflammasomes. *Eur J Immunol* 43:2240-2245.
73. Kayagaki, N., S. Warming, M. Lamkanfi, L. Vande Walle, S. Louie, J. Dong, K. Newton, Y. Qu, J. Liu, S. Heldens, J. Zhang, W. P. Lee, M. Roose-Girma, and V. M. Dixit. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature* 479:117-121.
74. Rathinam, V. A., S. K. Vanaja, L. Waggoner, A. Sokolovska, C. Becker, L. M. Stuart, J. M. Leong, and K. A. Fitzgerald. 2012. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 150:606-619.
75. Kang, S. J., S. Wang, H. Hara, E. P. Peterson, S. Namura, S. Amin-Hanjani, Z. Huang, A. Srinivasan, K. J. Tomaselli, N. A. Thornberry, M. A. Moskowitz, and J. Yuan. 2000. Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J Cell Biol* 149:613-622.
76. Brough, D., and N. J. Rothwell. 2007. Caspase-1-dependent processing of pro-interleukin-1 $\beta$  is cytosolic and precedes cell death. *J Cell Sci* 120:772-781.
77. Liu, T., Y. Yamaguchi, Y. Shirasaki, K. Shikada, M. Yamagishi, K. Hoshino, T. Kaisho, K. Takemoto, T. Suzuki, E. Kuranaga, O. Ohara, and M. Miura. 2014. Single-cell imaging of caspase-1 dynamics reveals an all-or-none inflammasome signaling response. *Cell Rep* 8:974-982.
78. Baroja-Mazo, A., F. Martin-Sanchez, A. I. Gomez, C. M. Martinez, J. Amores-Iniesta, V. Compan, M. Barbera-Cremades, J. Yague, E. Ruiz-Ortiz, J. Anton, S. Bujan, I. Couillin, D. Brough, J. I. Arostegui, and P. Pelegrin. 2014. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol* 15:738-748.
79. Franklin, B. S., L. Bossaller, D. De Nardo, J. M. Ratter, A. Stutz, G. Engels, C. Brenker, M. Nordhoff, S. R. Mirandola, A. Al-Amoudi, M. S. Mangan, S. Zimmer, B. G. Monks, M. Fricke, R. E. Schmidt, T. Espevik, B. Jones, A. G. Jarnicki, P. M. Hansbro, P. Busto, A. Marshak-Rothstein, S. Hornemann, A. Aguzzi, W. Kastentmuller, and E. Latz. 2014. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol* 15:727-737.
80. Majno, G., M. La Gattuta, and T. E. Thompson. 1960. Cellular death and necrosis: chemical, physical and morphologic changes in rat liver. *Virchows Arch Pathol Anat Physiol Klin Med* 333:421-465.
81. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991-1045.



82. Shi, Y., W. Zheng, and K. L. Rock. 2000. Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. *Proc Natl Acad Sci U S A* 97:14590-14595.
83. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5:1249-1255.
84. Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature* 454:428-435.
85. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418:191-195.
86. Andersson, U., H. Wang, K. Palmblad, A. C. Aveberger, O. Bloom, H. Erlandsson-Harris, A. Janson, R. Kokkola, M. Zhang, H. Yang, and K. J. Tracey. 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 192:565-570.
87. Tian, J., A. M. Avalos, S. Y. Mao, B. Chen, K. Senthil, H. Wu, P. Parroche, S. Drabic, D. Golenbock, C. Sirois, J. Hua, L. L. An, L. Audoly, G. La Rosa, A. Bierhaus, P. Naworth, A. Marshak-Rothstein, M. K. Crow, K. A. Fitzgerald, E. Latz, P. A. Kiener, and A. J. Coyle. 2007. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 8:487-496.
88. Sha, Y., J. Zmijewski, Z. Xu, and E. Abraham. 2008. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol* 180:2531-2537.
89. Rouhiainen, A., S. Tumova, L. Valmu, N. Kalkkinen, and H. Rauvala. 2007. Pivotal advance: analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1 (amphoterin). *J Leukoc Biol* 81:49-58.
90. Bianchi, M. E. 2009. HMGB1 loves company. *J Leukoc Biol* 86:573-576.
91. Coban, C., K. J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, T. Horii, and S. Akira. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med* 201:19-25.
92. Parroche, P., F. N. Lauw, N. Goutagny, E. Latz, B. G. Monks, A. Visintin, K. A. Halmen, M. Lamphier, M. Olivier, D. C. Bartholomeu, R. T. Gazzinelli, and D. T. Golenbock. 2007. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proc Natl Acad Sci U S A* 104:1919-1924.
93. Enoksson, M., K. Lyberg, C. Moller-Westerberg, P. G. Fallon, G. Nilsson, and C. Lunderius-Andersson. 2011. Mast cells as sensors of cell injury through IL-33 recognition. *J Immunol* 186:2523-2528.
94. Enoksson, M., C. Moller-Westerberg, G. Wicher, P. G. Fallon, K. Forsberg-Nilsson, C. Lunderius-Andersson, and G. Nilsson. 2013. Intraperitoneal influx of neutrophils in response to IL-33 is mast cell-dependent. *Blood* 121:530-536.
95. Chen, C. J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 13:851-856.

96. Kono, H., A. Onda, and T. Yanagida. 2014. Molecular determinants of sterile inflammation. *Curr Opin Immunol* 26:147-156.
97. Matzinger, P., and T. Kamala. 2011. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol* 11:221-230.
98. Kahlenberg, J. M., and G. R. Dubyak. 2004. Mechanisms of caspase-1 activation by P2X7 receptor-mediated K<sup>+</sup> release. *Am J Physiol Cell Physiol* 286:C1100-1108.
99. Kanneganti, T. D., M. Lamkanfi, Y. G. Kim, G. Chen, J. H. Park, L. Franchi, P. Vandenabeele, and G. Nunez. 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 26:433-443.
100. Iyer, S. S., W. P. Pulskens, J. J. Sadler, L. M. Butter, G. J. Teske, T. K. Ulland, S. C. Eisenbarth, S. Florquin, R. A. Flavell, J. C. Leemans, and F. S. Sutterwala. 2009. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 106:20388-20393.
101. McDonald, B., K. Pittman, G. B. Menezes, S. A. Hirota, I. Slaba, C. C. Waterhouse, P. L. Beck, D. A. Muruve, and P. Kubes. 2010. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 330:362-366.
102. Elliott, M. R., F. B. Chekeni, P. C. Trampont, E. R. Lazarowski, A. Kadl, S. F. Walk, D. Park, R. I. Woodson, M. Ostankovich, P. Sharma, J. J. Lysiak, T. K. Harden, N. Leitinger, and K. S. Ravichandran. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 461:282-286.
103. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425:516-521.
104. McCarty, D. J., and J. L. Hollander. 1961. Identification of urate crystals in gouty synovial fluid. *Ann Intern Med* 54:452-460.
105. Kobayashi, T., H. Kouzaki, and H. Kita. 2010. Human eosinophils recognize endogenous danger signal crystalline uric acid and produce proinflammatory cytokines mediated by autocrine ATP. *J Immunol* 184:6350-6358.
106. Gasse, P., N. Riteau, S. Charron, S. Girre, L. Fick, V. Petrilli, J. Tschopp, V. Lagente, V. F. Quesniaux, B. Ryffel, and I. Couillin. 2009. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. *Am J Respir Crit Care Med* 179:903-913.
107. van der Meer, J. W., M. Barza, S. M. Wolff, and C. A. Dinarello. 1988. A low dose of recombinant interleukin 1 protects granulocytopenic mice from lethal gram-negative infection. *Proc Natl Acad Sci U S A* 85:1620-1623.
108. Eigenbrod, T., J. H. Park, J. Harder, Y. Iwakura, and G. Nunez. 2008. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J Immunol* 181:8194-8198.
109. Kono, H., D. Karmarkar, Y. Iwakura, and K. L. Rock. 2010. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J Immunol* 184:4470-4478.

110. Huang, H., H. W. Chen, J. Evankovich, W. Yan, B. R. Rosborough, G. W. Nace, Q. Ding, P. Loughran, D. Beer-Stolz, T. R. Billiar, C. T. Esmon, and A. Tsung. 2013. Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. *J Immunol* 191:2665-2679.
111. Mosley, B., D. L. Urdal, K. S. Prickett, A. Larsen, D. Cosman, P. J. Conlon, S. Gillis, and S. K. Dower. 1987. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem* 262:2941-2944.
112. Fuhlbrigge, R. C., S. M. Fine, E. R. Unanue, and D. D. Chaplin. 1988. Expression of membrane interleukin 1 by fibroblasts transfected with murine pro-interleukin 1 alpha cDNA. *Proc Natl Acad Sci U S A* 85:5649-5653.
113. Kurt-Jones, E. A., D. I. Beller, S. B. Mizel, and E. R. Unanue. 1985. Identification of a membrane-associated interleukin 1 in macrophages. *Proc Natl Acad Sci U S A* 82:1204-1208.
114. Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267:2000-2003.
115. Lamkanfi, M., and V. M. Dixit. 2012. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* 28:137-161.
116. Vande Walle, L., T. D. Kanneganti, and M. Lamkanfi. 2011. HMGB1 release by inflammasomes. *Virulence* 2:162-165.
117. Lamkanfi, M., A. Sarkar, L. Vande Walle, A. C. Vitari, A. O. Amer, M. D. Wewers, K. J. Tracey, T. D. Kanneganti, and V. M. Dixit. 2010. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol* 185:4385-4392.
118. Cohen, I., P. Rider, Y. Carmi, A. Braiman, S. Dotan, M. R. White, E. Voronov, M. U. Martin, C. A. Dinarello, and R. N. Apte. 2010. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc Natl Acad Sci U S A* 107:2574-2579.
119. Kobayashi, Y., K. Yamamoto, T. Saido, H. Kawasaki, J. J. Oppenheim, and K. Matsushima. 1990. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. *Proc Natl Acad Sci U S A* 87:5548-5552.
120. Zheng, Y., M. Humphry, J. J. Maguire, M. R. Bennett, and M. C. Clarke. 2013. Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. *Immunity* 38:285-295.
121. Kono, H., G. M. Orłowski, Z. Patel, and K. L. Rock. 2012. The IL-1-dependent sterile inflammatory response has a substantial caspase-1-independent component that requires cathepsin C. *J Immunol* 189:3734-3740.
122. Fettelschoss, A., M. Kistowska, S. LeibundGut-Landmann, H. D. Beer, P. Johansen, G. Senti, E. Contassot, M. F. Bachmann, L. E. French, A. Oxenius, and T. M. Kundig. 2011. Inflammasome activation and IL-1beta target IL-1alpha for

- secretion as opposed to surface expression. *Proc Natl Acad Sci U S A* 108:18055-18060.
123. Schroder, K., and J. Tschopp. The inflammasomes. *Cell* 140:821-832.
  124. Latz, E. The inflammasomes: mechanisms of activation and function. *Curr Opin Immunol* 22:28-33.
  125. Hornung, V., and E. Latz. Critical functions of priming and lysosomal damage for NLRP3 activation. *Eur J Immunol* 40:620-623.
  126. Chu, J., L. M. Thomas, S. C. Watkins, L. Franchi, G. Nunez, and R. D. Salter. 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.
  127. Said-Sadier, N., E. Padilla, G. Langsley, and D. M. Ojcius. *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS One* 5:e10008.
  128. Kankkunen, P., L. Teirila, J. Rintahaka, H. Alenius, H. Wolff, and S. Matikainen. (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J Immunol* 184:6335-6342.
  129. Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hanneschlagel, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459:433-436.
  130. Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B. T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677.
  131. Cassel, S. L., S. C. Eisenbarth, S. S. Iyer, J. J. Sadler, O. R. Colegio, L. A. Tephly, A. B. Carter, P. B. Rothman, R. A. Flavell, and F. S. Sutterwala. 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105:9035-9040.
  132. Eisenbarth, S. C., O. R. Colegio, W. O'Connor, F. S. Sutterwala, and R. A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453:1122-1126.
  133. Petrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589.
  134. Petrovski, G., G. Ayna, G. Majai, J. Hodrea, S. Benko, A. Madi, and L. Fesus. Phagocytosis of cells dying through autophagy induces inflammasome activation and IL-1 $\beta$  release in human macrophages. *Autophagy* 7.
  135. Lindauer, M., J. Wong, and B. Magun. Ricin Toxin Activates the NALP3 Inflammasome. *Toxins (Basel)* 2:1500-1514.
  136. Rajamaki, K., J. Lappalainen, K. Oorni, E. Valimaki, S. Matikainen, P. T. Kovanen, and K. K. Eklund. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One* 5:e11765.

137. Tschopp, J., and K. Schroder. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* 10:210-215.
138. Cruz, C. M., A. Rinna, H. J. Forman, A. L. Ventura, P. M. Persechini, and D. M. Ojcius. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282:2871-2879.
139. Abdul-Sater, A. A., N. Said-Sadier, E. V. Padilla, and D. M. Ojcius. Chlamydial infection of monocytes stimulates IL-1beta secretion through activation of the NLRP3 inflammasome. *Microbes Infect* 12:652-661.
140. Barlan, A. U., T. M. Griffin, K. A. McGuire, and C. M. Wiethoff. Adenovirus membrane penetration activates the NLRP3 inflammasome. *J Virol* 85:146-155.
141. Lopez-Castejon, G., J. Theaker, P. Pelegrin, A. D. Clifton, M. Braddock, and A. Surprenant. P2X(7) receptor-mediated release of cathepsins from macrophages is a cytokine-independent mechanism potentially involved in joint diseases. *J Immunol* 185:2611-2619.
142. Hentze, H., X. Y. Lin, M. S. Choi, and A. G. Porter. 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.
143. Fujisawa, A., N. Kambe, M. Saito, R. Nishikomori, H. Tanizaki, N. Kanazawa, S. Adachi, T. Heike, J. Sagara, T. Suda, T. Nakahata, and Y. Miyachi. 2007. Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood* 109:2903-2911.
144. Hornung, V., F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9:847-856.
145. Duewell, P., H. Kono, K. J. Rayner, C. M. Sirois, G. Vladimer, F. G. Bauernfeind, G. S. Abela, L. Franchi, G. Nunez, M. Schnurr, T. Espevik, E. Lien, K. A. Fitzgerald, K. L. Rock, K. J. Moore, S. D. Wright, V. Hornung, and E. Latz. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357-1361.
146. Morishige, T., Y. Yoshioka, A. Tanabe, X. Yao, S. Tsunoda, Y. Tsutsumi, Y. Mukai, N. Okada, and S. Nakagawa. Titanium dioxide induces different levels of IL-1beta production dependent on its particle characteristics through caspase-1 activation mediated by reactive oxygen species and cathepsin B. *Biochem Biophys Res Commun* 392:160-165.
147. Duncan, J. A., X. Gao, M. T. Huang, B. P. O'Connor, C. E. Thomas, S. B. Willingham, D. T. Bergstralh, G. A. Jarvis, P. F. Sparling, and J. P. Ting. 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.

148. Meixenberger, K., F. Pache, J. Eitel, B. Schmeck, S. Hippenstiel, H. Slevogt, P. N'Guessan, M. Witzenth, M. G. Netea, T. Chakraborty, N. Suttorp, and B. Opitz. *Listeria monocytogenes*-infected human peripheral blood mononuclear cells produce IL-1 $\beta$ , depending on listeriolysin O and NLRP3. *J Immunol* 184:922-930.
149. Barlan, A. U., P. Danthi, and C. M. Wiethoff. Lysosomal localization and mechanism of membrane penetration influence nonenveloped virus activation of the NLRP3 inflammasome. *Virology* 412:306-314.
150. Bauer, C., P. Duewell, C. Mayer, H. A. Lehr, K. A. Fitzgerald, M. Dauer, J. Tschopp, S. Endres, E. Latz, and M. Schnurr. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* 59:1192-1199.
151. Rintahaka, J., N. Lietzen, T. Ohman, T. A. Nyman, and S. Matikainen. Recognition of cytoplasmic RNA results in cathepsin-dependent inflammasome activation and apoptosis in human macrophages. *J Immunol* 186:3085-3092.
152. Terada, K., J. Yamada, Y. Hayashi, Z. Wu, Y. Uchiyama, C. Peters, and H. Nakanishi. Involvement of cathepsin B in the processing and secretion of interleukin-1 $\beta$  in chromogranin A-stimulated microglia. *Glia* 58:114-124.
153. Muruve, D. A., V. Petrilli, A. K. Zaiss, L. R. White, S. A. Clark, P. J. Ross, R. J. Parks, and J. Tschopp. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452:103-107.
154. Munoz-Planillo, R., P. Kuffa, G. Martinez-Colon, B. L. Smith, T. M. Rajendiran, and G. Nunez. 2013. K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38:1142-1153.
155. Jacobson, L. S., H. Lima, Jr., M. F. Goldberg, V. Gocheva, V. Tsiperson, F. S. Sutterwala, J. A. Joyce, B. V. Gapp, V. A. Blomen, K. Chandran, T. R. Brummelkamp, F. Diaz-Griffero, and J. Brojatsch. 2013. Cathepsin-mediated necrosis controls the adaptive immune response by Th2 (T helper type 2)-associated adjuvants. *J Biol Chem* 288:7481-7491.
156. Montaser, M., G. Lalmanach, and L. Mach. 2002. CA-074, but not its methyl ester CA-074Me, is a selective inhibitor of cathepsin B within living cells. *Biol Chem* 383:1305-1308.
157. Mihalik, R., G. Imre, I. Petak, B. Szende, and L. Kopper. 2004. Cathepsin B-independent abrogation of cell death by CA-074-OMe upstream of lysosomal breakdown. *Cell Death Differ* 11:1357-1360.
158. Klemencic, I., A. K. Carmona, M. H. Cezari, M. A. Juliano, L. Juliano, G. Guncar, D. Turk, I. Krizaj, V. Turk, and B. Turk. 2000. Biochemical characterization of human cathepsin X revealed that the enzyme is an exopeptidase, acting as carboxymonopeptidase or carboxydipeptidase. *Eur J Biochem* 267:5404-5412.
159. Bogoy, M., S. Verhelst, V. Bellingard-Dubouchaud, S. Toba, and D. Greenbaum. 2000. Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs. *Chem Biol* 7:27-38.

160. Bruchard, M., G. Mignot, V. Derangere, F. Chalmin, A. Chevriaux, F. Vegran, W. Boireau, B. Simon, B. Ryffel, J. L. Connat, J. Kanellopoulos, F. Martin, C. Rebe, L. Apetoh, and F. Ghiringhelli. 2012. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* 19:57-64.
161. Terada, K., J. Yamada, Y. Hayashi, Z. Wu, Y. Uchiyama, C. Peters, and H. Nakanishi. 2009. Involvement of cathepsin B in the processing and secretion of interleukin-1beta in chromogranin A-stimulated microglia. *Glia* 58:114-124.
162. Newman, Z. L., S. H. Leppla, and M. Moayeri. 2009. CA-074Me protection against anthrax lethal toxin. *Infect Immun* 77:4327-4336.
163. Lima, H., Jr., L. S. Jacobson, M. F. Goldberg, K. Chandran, F. Diaz-Griffero, M. P. Lisanti, and J. Brojatsch. 2013. Role of lysosome rupture in controlling Nlrp3 signaling and necrotic cell death. *Cell Cycle* 12:1868-1878.
164. Shukla, A., M. Gulumian, T. K. Hei, D. Kamp, Q. Rahman, and B. T. Mossman. 2003. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. *Free Radic Biol Med* 34:1117-1129.
165. Costantini, L. M., R. M. Gilberti, and D. A. Knecht. 2011. The phagocytosis and toxicity of amorphous silica. *PLoS One* 6:e14647.
166. Morishige, T., Y. Yoshioka, H. Inakura, A. Tanabe, X. Yao, S. Narimatsu, Y. Monobe, T. Imazawa, S. Tsunoda, Y. Tsutsumi, Y. Mukai, N. Okada, and S. Nakagawa. 2010. The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1beta production, ROS production and endosomal rupture. *Biomaterials* 31:6833-6842.
167. Nel, A. E., L. Madler, D. Velegol, T. Xia, E. M. Hoek, P. Somasundaran, F. Klaessig, V. Castranova, and M. Thompson. 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nat Mater* 8:543-557.
168. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol* 21:335-376.
169. Latz, E. 2010. NOX-free inflammasome activation. *Blood* 116:1393-1394.
170. Meissner, F., K. Molawi, and A. Zychlinsky. 2008. Superoxide dismutase 1 regulates caspase-1 and endotoxic shock. *Nat Immunol* 9:866-872.
171. Segal, B. H., W. Han, J. J. Bushey, M. Joo, Z. Bhatti, J. Feminella, C. G. Dennis, R. R. Vethanayagam, F. E. Yull, M. Capitano, P. K. Wallace, H. Minderman, J. W. Christman, M. B. Sporn, J. Chan, D. C. Vinh, S. M. Holland, L. R. Romani, S. L. Gaffen, M. L. Freeman, and T. S. Blackwell. 2010. NADPH oxidase limits innate immune responses in the lungs in mice. *PLoS One* 5:e9631.
172. Sutterwala, F. S., S. Haasken, and S. L. Cassel. 2014. Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci* 1319:82-95.
173. Zhou, R., A. S. Yazdi, P. Menu, and J. Tschopp. 2010. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469:221-225.
174. Nakahira, K., J. A. Haspel, V. A. Rathinam, S. J. Lee, T. Dolinay, H. C. Lam, J. A. Englert, M. Rabinovitch, M. Cernadas, H. P. Kim, K. A. Fitzgerald, S. W. Ryter, and A. M. Choi. 2011. Autophagy proteins regulate innate immune

- responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 12:222-230.
175. Zhou, R., A. Tardivel, B. Thorens, I. Choi, and J. Tschopp. 2009. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11:136-140.
  176. Zhou, R., A. S. Yazdi, P. Menu, and J. Tschopp. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469:221-225.
  177. Schroder, K., R. Zhou, and J. Tschopp. The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 327:296-300.
  178. Zhou, R., A. Tardivel, B. Thorens, I. Choi, and J. Tschopp. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11:136-140.
  179. Lunov, O., T. Syrovets, C. Loos, G. U. Nienhaus, V. Mailander, K. Landfester, M. Rouis, and T. Simmet. 2011. Amino-functionalized polystyrene nanoparticles activate the NLRP3 inflammasome in human macrophages. *ACS Nano* 5:9648-9657.
  180. Colombini, M. 2004. VDAC: the channel at the interface between mitochondria and the cytosol. *Mol Cell Biochem* 256-257:107-115.
  181. Hsu, H. Y., and M. H. Wen. 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem* 277:22131-22139.
  182. Asehnoune, K., D. Strassheim, S. Mitra, J. Y. Kim, and E. Abraham. 2004. Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J Immunol* 172:2522-2529.
  183. Stosic-Grujicic, S. D., D. M. Miljkovic, I. D. Cvetkovic, D. D. Maksimovic-Ivanic, and V. Trajkovic. 2004. Immunosuppressive and anti-inflammatory action of antioxidants in rat autoimmune diabetes. *J Autoimmun* 22:267-276.
  184. Bauernfeind, F., E. Bartok, A. Rieger, L. Franchi, G. Nunez, and V. Hornung. 2011. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *J Immunol* 187:613-617.
  185. Hornung, V., and E. Latz. 2010. Critical functions of priming and lysosomal damage for NLRP3 activation. *Eur J Immunol* 40:620-623.
  186. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.
  187. Lamkanfi, M., J. L. Mueller, A. C. Vitari, S. Misaghi, A. Fedorova, K. Deshayes, W. P. Lee, H. M. Hoffman, and V. M. Dixit. 2009. Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J Cell Biol* 187:61-70.
  188. Yakubovich, N., J. R. Eldstrom, and D. A. Mathers. 2001. Lipopolysaccharide can activate BK channels of arterial smooth muscle in the absence of iNOS expression. *Biochim Biophys Acta* 1514:239-252.
  189. Blunck, R., O. Scheel, M. Muller, K. Brandenburg, U. Seitzer, and U. Seydel. 2001. New insights into endotoxin-induced activation of macrophages:



- involvement of a K<sup>+</sup> channel in transmembrane signaling. *J Immunol* 166:1009-1015.
190. Hoang, L. M., C. Chen, and D. A. Mathers. 1997. Lipopolysaccharide rapidly activates K<sup>+</sup> channels at the intracellular membrane face of rat cerebral artery smooth muscle cells. *Neurosci Lett* 231:25-28.
  191. McKinney, L. C., and E. K. Gallin. 1990. Effect of adherence, cell morphology, and lipopolysaccharide on potassium conductance and passive membrane properties of murine macrophage J774.1 cells. *J Membr Biol* 116:47-56.
  192. Vicente, R., A. Escalada, M. Coma, G. Fuster, E. Sanchez-Tillo, C. Lopez-Iglesias, C. Soler, C. Solsona, A. Celada, and A. Felipe. 2003. Differential voltage-dependent K<sup>+</sup> channel responses during proliferation and activation in macrophages. *J Biol Chem* 278:46307-46320.
  193. Chen, Y. J., M. Y. Hsieh, M. Y. Chang, H. C. Chen, M. S. Jan, M. C. Maa, and T. H. Leu. 2012. Eps8 protein facilitates phagocytosis by increasing TLR4-MyD88 protein interaction in lipopolysaccharide-stimulated macrophages. *J Biol Chem* 287:18806-18819.
  194. Peppelenbosch, M. P., M. DeSmedt, T. ten Hove, S. J. van Deventer, and J. Grooten. 1999. Lipopolysaccharide regulates macrophage fluid phase pinocytosis via CD14-dependent and CD14-independent pathways. *Blood* 93:4011-4018.
  195. Schorn, C., B. Frey, K. Lauber, C. Janko, M. Stryio, H. Keppeler, U. S. Gaipf, R. E. Voll, E. Springer, L. E. Munoz, G. Schett, and M. Herrmann. Sodium overload and water influx activate the NALP3 inflammasome. *J Biol Chem* 286:35-41.
  196. Jin, C., and R. A. Flavell. Molecular mechanism of NLRP3 inflammasome activation. *J Clin Immunol* 30:628-631.
  197. Michallet, M. C., F. Saltel, M. Flacher, J. P. Revillard, and L. Genestier. 2004. Cathepsin-dependent apoptosis triggered by supraoptimal activation of T lymphocytes: a possible mechanism of high dose tolerance. *J Immunol* 172:5405-5414.
  198. Blomgran, R., L. Zheng, and O. Stendahl. 2007. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. *J Leukoc Biol* 81:1213-1223.
  199. Windelborn, J. A., and P. Lipton. 2008. Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. *J Neurochem* 106:56-69.
  200. Conus, S., and H. U. Simon. 2008. Cathepsins: key modulators of cell death and inflammatory responses. *Biochem Pharmacol* 76:1374-1382.
  201. Furlong, I. J., R. Ascaso, A. Lopez Rivas, and M. K. Collins. 1997. Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. *J Cell Sci* 110 ( Pt 5):653-661.
  202. Aronson, P. S., and G. Giebisch. 2011. Effects of pH on potassium: new explanations for old observations. *J Am Soc Nephrol* 22:1981-1989.
  203. Lee, G. S., N. Subramanian, A. I. Kim, I. Aksentijevich, R. Goldbach-Mansky, D. B. Sacks, R. N. Germain, D. L. Kastner, and J. J. Chae. 2012. The calcium-

- sensing receptor regulates the NLRP3 inflammasome through  $\text{Ca}^{2+}$  and cAMP. *Nature* 492:123-127.
204. Rossol, M., M. Pierer, N. Raulien, D. Quandt, U. Meusch, K. Rothe, K. Schubert, T. Schoneberg, M. Schaefer, U. Krugel, S. Smajilovic, H. Brauner-Osborne, C. Baerwald, and U. Wagner. 2012. Extracellular  $\text{Ca}^{2+}$  is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nat Commun* 3:1329.
  205. Compan, V., A. Baroja-Mazo, G. Lopez-Castejon, A. I. Gomez, C. M. Martinez, D. Angosto, M. T. Montero, A. S. Herranz, E. Bazan, D. Reimers, V. Mulero, and P. Pelegrin. 2012. Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity* 37:487-500.
  206. Zhong, Z., Y. Zhai, S. Liang, Y. Mori, R. Han, F. S. Sutterwala, and L. Qiao. 2013. TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nat Commun* 4:1611.
  207. Murakami, T., J. Ockinger, J. Yu, V. Byles, A. McColl, A. M. Hofer, and T. Horng. 2012. Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 109:11282-11287.
  208. Triantafilou, K., T. R. Hughes, M. Triantafilou, and B. P. Morgan. 2013. The complement membrane attack complex triggers intracellular  $\text{Ca}^{2+}$  fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126:2903-2913.
  209. Abdul-Sater, A. A., I. Tattoli, L. Jin, A. Grajkowski, A. Levi, B. H. Koller, I. C. Allen, S. L. Beaucage, K. A. Fitzgerald, J. P. Ting, J. C. Cambier, S. E. Girardin, and C. Schindler. 2013. Cyclic-di-GMP and cyclic-di-AMP activate the NLRP3 inflammasome. *EMBO Rep* 14:900-906.
  210. Gross, O., A. S. Yazdi, C. J. Thomas, M. Masin, L. X. Heinz, G. Guarda, M. Quadroni, S. K. Drexler, and J. Tschopp. 2012. Inflammasome activators induce interleukin-1 $\alpha$  secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* 36:388-400.
  211. Watanabe, N., J. Suzuki, and Y. Kobayashi. 1996. Role of calcium in tumor necrosis factor- $\alpha$  production by activated macrophages. *J Biochem* 120:1190-1195.
  212. Jin, S. W., L. Zhang, Q. Q. Lian, S. L. Yao, P. Wu, X. Y. Zhou, W. Xiong, and D. Y. Ye. 2006. Close functional coupling between  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels and reactive oxygen species production in murine macrophages. *Mediators Inflamm* 2006:36192.
  213. Zhou, X., W. Yang, and J. Li. 2006.  $\text{Ca}^{2+}$ - and protein kinase C-dependent signaling pathway for nuclear factor- $\kappa$ B activation, inducible nitric-oxide synthase expression, and tumor necrosis factor- $\alpha$  production in lipopolysaccharide-stimulated rat peritoneal macrophages. *J Biol Chem* 281:31337-31347.
  214. Aki, D., Y. Minoda, H. Yoshida, S. Watanabe, R. Yoshida, G. Takaesu, T. Chinen, T. Inaba, M. Hikida, T. Kurosaki, K. Saeki, and A. Yoshimura. 2008. Peptidoglycan and lipopolysaccharide activate PLC $\gamma$ 2, leading to enhanced cytokine production in macrophages and dendritic cells. *Genes Cells* 13:199-208.

215. Zanoni, I., R. Ostuni, G. Capuano, M. Collini, M. Caccia, A. E. Ronchi, M. Rocchetti, F. Mingozzi, M. Foti, G. Chirico, B. Costa, A. Zaza, P. Ricciardi-Castagnoli, and F. Granucci. 2009. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. *Nature* 460:264-268.
216. Chiang, C. Y., V. Veckman, K. Limmer, and M. David. 2012. Phospholipase Cgamma-2 and intracellular calcium are required for lipopolysaccharide-induced Toll-like receptor 4 (TLR4) endocytosis and interferon regulatory factor 3 (IRF3) activation. *J Biol Chem* 287:3704-3709.
217. Zhang, Q., M. Raoof, Y. Chen, Y. Sumi, T. Sursal, W. Junger, K. Brohi, K. Itagaki, and C. J. Hauser. 2010. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 464:104-107.
218. Shimada, K., T. R. Crother, J. Karlin, J. Dagvadorj, N. Chiba, S. Chen, V. K. Ramanujan, A. J. Wolf, L. Vergnes, D. M. Ojcius, A. Rentsendorj, M. Vargas, C. Guerrero, Y. Wang, K. A. Fitzgerald, D. M. Underhill, T. Town, and M. Ardit. 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36:401-414.
219. Johansson, A. C., H. Appelqvist, C. Nilsson, K. Kagedal, K. Roberg, and K. Ollinger. 2010. Regulation of apoptosis-associated lysosomal membrane permeabilization. *Apoptosis* 15:527-540.
220. Iyer, S. S., Q. He, J. R. Janczy, E. I. Elliott, Z. Zhong, A. K. Olivier, J. J. Sadler, V. Knepper-Adrian, R. Han, L. Qiao, S. C. Eisenbarth, W. M. Nauseef, S. L. Cassel, and F. S. Sutterwala. 2013. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39:311-323.
221. Gonzalez, F., Z. T. Schug, R. H. Houtkooper, E. D. MacKenzie, D. G. Brooks, R. J. Wanders, P. X. Petit, F. M. Vaz, and E. Gottlieb. 2008. Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. *J Cell Biol* 183:681-696.
222. Liu, J., R. F. Epand, D. Durrant, D. Grossman, N. W. Chi, R. M. Epand, and R. M. Lee. 2008. Role of phospholipid scramblase 3 in the regulation of tumor necrosis factor-alpha-induced apoptosis. *Biochemistry* 47:4518-4529.
223. Garcia Fernandez, M., L. Troiano, L. Moretti, M. Nasi, M. Pinti, S. Salvioli, J. Dobrucki, and A. Cossarizza. 2002. Early changes in intramitochondrial cardiolipin distribution during apoptosis. *Cell Growth Differ* 13:449-455.
224. Ichinohe, T., T. Yamazaki, T. Koshiba, and Y. Yanagi. 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.
225. Schroder, K., V. Sagulenko, A. Zamoshnikova, A. A. Richards, J. A. Cridland, K. M. Irvine, K. J. Stacey, and M. J. Sweet. 2012. Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. *Immunobiology* 217:1325-1329.
226. Juliana, C., T. Fernandes-Alnemri, S. Kang, A. Farias, F. Qin, and E. S. Alnemri. 2012. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem* 287:36617-36622.

227. Fernandes-Alnemri, T., S. Kang, C. Anderson, J. Sagara, K. A. Fitzgerald, and E. S. Alnemri. 2013. Cutting edge: TLR signaling licenses IRAK1 for rapid activation of the NLRP3 inflammasome. *J Immunol* 191:3995-3999.
228. Lin, K. M., W. Hu, T. D. Troutman, M. Jennings, T. Brewer, X. Li, S. Nanda, P. Cohen, J. A. Thomas, and C. Pasare. 2014. IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proc Natl Acad Sci U S A* 111:775-780.
229. Py, B. F., M. S. Kim, H. Vakifahmetoglu-Norberg, and J. Yuan. 2013. Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol Cell* 49:331-338.
230. Lopez-Castejon, G., N. M. Luheshi, V. Compan, S. High, R. C. Whitehead, S. Flitsch, A. Kirov, I. Prudovsky, E. Swanton, and D. Brough. 2013. Deubiquitinases regulate the activity of caspase-1 and interleukin-1beta secretion via assembly of the inflammasome. *J Biol Chem* 288:2721-2733.
231. Mayor, A., F. Martinon, T. De Smedt, V. Petrilli, and J. Tschopp. 2007. A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses. *Nat Immunol* 8:497-503.
232. Chen, C. J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J Clin Invest* 116:2262-2271.
233. Netea, M. G., C. A. Nold-Petry, M. F. Nold, L. A. Joosten, B. Opitz, J. H. van der Meer, F. L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C. J. Funk, R. J. Mason, B. J. Kullberg, A. Rubartelli, J. W. van der Meer, and C. A. Dinarello. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113:2324-2335.
234. Kahlenberg, J. M., and G. R. Dubyak. 2004. Differing caspase-1 activation states in monocyte versus macrophage models of IL-1beta processing and release. *J Leukoc Biol* 76:676-684.
235. Vanden Berghe, T., D. Demon, P. Bogaert, B. Vandendriessche, A. Goethals, B. Depuydt, M. Vuylsteke, R. Roelandt, E. Van Wonterghem, J. Vandembroecke, S. M. Choi, E. Meyer, S. Krautwald, W. Declercq, N. Takahashi, A. Cauwels, and P. Vandenabeele. 2014. Simultaneous targeting of IL-1 and IL-18 is required for protection against inflammatory and septic shock. *Am J Respir Crit Care Med* 189:282-291.
236. Gattorno, M., S. Tassi, S. Carta, L. Delfino, F. Ferlito, M. A. Pelagatti, A. D'Osualdo, A. Buoncompagni, M. G. Alpigliani, M. Alessio, A. Martini, and A. Rubartelli. 2007. Pattern of interleukin-1beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum* 56:3138-3148.
237. Moriwaki, K., J. Bertin, P. J. Gough, and F. K. Chan. 2015. A RIPK3-Caspase 8 Complex Mediates Atypical Pro-IL-1beta Processing. *J Immunol* 194:1938-1944.

238. Maelfait, J., E. Vercammen, S. Janssens, P. Schotte, M. Haegman, S. Magez, and R. Beyaert. 2008. Stimulation of Toll-like receptor 3 and 4 induces interleukin-1 $\beta$  maturation by caspase-8. *J Exp Med* 205:1967-1973.
239. Guarda, G., M. Braun, F. Staehli, A. Tardivel, C. Mattmann, I. Forster, M. Farlik, T. Decker, R. A. Du Pasquier, P. Romero, and J. Tschopp. 2011. Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34:213-223.
240. Bossaller, L., P. I. Chiang, C. Schmidt-Lauber, S. Ganesan, W. J. Kaiser, V. A. Rathinam, E. S. Mocarski, D. Subramanian, D. R. Green, N. Silverman, K. A. Fitzgerald, A. Marshak-Rothstein, and E. Latz. 2012. Cutting edge: FAS (CD95) mediates noncanonical IL-1 $\beta$  and IL-18 maturation via caspase-8 in an RIP3-independent manner. *J Immunol* 189:5508-5512.
241. Gringhuis, S. I., T. M. Kaptein, B. A. Wevers, B. Theelen, M. van der Vlist, T. Boekhout, and T. B. Geijtenbeek. 2012. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1 $\beta$  via a noncanonical caspase-8 inflammasome. *Nat Immunol* 13:246-254.
242. Ganesan, S., V. A. Rathinam, L. Bossaller, K. Army, W. J. Kaiser, E. S. Mocarski, C. P. Dillon, D. R. Green, T. N. Mayadas, S. M. Levitz, A. G. Hise, N. Silverman, and K. A. Fitzgerald. 2014. Caspase-8 modulates dectin-1 and complement receptor 3-driven IL-1 $\beta$  production in response to beta-glucans and the fungal pathogen, *Candida albicans*. *J Immunol* 193:2519-2530.
243. Antonopoulos, C., C. El Sanadi, W. J. Kaiser, E. S. Mocarski, and G. R. Dubyak. 2013. Proapoptotic chemotherapeutic drugs induce noncanonical processing and release of IL-1 $\beta$  via caspase-8 in dendritic cells. *J Immunol* 191:4789-4803.
244. England, H., H. R. Summersgill, M. E. Edye, N. J. Rothwell, and D. Brough. 2014. Release of interleukin-1 $\alpha$  or interleukin-1 $\beta$  depends on mechanism of cell death. *J Biol Chem* 289:15942-15950.
245. Shenderov, K., N. Riteau, R. Yip, K. D. Mayer-Barber, S. Oland, S. Hieny, P. Fitzgerald, A. Oberst, C. P. Dillon, D. R. Green, V. Cerundolo, and A. Sher. 2014. Cutting edge: Endoplasmic reticulum stress licenses macrophages to produce mature IL-1 $\beta$  in response to TLR4 stimulation through a caspase-8- and TRIF-dependent pathway. *J Immunol* 192:2029-2033.
246. Man, S. M., P. Toulomousis, L. Hopkins, T. P. Monie, K. A. Fitzgerald, and C. E. Bryant. 2013. *Salmonella* infection induces recruitment of Caspase-8 to the inflammasome to modulate IL-1 $\beta$  production. *J Immunol* 191:5239-5246.
247. Gurung, P., P. K. Anand, R. K. Malireddi, L. Vande Walle, N. Van Opdenbosch, C. P. Dillon, R. Weinlich, D. R. Green, M. Lamkanfi, and T. D. Kanneganti. 2014. FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol* 192:1835-1846.
248. Weng, D., R. Marty-Roix, S. Ganesan, M. K. Proulx, G. I. Vladimer, W. J. Kaiser, E. S. Mocarski, K. Pouliot, F. K. Chan, M. A. Kelliher, P. A. Harris, J. Bertin, P. J. Gough, D. M. Shayakhmetov, J. D. Goguen, K. A. Fitzgerald, N. Silverman, and E. Lien. 2014. Caspase-8 and RIP kinases regulate bacteria-

- induced innate immune responses and cell death. *Proc Natl Acad Sci U S A* 111:7391-7396.
249. Moriwaki, K., and F. K. Chan. 2013. RIP3: a molecular switch for necrosis and inflammation. *Genes Dev* 27:1640-1649.
  250. Feoktistova, M., P. Geserick, B. Kellert, D. P. Dimitrova, C. Langlais, M. Hupe, K. Cain, M. MacFarlane, G. Hacker, and M. Leverkus. 2011. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell* 43:449-463.
  251. Dondelinger, Y., M. A. Aguilera, V. Goossens, C. Dubuisson, S. Grootjans, E. Dejardin, P. Vandenabeele, and M. J. Bertrand. 2013. RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. *Cell Death Differ* 20:1381-1392.
  252. Yabal, M., N. Muller, H. Adler, N. Knies, C. J. Gross, R. B. Damgaard, H. Kanegane, M. Ringelhan, T. Kaufmann, M. Heikenwalder, A. Strasser, O. Gross, J. Ruland, C. Peschel, M. Gyrd-Hansen, and P. J. Jost. 2014. XIAP restricts TNF- and RIP3-dependent cell death and inflammasome activation. *Cell Rep* 7:1796-1808.
  253. Vince, J. E., W. W. Wong, I. Gentle, K. E. Lawlor, R. Allam, L. O'Reilly, K. Mason, O. Gross, S. Ma, G. Guarda, H. Anderton, R. Castillo, G. Hacker, J. Silke, and J. Tschopp. 2012. Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. *Immunity* 36:215-227.
  254. Kaiser, W. J., J. W. Upton, A. B. Long, D. Livingston-Rosanoff, L. P. Daley-Bauer, R. Hakem, T. Caspary, and E. S. Mocarski. 2011. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 471:368-372.
  255. Newton, K., D. L. Dugger, K. E. Wickliffe, N. Kapoor, M. C. de Almagro, D. Vucic, L. Komuves, R. E. Ferrando, D. M. French, J. Webster, M. Roose-Girma, S. Warming, and V. M. Dixit. 2014. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. *Science* 343:1357-1360.
  256. Kang, T. B., S. H. Yang, B. Toth, A. Kovalenko, and D. Wallach. 2012. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* 38:27-40.
  257. Aits, S., and M. Jaattela. 2013. Lysosomal cell death at a glance. *J Cell Sci* 126:1905-1912.
  258. De Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* 60:604-617.
  259. Turk, V., V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, and D. Turk. 2012. Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim Biophys Acta* 1824:68-88.
  260. Luzio, J. P., P. R. Pryor, and N. A. Bright. 2007. Lysosomes: fusion and function. *Nat Rev Mol Cell Biol* 8:622-632.
  261. Puente, X. S., L. M. Sanchez, C. M. Overall, and C. Lopez-Otin. 2003. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 4:544-558.

262. Dahl, S. W., T. Halkier, C. Lauritzen, I. Dolenc, J. Pedersen, V. Turk, and B. Turk. 2001. Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. *Biochemistry* 40:1671-1678.
263. Caglic, D., J. R. Pungercar, G. Pejler, V. Turk, and B. Turk. 2007. Glycosaminoglycans facilitate procathepsin B activation through disruption of propeptide-mature enzyme interactions. *J Biol Chem* 282:33076-33085.
264. Vasiljeva, O., M. Dolinar, J. R. Pungercar, V. Turk, and B. Turk. 2005. Recombinant human procathepsin S is capable of autocatalytic processing at neutral pH in the presence of glycosaminoglycans. *FEBS Lett* 579:1285-1290.
265. Turk, B., D. Turk, and G. S. Salvesen. 2002. Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. *Curr Pharm Des* 8:1623-1637.
266. Turk, B., and V. Turk. 2009. Lysosomes as "suicide bags" in cell death: myth or reality? *J Biol Chem* 284:21783-21787.
267. Turk, B., J. G. Bieth, I. Bjork, I. Dolenc, D. Turk, N. Cimerman, J. Kos, A. Colic, V. Stoka, and V. Turk. 1995. Regulation of the activity of lysosomal cysteine proteinases by pH-induced inactivation and/or endogenous protein inhibitors, cystatins. *Biol Chem Hoppe Seyler* 376:225-230.
268. Turk, B., I. Dolenc, V. Turk, and J. G. Bieth. 1993. Kinetics of the pH-induced inactivation of human cathepsin L. *Biochemistry* 32:375-380.
269. Boya, P., and G. Kroemer. 2008. Lysosomal membrane permeabilization in cell death. *Oncogene* 27:6434-6451.
270. Duncan, E. M., T. L. Muratore-Schroeder, R. G. Cook, B. A. Garcia, J. Shabanowitz, D. F. Hunt, and C. D. Allis. 2008. Cathepsin L proteolytically processes histone H3 during mouse embryonic stem cell differentiation. *Cell* 135:284-294.
271. Jevnikar, Z., N. Obermajer, B. Doljak, S. Turk, S. Gobec, U. Svajger, S. Hailfinger, M. Thome, and J. Kos. 2011. Cathepsin X cleavage of the beta2 integrin regulates talin-binding and LFA-1 affinity in T cells. *J Leukoc Biol* 90:99-109.
272. Jevnikar, Z., N. Obermajer, U. Pecar-Fonovic, A. Karaoglanovic-Carmona, and J. Kos. 2009. Cathepsin X cleaves the beta2 cytoplasmic tail of LFA-1 inducing the intermediate affinity form of LFA-1 and alpha-actinin-1 binding. *Eur J Immunol* 39:3217-3227.
273. Lechner, A. M., I. Assfalg-Machleidt, S. Zahler, M. Stoeckelhuber, W. Machleidt, M. Jochum, and D. K. Nagler. 2006. RGD-dependent binding of procathepsin X to integrin alphavbeta3 mediates cell-adhesive properties. *J Biol Chem* 281:39588-39597.
274. Obermajer, N., U. Repnik, Z. Jevnikar, B. Turk, M. Kreft, and J. Kos. 2008. Cysteine protease cathepsin X modulates immune response via activation of beta2 integrins. *Immunology* 124:76-88.
275. Nakagawa, T., W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J. A. Villadangos, H. Ploegh, C. Peters, and A. Y. Rudensky. 1998. Cathepsin L:

- critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* 280:450-453.
276. Honey, K., T. Nakagawa, C. Peters, and A. Rudensky. 2002. Cathepsin L regulates CD4<sup>+</sup> T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J Exp Med* 195:1349-1358.
  277. Pham, C. T., and T. J. Ley. 1999. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. *Proc Natl Acad Sci U S A* 96:8627-8632.
  278. Hazuda, D. J., J. Strickler, F. Kueppers, P. L. Simon, and P. R. Young. 1990. Processing of precursor interleukin 1 beta and inflammatory disease. *J Biol Chem* 265:6318-6322.
  279. Mizutani, H., N. Schechter, G. Lazarus, R. A. Black, and T. S. Kupper. 1991. Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *J Exp Med* 174:821-825.
  280. Schonbeck, U., F. Mach, and P. Libby. 1998. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 161:3340-3346.
  281. Coeshott, C., C. Ohnemus, A. Pilyavskaya, S. Ross, M. Wieczorek, H. Kroona, A. H. Leimer, and J. Cheronis. 1999. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96:6261-6266.
  282. Turk, B., D. Turk, and V. Turk. 2000. Lysosomal cysteine proteases: more than scavengers. *Biochim Biophys Acta* 1477:98-111.
  283. Polgar, L., and C. Csoma. 1987. Dissociation of ionizing groups in the binding cleft inversely controls the endo- and exopeptidase activities of cathepsin B. *J Biol Chem* 262:14448-14453.
  284. Jordans, S., S. Jenko-Kokalj, N. M. Kuhl, S. Tedelind, W. Sendt, D. Bromme, D. Turk, and K. Brix. 2009. Monitoring compartment-specific substrate cleavage by cathepsins B, K, L, and S at physiological pH and redox conditions. *BMC Biochem* 10:23.
  285. Godat, E., F. Lecaille, C. Desmazes, S. Duchene, E. Weidauer, P. Saftig, D. Bromme, C. Vandier, and G. Lalmanach. 2004. Cathepsin K: a cysteine protease with unique kinin-degrading properties. *Biochem J* 383:501-506.
  286. Lecaille, F., C. Vandier, E. Godat, V. Herve-Grepinet, D. Bromme, and G. Lalmanach. 2007. Modulation of hypotensive effects of kinins by cathepsin K. *Arch Biochem Biophys* 459:129-136.
  287. Cravatt, B. F., A. T. Wright, and J. W. Kozarich. 2008. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu Rev Biochem* 77:383-414.
  288. Caglic, D., A. Globisch, M. Kindermann, N. H. Lim, V. Jeske, H. P. Juretschke, E. Bartnik, K. U. Weithmann, H. Nagase, B. Turk, and K. U. Wendt. 2011.



- Functional in vivo imaging of cysteine cathepsin activity in murine model of inflammation. *Bioorg Med Chem* 19:1055-1061.
289. Watzke, A., G. Kosec, M. Kindermann, V. Jeske, H. P. Nestler, V. Turk, B. Turk, and K. U. Wendt. 2008. Selective activity-based probes for cysteine cathepsins. *Angew Chem Int Ed Engl* 47:406-409.
  290. Blum, G., G. von Degenfeld, M. J. Merchant, H. M. Blau, and M. Bogyo. 2007. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat Chem Biol* 3:668-677.
  291. Blum, G., S. R. Mullins, K. Keren, M. Fonovic, C. Jedeszko, M. J. Rice, B. F. Sloane, and M. Bogyo. 2005. Dynamic imaging of protease activity with fluorescently quenched activity-based probes. *Nat Chem Biol* 1:203-209.
  292. Paulick, M. G., and M. Bogyo. 2011. Development of activity-based probes for cathepsin X. *ACS Chem Biol* 6:563-572.
  293. Verdoes, M., K. Oresic Bender, E. Segal, W. A. van der Linden, S. Syed, N. P. Withana, L. E. Sanman, and M. Bogyo. 2013. Improved quenched fluorescent probe for imaging of cysteine cathepsin activity. *J Am Chem Soc* 135:14726-14730.
  294. Cesen, M. H., K. Pegan, A. Spes, and B. Turk. 2012. Lysosomal pathways to cell death and their therapeutic applications. *Exp Cell Res* 318:1245-1251.
  295. Kaczmarek, A., P. Vandenabeele, and D. V. Krysko. 2013. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 38:209-223.
  296. Brenner, D., and T. W. Mak. 2009. Mitochondrial cell death effectors. *Curr Opin Cell Biol* 21:871-877.
  297. Bayir, H., B. Fadeel, M. J. Palladino, E. Witas, I. V. Kurnikov, Y. Y. Tyurina, V. A. Tyurin, A. A. Amoscato, J. Jiang, P. M. Kochanek, S. T. DeKosky, J. S. Greenberger, A. A. Shvedova, and V. E. Kagan. 2006. Apoptotic interactions of cytochrome c: redox flirting with anionic phospholipids within and outside of mitochondria. *Biochim Biophys Acta* 1757:648-659.
  298. Yin, X. M. 2006. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. *Gene* 369:7-19.
  299. Sandra, F., M. Degli Esposti, K. Ndebele, P. Gona, D. Knight, M. Rosenquist, and R. Khosravi-Far. 2005. Tumor necrosis factor-related apoptosis-inducing ligand alters mitochondrial membrane lipids. *Cancer Res* 65:8286-8297.
  300. Arora, A. S., B. J. Jones, T. C. Patel, S. F. Bronk, and G. J. Gores. 1997. Ceramide induces hepatocyte cell death through disruption of mitochondrial function in the rat. *Hepatology* 25:958-963.
  301. Repnik, U., and B. Turk. 2010. Lysosomal-mitochondrial cross-talk during cell death. *Mitochondrion* 10:662-669.
  302. de Duve, C. 1983. Lysosomes revisited. *Eur J Biochem* 137:391-397.
  303. Firestone, R. A., J. M. Pisano, and R. J. Bonney. 1979. Lysosomotropic agents. 1. Synthesis and cytotoxic action of lysosomotropic detergents. *J Med Chem* 22:1130-1133.

304. Schotte, P., W. Declercq, S. Van Huffel, P. Vandenabeele, and R. Beyaert. 1999. Non-specific effects of methyl ketone peptide inhibitors of caspases. *FEBS Lett* 442:117-121.
305. Oberle, C., J. Huai, T. Reinheckel, M. Tacke, M. Rassner, P. G. Ekert, J. Buellesbach, and C. Borner. 2010. Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes. *Cell Death Differ* 17:1167-1178.
306. Fehrenbacher, N., M. Gyrd-Hansen, B. Poulsen, U. Felbor, T. Kallunki, M. Boes, E. Weber, M. Leist, and M. Jaattela. 2004. Sensitization to the lysosomal cell death pathway upon immortalization and transformation. *Cancer Res* 64:5301-5310.
307. Baskin-Bey, E. S., A. Canbay, S. F. Bronk, N. Werneburg, M. E. Guicciardi, S. L. Nyberg, and G. J. Gores. 2005. Cathepsin B inactivation attenuates hepatocyte apoptosis and liver damage in steatotic livers after cold ischemia-warm reperfusion injury. *Am J Physiol Gastrointest Liver Physiol* 288:G396-402.
308. Guicciardi, M. E., J. Deussing, H. Miyoshi, S. F. Bronk, P. A. Svingen, C. Peters, S. H. Kaufmann, and G. J. Gores. 2000. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest* 106:1127-1137.
309. Guicciardi, M. E., S. F. Bronk, N. W. Werneburg, and G. J. Gores. 2007. cFLIPL prevents TRAIL-induced apoptosis of hepatocellular carcinoma cells by inhibiting the lysosomal pathway of apoptosis. *Am J Physiol Gastrointest Liver Physiol* 292:G1337-1346.
310. Foghsgaard, L., D. Wissing, D. Mauch, U. Lademann, L. Bastholm, M. Boes, F. Elling, M. Leist, and M. Jaattela. 2001. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol* 153:999-1010.
311. Di Piazza, M., C. Mader, K. Geletneky, Y. C. M. Herrero, E. Weber, J. Schlehofer, L. Deleu, and J. Rommelaere. 2007. Cytosolic activation of cathepsins mediates parvovirus H-1-induced killing of cisplatin and TRAIL-resistant glioma cells. *J Virol* 81:4186-4198.
312. Zhang, H., C. Zhong, L. Shi, Y. Guo, and Z. Fan. 2009. Granulysin induces cathepsin B release from lysosomes of target tumor cells to attack mitochondria through processing of bid leading to Necroptosis. *J Immunol* 182:6993-7000.
313. Wu, G. S., P. Saftig, C. Peters, and W. S. El-Deiry. 1998. Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity. *Oncogene* 16:2177-2183.
314. Heinrich, M., J. Neumeyer, M. Jakob, C. Hallas, V. Tchikov, S. Winoto-Morbach, M. Wickel, W. Schneider-Brachert, A. Trauzold, A. Hethke, and S. Schutze. 2004. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell Death Differ* 11:550-563.
315. Conus, S., R. Perozzo, T. Reinheckel, C. Peters, L. Scapozza, S. Yousefi, and H. U. Simon. 2008. Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation. *J Exp Med* 205:685-698.

316. Bidere, N., H. K. Lorenzo, S. Carmona, M. Laforge, F. Harper, C. Dumont, and A. Senik. 2003. Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *J Biol Chem* 278:31401-31411.
317. Deiss, L. P., H. Galinka, H. Berissi, O. Cohen, and A. Kimchi. 1996. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO J* 15:3861-3870.
318. Laforge, M., F. Petit, J. Estaquier, and A. Senik. 2007. Commitment to apoptosis in CD4(+) T lymphocytes productively infected with human immunodeficiency virus type 1 is initiated by lysosomal membrane permeabilization, itself induced by the isolated expression of the viral protein Nef. *J Virol* 81:11426-11440.
319. Kagedal, K., M. Zhao, I. Svensson, and U. T. Brunk. 2001. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem J* 359:335-343.
320. Brunk, U. T., H. Dalen, K. Roberg, and H. B. Hellquist. 1997. Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. *Free Radic Biol Med* 23:616-626.
321. Zhou, Z. D., B. P. Yap, A. Y. Gung, S. M. Leong, S. T. Ang, and T. M. Lim. 2006. Dopamine-related and caspase-independent apoptosis in dopaminergic neurons induced by overexpression of human wild type or mutant alpha-synuclein. *Exp Cell Res* 312:156-170.
322. Nylandsted, J., M. Rohde, K. Brand, L. Bastholm, F. Elling, and M. Jaattela. 2000. Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proc Natl Acad Sci U S A* 97:7871-7876.
323. Ostenfeld, M. S., N. Fehrenbacher, M. Hoyer-Hansen, C. Thomsen, T. Farkas, and M. Jaattela. 2005. Effective tumor cell death by sigma-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress. *Cancer Res* 65:8975-8983.
324. Nylandsted, J., M. Gyrd-Hansen, A. Danielewicz, N. Fehrenbacher, U. Lademann, M. Hoyer-Hansen, E. Weber, G. Multhoff, M. Rohde, and M. Jaattela. 2004. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J Exp Med* 200:425-435.
325. Vanden Berghe, T., N. Vanlangenakker, E. Parthoens, W. Deckers, M. Devos, N. Festjens, C. J. Guerin, U. T. Brunk, W. Declercq, and P. Vandenabeele. 2010. Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. *Cell Death Differ* 17:922-930.
326. Brunk, U. T., and J. L. Ericsson. 1972. Cytochemical evidence for the leakage of acid phosphatase through ultrastructurally intact lysosomal membranes. *Histochem J* 4:479-491.
327. de Duve, C., T. de Barsey, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof. 1974. Commentary. Lysosomotropic agents. *Biochem Pharmacol* 23:2495-2531.
328. Thiele, D. L., and P. E. Lipsky. 1990. The action of leucyl-leucine methyl ester on cytotoxic lymphocytes requires uptake by a novel dipeptide-specific facilitated

- transport system and dipeptidyl peptidase I-mediated conversion to membranolytic products. *J Exp Med* 172:183-194.
329. Thiele, D. L., and P. E. Lipsky. 1990. Mechanism of L-leucyl-L-leucine methyl ester-mediated killing of cytotoxic lymphocytes: dependence on a lysosomal thiol protease, dipeptidyl peptidase I, that is enriched in these cells. *Proc Natl Acad Sci U S A* 87:83-87.
  330. Yap, Y. W., M. Whiteman, B. H. Bay, Y. Li, F. S. Sheu, R. Z. Qi, C. H. Tan, and N. S. Cheung. 2006. Hypochlorous acid induces apoptosis of cultured cortical neurons through activation of calpains and rupture of lysosomes. *J Neurochem* 98:1597-1609.
  331. Yamashima, T., Y. Kohda, K. Tsuchiya, T. Ueno, J. Yamashita, T. Yoshioka, and E. Kominami. 1998. Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. *Eur J Neurosci* 10:1723-1733.
  332. Yamashima, T., T. C. Saido, M. Takita, A. Miyazawa, J. Yamano, A. Miyakawa, H. Nishijyo, J. Yamashita, S. Kawashima, T. Ono, and T. Yoshioka. 1996. Transient brain ischaemia provokes Ca<sup>2+</sup>, PIP<sub>2</sub> and calpain responses prior to delayed neuronal death in monkeys. *Eur J Neurosci* 8:1932-1944.
  333. Yamashima, T. 2012. Hsp70.1 and related lysosomal factors for necrotic neuronal death. *J Neurochem* 120:477-494.
  334. Stoka, V., B. Turk, S. L. Schendel, T. H. Kim, T. Cirman, S. J. Snipas, L. M. Ellerby, D. Bredesen, H. Freeze, M. Abrahamson, D. Bromme, S. Krajewski, J. C. Reed, X. M. Yin, V. Turk, and G. S. Salvesen. 2001. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* 276:3149-3157.
  335. Cirman, T., K. Oresic, G. D. Mazovec, V. Turk, J. C. Reed, R. M. Myers, G. S. Salvesen, and B. Turk. 2004. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *J Biol Chem* 279:3578-3587.
  336. Droga-Mazovec, G., L. Bojic, A. Petelin, S. Ivanova, R. Romih, U. Repnik, G. S. Salvesen, V. Stoka, V. Turk, and B. Turk. 2008. Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *J Biol Chem* 283:19140-19150.
  337. Roberg, K., K. Kagedal, and K. Ollinger. 2002. Microinjection of cathepsin d induces caspase-dependent apoptosis in fibroblasts. *Am J Pathol* 161:89-96.
  338. Bivik, C. A., P. K. Larsson, K. M. Kagedal, I. K. Rosdahl, and K. M. Ollinger. 2006. UVA/B-induced apoptosis in human melanocytes involves translocation of cathepsins and Bcl-2 family members. *J Invest Dermatol* 126:1119-1127.
  339. McGuire, K. A., A. U. Barlan, T. M. Griffin, and C. M. Wiethoff. 2011. Adenovirus type 5 rupture of lysosomes leads to cathepsin B-dependent mitochondrial stress and production of reactive oxygen species. *J Virol* 85:10806-10813.
  340. Roberts, L. R., P. N. Adjei, and G. J. Gores. 1999. Cathepsins as effector proteases in hepatocyte apoptosis. *Cell Biochem Biophys* 30:71-88.

341. Guicciardi, M. E., H. Miyoshi, S. F. Bronk, and G. J. Gores. 2001. Cathepsin B knockout mice are resistant to tumor necrosis factor-alpha-mediated hepatocyte apoptosis and liver injury: implications for therapeutic applications. *Am J Pathol* 159:2045-2054.
342. Vancompernelle, K., F. Van Herreweghe, G. Pynaert, M. Van de Craen, K. De Vos, N. Totty, A. Sterling, W. Fiers, P. Vandenabeele, and J. Grooten. 1998. Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. *FEBS Lett* 438:150-158.
343. Schotte, P., W. Van Crielinge, M. Van de Craen, G. Van Loo, M. Desmedt, J. Grooten, M. Cornelissen, L. De Ridder, J. Vandekerckhove, W. Fiers, P. Vandenabeele, and R. Beyaert. 1998. Cathepsin B-mediated activation of the proinflammatory caspase-11. *Biochem Biophys Res Commun* 251:379-387.
344. Kagedal, K., A. C. Johansson, U. Johansson, G. Heimlich, K. Roberg, N. S. Wang, J. M. Jurgensmeier, and K. Ollinger. 2005. Lysosomal membrane permeabilization during apoptosis--involvement of Bax? *Int J Exp Pathol* 86:309-321.
345. Bove, J., M. Martinez-Vicente, B. Dehay, C. Perier, A. Recasens, A. Bombrun, B. Antonsson, and M. Vila. 2014. BAX channel activity mediates lysosomal disruption linked to Parkinson disease. *Autophagy* 10:889-900.
346. Werneburg, N. W., S. F. Bronk, M. E. Guicciardi, L. Thomas, J. D. Dikeakos, G. Thomas, and G. J. Gores. 2012. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein-induced lysosomal translocation of proapoptotic effectors is mediated by phosphofurin acidic cluster sorting protein-2 (PACS-2). *J Biol Chem* 287:24427-24437.
347. Werneburg, N. W., M. E. Guicciardi, S. F. Bronk, S. H. Kaufmann, and G. J. Gores. 2007. Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. *J Biol Chem* 282:28960-28970.
348. Gonzalez, P., I. Mader, A. Tchoghandjian, S. Enzenmuller, S. Cristofanon, F. Basit, K. M. Debatin, and S. Fulda. 2012. Impairment of lysosomal integrity by B10, a glycosylated derivative of betulinic acid, leads to lysosomal cell death and converts autophagy into a detrimental process. *Cell Death Differ* 19:1337-1346.
349. Boya, P., K. Andreau, D. Poncet, N. Zamzami, J. L. Perfettini, D. Metivier, D. M. Ojcius, M. Jaattela, and G. Kroemer. 2003. Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion. *J Exp Med* 197:1323-1334.
350. Rammer, P., L. Groth-Pedersen, T. Kirkegaard, M. Daugaard, A. Rytter, P. Szyniarowski, M. Hoyer-Hansen, L. K. Povlsen, J. Nylandsted, J. E. Larsen, and M. Jaattela. 2010. BAMLET activates a lysosomal cell death program in cancer cells. *Mol Cancer Ther* 9:24-32.
351. Kurz, T., A. Terman, B. Gustafsson, and U. T. Brunk. 2008. Lysosomes and oxidative stress in aging and apoptosis. *Biochim Biophys Acta* 1780:1291-1303.
352. Terman, A., B. Gustafsson, and U. T. Brunk. 2006. The lysosomal-mitochondrial axis theory of postmitotic aging and cell death. *Chem Biol Interact* 163:29-37.

353. Kurz, T., J. W. Eaton, and U. T. Brunk. 2009. Redox activity within the lysosomal compartment: implications for aging and apoptosis. *Antioxid Redox Signal* 13:511-523.
354. Kreuzaler, P. A., A. D. Staniszewska, W. Li, N. Omidvar, B. Kedjouar, J. Turkson, V. Poli, R. A. Flavell, R. W. Clarkson, and C. J. Watson. 2011. Stat3 controls lysosomal-mediated cell death in vivo. *Nat Cell Biol* 13:303-309.
355. Ghosh, M., F. Carlsson, A. Laskar, X. M. Yuan, and W. Li. 2011. Lysosomal membrane permeabilization causes oxidative stress and ferritin induction in macrophages. *FEBS Lett* 585:623-629.
356. Kurz, T., A. Terman, and U. T. Brunk. 2007. Autophagy, ageing and apoptosis: the role of oxidative stress and lysosomal iron. *Arch Biochem Biophys* 462:220-230.
357. Chwieralski, C. E., T. Welte, and F. Buhling. 2006. Cathepsin-regulated apoptosis. *Apoptosis* 11:143-149.
358. Liu, L., Z. Zhang, and D. Xing. 2011. Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage. *Free Radic Biol Med* 51:53-68.
359. Morgan, M. J., and Z. G. Liu. 2011. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* 21:103-115.
360. Zhao, M., F. Antunes, J. W. Eaton, and U. T. Brunk. 2003. Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis. *Eur J Biochem* 270:3778-3786.
361. Hawkins, P. N., H. J. Lachmann, and M. F. McDermott. 2003. Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N Engl J Med* 348:2583-2584.
362. Goldbach-Mansky, R., N. J. Dailey, S. W. Canna, A. Gelabert, J. Jones, B. I. Rubin, H. J. Kim, C. Brewer, C. Zalewski, E. Wiggs, S. Hill, M. L. Turner, B. I. Karp, I. Aksentjevich, F. Pucino, S. R. Penzak, M. H. Haverkamp, L. Stein, B. S. Adams, T. L. Moore, R. C. Fuhlbrigge, B. Shaham, J. N. Jarvis, K. O'Neil, R. K. Vehe, L. O. Beitz, G. Gardner, W. P. Hannan, R. W. Warren, W. Horn, J. L. Cole, S. M. Paul, P. N. Hawkins, T. H. Pham, C. Snyder, R. A. Wesley, S. C. Hoffmann, S. M. Holland, J. A. Butman, and D. L. Kastner. 2006. Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med* 355:581-592.
363. Hoffman, H. M., S. Rosengren, D. L. Boyle, J. Y. Cho, J. Nayar, J. L. Mueller, J. P. Anderson, A. A. Wanderer, and G. S. Firestein. 2004. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 364:1779-1785.
364. Toker, O., and P. J. Hashkes. Critical appraisal of canakinumab in the treatment of adults and children with cryopyrin-associated periodic syndrome (CAPS). *Biologics* 4:131-138.
365. Cocheme, H. M., and M. P. Murphy. 2010. Can antioxidants be effective therapeutics? *Curr Opin Investig Drugs* 11:426-431.

366. Severa, M., E. M. Coccia, and K. A. Fitzgerald. 2006. Toll-like receptor-dependent and -independent viperin gene expression and counter-regulation by PRDI-binding factor-1/BLIMP1. *J Biol Chem* 281:26188-26195.
367. Jensen, B. M., E. J. Swindle, S. Iwaki, and A. M. Gilfillan. 2006. Generation, isolation, and maintenance of rodent mast cells and mast cell lines. *Curr Protoc Immunol* Chapter 3:Unit 3 23.
368. Kanneganti, T. D., N. Ozoren, M. Body-Malapel, A. Amer, J. H. Park, L. Franchi, J. Whitfield, W. Barchet, M. Colonna, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant, S. Akira, and G. Nunez. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440:233-236.
369. Shi, G. P., J. A. Villadangos, G. Dranoff, C. Small, L. Gu, K. J. Haley, R. Riese, H. L. Ploegh, and H. A. Chapman. 1999. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* 10:197-206.
370. Deussing, J., W. Roth, P. Saftig, C. Peters, H. L. Ploegh, and J. A. Villadangos. 1998. Cathepsins B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. *Proc Natl Acad Sci U S A* 95:4516-4521.
371. Gullapalli, R., A. Wong, E. Brigham, G. Kwong, A. Wadsworth, C. Willits, K. Quinn, E. Goldbach, and B. Samant. 2012. Development of ALZET(R) osmotic pump compatible solvent compositions to solubilize poorly soluble compounds for preclinical studies. *Drug Deliv* 19:239-246.
372. Dinarello, C. A. 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 117:3720-3732.
373. Heid, M. E., P. A. Keyel, C. Kamga, S. Shiva, S. C. Watkins, and R. D. Salter. 2013. Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol* 191:5230-5238.
374. Sun, L., Z. Wu, Y. Hayashi, C. Peters, M. Tsuda, K. Inoue, and H. Nakanishi. 2012. Microglial cathepsin B contributes to the initiation of peripheral inflammation-induced chronic pain. *J Neurosci* 32:11330-11342.
375. Meixenberger, K., F. Pache, J. Eitel, B. Schmeck, S. Hippenstiel, H. Slevogt, P. N'Guessan, M. Witzernath, M. G. Netea, T. Chakraborty, N. Suttorp, and B. Opitz. 2009. *Listeria monocytogenes*-infected human peripheral blood mononuclear cells produce IL-1 $\beta$ , depending on listeriolysin O and NLRP3. *J Immunol* 184:922-930.
376. Barlan, A. U., T. M. Griffin, K. A. McGuire, and C. M. Wiethoff. 2010. Adenovirus membrane penetration activates the NLRP3 inflammasome. *J Virol* 85:146-155.
377. Rintahaka, J., N. Lietzen, T. Ohman, T. A. Nyman, and S. Matikainen. 2011. Recognition of cytoplasmic RNA results in cathepsin-dependent inflammasome activation and apoptosis in human macrophages. *J Immunol* 186:3085-3092.
378. Kankkunen, P., L. Teirila, J. Rintahaka, H. Alenius, H. Wolff, and S. Matikainen. 2010. (1,3)- $\beta$ -glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J Immunol* 184:6335-6342.

379. Felbor, U., B. Kessler, W. Mothes, H. H. Goebel, H. L. Ploegh, R. T. Bronson, and B. R. Olsen. 2002. Neuronal loss and brain atrophy in mice lacking cathepsins B and L. *Proc Natl Acad Sci U S A* 99:7883-7888.
380. Vasiljeva, O., A. Papazoglou, A. Kruger, H. Brodoefel, M. Korovin, J. Deussing, N. Augustin, B. S. Nielsen, K. Almholt, M. Bogyo, C. Peters, and T. Reinheckel. 2006. Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. *Cancer Res* 66:5242-5250.
381. Sevenich, L., U. Schurigt, K. Sachse, M. Gajda, F. Werner, S. Muller, O. Vasiljeva, A. Schwinde, N. Klemm, J. Deussing, C. Peters, and T. Reinheckel. 2010. Synergistic antitumor effects of combined cathepsin B and cathepsin Z deficiencies on breast cancer progression and metastasis in mice. *Proc Natl Acad Sci U S A* 107:2497-2502.
382. Friedrichs, B., C. Tepel, T. Reinheckel, J. Deussing, K. von Figura, V. Herzog, C. Peters, P. Saftig, and K. Brix. 2003. Thyroid functions of mouse cathepsins B, K, and L. *J Clin Invest* 111:1733-1745.
383. Chen, Y. T., L. S. Brinen, I. D. Kerr, E. Hansell, P. S. Doyle, J. H. McKerrow, and W. R. Roush. 2009. In vitro and in vivo studies of the trypanocidal properties of WRR-483 against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 4.
384. Ochieng, J., and G. Chaudhuri. 2010. Cystatin superfamily. *J Health Care Poor Underserved* 21:51-70.
385. Jacobsen, W., U. Christians, and L. Z. Benet. 2000. In vitro evaluation of the disposition of A novel cysteine protease inhibitor. *Drug Metab Dispos* 28:1343-1351.
386. Engel, J. C., P. S. Doyle, I. Hsieh, and J. H. McKerrow. 1998. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. *J Exp Med* 188:725-734.
387. Barr, S. C., K. L. Warner, B. G. Kornreic, J. Piscitelli, A. Wolfe, L. Benet, and J. H. McKerrow. 2005. A cysteine protease inhibitor protects dogs from cardiac damage during infection by *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 49:5160-5161.
388. Doyle, P. S., Y. M. Zhou, J. C. Engel, and J. H. McKerrow. 2007. A cysteine protease inhibitor cures Chagas' disease in an immunodeficient-mouse model of infection. *Antimicrob Agents Chemother* 51:3932-3939.
389. Abdulla, M. H., K. C. Lim, M. Sajid, J. H. McKerrow, and C. R. Caffrey. 2007. Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. *PLoS Med* 4:e14.
390. McKerrow, J. H., P. S. Doyle, J. C. Engel, L. M. Podust, S. A. Robertson, R. Ferreira, T. Saxton, M. Arkin, I. D. Kerr, L. S. Brinen, and C. S. Craik. 2009. Two approaches to discovering and developing new drugs for Chagas disease. *Mem Inst Oswaldo Cruz* 104 Suppl 1:263-269.
391. Chen, Y. T., L. S. Brinen, I. D. Kerr, E. Hansell, P. S. Doyle, J. H. McKerrow, and W. R. Roush. In vitro and in vivo studies of the trypanocidal properties of WRR-483 against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 4.



392. Turk, V., V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, and D. Turk. 2011. Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim Biophys Acta* 1824:68-88.
393. Chapman, H. A., Jr., J. J. Reilly, Jr., R. Yee, and A. Grubb. 1990. Identification of cystatin C, a cysteine proteinase inhibitor, as a major secretory product of human alveolar macrophages in vitro. *Am Rev Respir Dis* 141:698-705.
394. Schulte, S., J. Sun, P. Libby, L. Macfarlane, C. Sun, M. Lopez-Illasaca, G. P. Shi, and G. K. Sukhova. 2010. Cystatin C deficiency promotes inflammation in angiotensin II-induced abdominal aortic aneurisms in atherosclerotic mice. *Am J Pathol* 177:456-463.
395. Maher, K., B. Jeric Kokelj, M. Butinar, G. Mikhaylov, M. Mancek-Keber, V. Stoka, O. Vasiljeva, B. Turk, S. A. Grigoryev, and N. Kopitar-Jerala. 2014. A role for stefin B (cystatin B) in inflammation and endotoxemia. *J Biol Chem* 289:31736-31750.
396. Ceru, S., S. Konjar, K. Maher, U. Repnik, I. Krizaj, M. Bencina, M. Renko, A. Nepveu, E. Zerovnik, B. Turk, and N. Kopitar-Jerala. 2010. Stefin B interacts with histones and cathepsin L in the nucleus. *J Biol Chem* 285:10078-10086.
397. Wang, Y. R., S. Qin, R. Han, J. C. Wu, Z. Q. Liang, Z. H. Qin, and Y. Wang. 2013. Cathepsin L plays a role in quinolinic acid-induced NF-KappaB activation and excitotoxicity in rat striatal neurons. *PLoS One* 8:e75702.
398. Maher, K., J. Zavrsnik, B. Jeric-Kokelj, O. Vasiljeva, B. Turk, and N. Kopitar-Jerala. 2014. Decreased IL-10 expression in stefin B-deficient macrophages is regulated by the MAP kinase and STAT-3 signaling pathways. *FEBS Lett* 588:720-726.
399. Schotte, P., R. Schauvliege, S. Janssens, and R. Beyaert. 2001. The cathepsin B inhibitor z-FA.fmk inhibits cytokine production in macrophages stimulated by lipopolysaccharide. *J Biol Chem* 276:21153-21157.
400. Park, B., M. M. Brinkmann, E. Spooner, C. C. Lee, Y. M. Kim, and H. L. Ploegh. 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol* 9:1407-1414.
401. Ewald, S. E., A. Engel, J. Lee, M. Wang, M. Bogyo, and G. M. Barton. 2011. Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J Exp Med* 208:643-651.
402. Frew, B. C., V. R. Joag, and J. Mogridge. 2012. Proteolytic processing of Nlrp1b is required for inflammasome activity. *PLoS Pathog* 8:e1002659.
403. Finger, J. N., J. D. Lich, L. C. Dare, M. N. Cook, K. K. Brown, C. Duraiswami, J. Bertin, and P. J. Gough. 2012. Autolytic proteolysis within the function to find domain (FIIND) is required for NLRP1 inflammasome activity. *J Biol Chem* 287:25030-25037.
404. Martinon, F., L. Agostini, E. Meylan, and J. Tschopp. 2004. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr Biol* 14:1929-1934.
405. Brojatsch, J., H. Lima, A. K. Kar, L. S. Jacobson, S. M. Muehlbauer, K. Chandran, and F. Diaz-Griffero. 2014. A proteolytic cascade controls lysosome

- rupture and necrotic cell death mediated by lysosome-destabilizing adjuvants. *PLoS One* 9:e95032.
406. Orłowski, G. M., J. D. Colbert, S. Sharma, M. Bogoy, S. A. Robertson, and K. L. Rock. 2015. Multiple Cathepsins Promote Pro-IL-1 $\beta$  Synthesis and NLRP3-Mediated IL-1 $\beta$  Activation. *Journal of Immunology (IN REVIEW)*.
  407. Stehlik, C. 2009. Multiple interleukin-1 $\beta$ -converting enzymes contribute to inflammatory arthritis. *Arthritis Rheum* 60:3524-3530.
  408. Joosten, L. A., M. G. Netea, G. Fantuzzi, M. I. Koenders, M. M. Helsen, H. Sparrer, C. T. Pham, J. W. van der Meer, C. A. Dinarello, and W. B. van den Berg. 2009. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1 $\beta$ . *Arthritis Rheum* 60:3651-3662.
  409. Fantuzzi, G., G. Ku, M. W. Harding, D. J. Livingston, J. D. Sipe, K. Kuida, R. A. Flavell, and C. A. Dinarello. 1997. Response to local inflammation of IL-1  $\beta$ -converting enzyme- deficient mice. *J Immunol* 158:1818-1824.
  410. Guma, M., L. Ronacher, R. Liu-Bryan, S. Takai, M. Karin, and M. Corr. 2009. Caspase 1-independent activation of interleukin-1 $\beta$  in neutrophil-predominant inflammation. *Arthritis Rheum* 60:3642-3650.
  411. Yoshida, T., I. Tomioka, T. Nagahara, T. Holyst, M. Sawada, P. Hayes, V. Gama, M. Okuno, Y. Chen, Y. Abe, T. Kanouchi, H. Sasada, D. Wang, T. Yokota, E. Sato, and S. Matsuyama. 2004. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem Biophys Res Commun* 321:961-966.
  412. Vandenberg, C. J., and S. Cory. 2013. ABT-199, a new Bcl-2-specific BH3 mimetic, has in vivo efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. *Blood* 121:2285-2288.
  413. Ko, T. K., C. T. Chuah, J. W. Huang, K. P. Ng, and S. T. Ong. 2014. The BCL2 inhibitor ABT-199 significantly enhances imatinib-induced cell death in chronic myeloid leukemia progenitors. *Oncotarget* 5:9033-9038.
  414. Vince, J. E., W. W. Wong, I. Gentle, K. E. Lawlor, R. Allam, L. O'Reilly, K. Mason, O. Gross, S. Ma, G. Guarda, H. Anderton, R. Castillo, G. Hacker, J. Silke, and J. Tschopp. 2014. Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. *Immunity* 36:215-227.
  415. Tse, C., A. R. Shoemaker, J. Adickes, M. G. Anderson, J. Chen, S. Jin, E. F. Johnson, K. C. Marsh, M. J. Mitten, P. Nimmer, L. Roberts, S. K. Tahir, Y. Xiao, X. Yang, H. Zhang, S. Fesik, S. H. Rosenberg, and S. W. Elmore. 2008. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* 68:3421-3428.
  416. Saito, M., R. Nishikomori, N. Kambe, A. Fujisawa, H. Tanizaki, K. Takeichi, T. Imagawa, T. Iehara, H. Takada, T. Matsubayashi, H. Tanaka, H. Kawashima, K. Kawakami, S. Kagami, I. Okafuji, T. Yoshioka, S. Adachi, T. Heike, Y. Miyachi, and T. Nakahata. 2008. Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. *Blood* 111:2132-2141.

417. Moore, S. F., and A. B. MacKenzie. 2009. NADPH oxidase NOX2 mediates rapid cellular oxidation following ATP stimulation of endotoxin-primed macrophages. *J Immunol* 183:3302-3308.
418. Willingham, S. B., I. C. Allen, D. T. Bergstralh, W. J. Brickey, M. T. Huang, D. J. Taxman, J. A. Duncan, and J. P. Ting. 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.
419. Gelb, B. D., G. P. Shi, H. A. Chapman, and R. J. Desnick. 1996. Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 273:1236-1238.
420. Koike, M., H. Nakanishi, P. Saftig, J. Ezaki, K. Isahara, Y. Ohsawa, W. Schulz-Schaeffer, T. Watanabe, S. Waguri, S. Kametaka, M. Shibata, K. Yamamoto, E. Kominami, C. Peters, K. von Figura, and Y. Uchiyama. 2000. Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons. *J Neurosci* 20:6898-6906.
421. Yanagawa, M., T. Tsukuba, T. Nishioku, Y. Okamoto, K. Okamoto, R. Takii, Y. Terada, K. I. Nakayama, T. Kadowaki, and K. Yamamoto. 2007. Cathepsin E deficiency induces a novel form of lysosomal storage disorder showing the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages. *J Biol Chem* 282:1851-1862.
422. Falgoutyret, J. P., S. Desmarais, R. Oballa, W. C. Black, W. Cromlish, K. Khougaz, S. Lamontagne, F. Masse, D. Riendeau, S. Toulmond, and M. D. Percival. 2005. Lysosomotropism of basic cathepsin K inhibitors contributes to increased cellular potencies against off-target cathepsins and reduced functional selectivity. *J Med Chem* 48:7535-7543.
423. Palmer, J. T., C. Bryant, D. X. Wang, D. E. Davis, E. L. Setti, R. M. Rydzewski, S. Venkatraman, Z. Q. Tian, L. C. Burrill, R. V. Mendonca, E. Springman, J. McCarter, T. Chung, H. Cheung, J. W. Janc, M. McGrath, J. R. Somoza, P. Enriquez, Z. W. Yu, R. M. Strickley, L. Liu, M. C. Venuti, M. D. Percival, J. P. Falgoutyret, P. Prasit, R. Oballa, D. Riendeau, R. N. Young, G. Wesolowski, S. B. Rodan, C. Johnson, D. B. Kimmel, and G. Rodan. 2005. Design and synthesis of tri-ring P3 benzamide-containing aminonitriles as potent, selective, orally effective inhibitors of cathepsin K. *J Med Chem* 48:7520-7534.
424. Robichaud, J., W. C. Black, M. Therien, J. Paquet, R. M. Oballa, C. I. Bayly, D. J. McKay, Q. Wang, E. Isabel, S. Leger, C. Mellon, D. B. Kimmel, G. Wesolowski, M. D. Percival, F. Masse, S. Desmarais, J. P. Falgoutyret, and S. N. Crane. 2008. Identification of a nonbasic, nitrile-containing cathepsin K inhibitor (MK-1256) that is efficacious in a monkey model of osteoporosis. *J Med Chem* 51:6410-6420.
425. McKerrow, J. H., J. C. Engel, and C. R. Caffrey. 1999. Cysteine protease inhibitors as chemotherapy for parasitic infections. *Bioorg Med Chem* 7:639-644.
426. Knox, D. P. 2007. Proteinase inhibitors and helminth parasite infection. *Parasite Immunol* 29:57-71.
427. Leid, R. W., C. M. Suquet, H. G. Bouwer, and D. J. Hinrichs. 1986. Interleukin inhibition by a parasite proteinase inhibitor, taeniaestatin. *J Immunol* 137:2700-2702.

428. Lyo, V., F. Cattaruzza, T. N. Kim, A. W. Walker, M. Paulick, D. Cox, J. Cloyd, J. Buxbaum, J. Ostroff, M. Bogyo, E. F. Grady, N. W. Bunnett, and K. S. Kirkwood. 2012. Active cathepsins B, L, and S in murine and human pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 303:G894-903.
429. Choy, J. W., C. Bryant, C. M. Calvet, P. S. Doyle, S. S. Gunatilleke, S. S. Leung, K. K. Ang, S. Chen, J. Gut, J. A. Osés-Prieto, J. B. Johnston, M. R. Arkin, A. L. Burlingame, J. Taunton, M. P. Jacobson, J. M. McKerrow, L. M. Podust, and A. R. Renslo. 2013. Chemical-biological characterization of a cruzain inhibitor reveals a second target and a mammalian off-target. *Beilstein J Org Chem* 9:15-25.
430. Benchoua, A., J. Braudeau, A. Reis, C. Couriaud, and B. Onteniente. 2004. Activation of proinflammatory caspases by cathepsin B in focal cerebral ischemia. *J Cereb Blood Flow Metab* 24:1272-1279.
431. Hook, G., V. Y. Hook, and M. Kindy. 2007. Cysteine protease inhibitors reduce brain beta-amyloid and beta-secretase activity in vivo and are potential Alzheimer's disease therapeutics. *Biol Chem* 388:979-983.
432. Hook, V. Y., M. Kindy, and G. Hook. 2008. Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. *J Biol Chem* 283:7745-7753.
433. Zhang, L., X. H. Fu, Y. Yu, R. H. Shui, C. Li, H. Y. Zeng, Y. L. Qiao, L. Y. Ni, and Q. Wang. 2015. Treatment with CA-074Me, a Cathepsin B inhibitor, reduces lung interstitial inflammation and fibrosis in a rat model of polymyositis. *Lab Invest* 95:65-77.
434. Feng, Y., L. Ni, and Q. Wang. 2013. Administration of cathepsin B inhibitor CA-074Me reduces inflammation and apoptosis in polymyositis. *J Dermatol Sci* 72:158-167.
435. Biroc, S. L., S. Gay, K. Hummel, C. Magill, J. T. Palmer, D. R. Spencer, S. Sa, J. L. Klaus, B. A. Michel, D. Rasnick, and R. E. Gay. 2001. Cysteine protease activity is up-regulated in inflamed ankle joints of rats with adjuvant-induced arthritis and decreases with in vivo administration of a vinyl sulfone cysteine protease inhibitor. *Arthritis Rheum* 44:703-711.
436. Reiser, J., B. Adair, and T. Reinheckel. Specialized roles for cysteine cathepsins in health and disease. *J Clin Invest* 120:3421-3431.
437. Lutgens, S. P., K. B. Cleutjens, M. J. Daemen, and S. Heeneman. 2007. Cathepsin cysteine proteases in cardiovascular disease. *FASEB J* 21:3029-3041.
438. Kitamoto, S., G. K. Sukhova, J. Sun, M. Yang, P. Libby, V. Love, P. Duramad, C. Sun, Y. Zhang, X. Yang, C. Peters, and G. P. Shi. 2007. Cathepsin L deficiency reduces diet-induced atherosclerosis in low-density lipoprotein receptor-knockout mice. *Circulation* 115:2065-2075.
439. Gray, J., M. M. Haran, K. Schneider, S. Vesce, A. M. Ray, D. Owen, I. R. White, P. Cutler, and J. B. Davis. 2001. Evidence that inhibition of cathepsin-B contributes to the neuroprotective properties of caspase inhibitor Tyr-Val-Ala-Asp-chloromethyl ketone. *J Biol Chem* 276:32750-32755.

440. Rozman-Pungercar, J., N. Kopitar-Jerala, M. Bogyo, D. Turk, O. Vasiljeva, I. Stefe, P. Vandenabeele, D. Bromme, V. Puizdar, M. Fonovic, M. Trstenjak-Prebanda, I. Dolenc, V. Turk, and B. Turk. 2003. Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ* 10:881-888.
441. Caserta, T. M., A. N. Smith, A. D. Gultice, M. A. Reedy, and T. L. Brown. 2003. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis* 8:345-352.
442. Chen, M., V. O. Ona, M. Li, R. J. Ferrante, K. B. Fink, S. Zhu, J. Bian, L. Guo, L. A. Farrell, S. M. Hersch, W. Hobbs, J. P. Vonsattel, J. H. Cha, and R. M. Friedlander. 2000. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med* 6:797-801.
443. Kim, H. S., and Y. H. Suh. 2009. Minocycline and neurodegenerative diseases. *Behav Brain Res* 196:168-179.
444. Cotea, C. K., K. M. Reaa, S. L. Norrisb, N. v. Rooijenc, and S. L. Welkosa. 2004. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with *Bacillus anthracis* spores. *Microbial Pathogenesis* 37:6.
445. van Rooijen, N., and A. Sanders. 1997. Elimination, blocking, and activation of macrophages: three of a kind? *J Leukoc Biol* 62:702-709.
446. Kagan, E., and D. P. Hartmann. 1984. Elimination of macrophages with silica and asbestos. *Methods Enzymol* 108:325-335.
447. Thinwa, J., J. A. Segovia, S. Bose, and P. H. Dube. 2014. Integrin-mediated first signal for inflammasome activation in intestinal epithelial cells. *J Immunol* 193:1373-1382.
448. Hoesel, B., and J. A. Schmid. 2013. The complexity of NF-kappaB signaling in inflammation and cancer. *Mol Cancer* 12:86.
449. O'Neill, L. A., and D. G. Hardie. 2013. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* 493:346-355.
450. Gloire, G., S. Legrand-Poels, and J. Piette. 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol* 72:1493-1505.
451. Tannahill, G. M., A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C. Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K. Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier, and L. A. O'Neill. 2013. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* 496:238-242.
452. Bonello, S., C. Zahringer, R. S. BelAiba, T. Djordjevic, J. Hess, C. Michiels, T. Kietzmann, and A. Grolach. 2007. Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. *Arterioscler Thromb Vasc Biol* 27:755-761.

453. Wang, D., D. Malo, and S. Hekimi. 2010. Elevated mitochondrial reactive oxygen species generation affects the immune response via hypoxia-inducible factor-1alpha in long-lived Mcl1<sup>+/-</sup> mouse mutants. *J Immunol* 184:582-590.
454. Fenton, M. J. 1992. Review: transcriptional and post-transcriptional regulation of interleukin 1 gene expression. *Int J Immunopharmacol* 14:401-411.
455. Hari, A., Y. Zhang, Z. Tu, P. Detampel, M. Stenner, A. Ganguly, and Y. Shi. 2014. Activation of NLRP3 inflammasome by crystalline structures via cell surface contact. *Sci Rep* 4:7281.
456. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annu Rev Immunol* 27:229-265.
457. Nilsson, C., K. Kagedal, U. Johansson, and K. Ollinger. 2003. Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. *Methods Cell Sci* 25:185-194.
458. Matsuyama, S., J. Llopis, Q. L. Deveraux, R. Y. Tsien, and J. C. Reed. 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat Cell Biol* 2:318-325.
459. Imahashi, K., M. D. Schneider, C. Steenbergen, and E. Murphy. 2004. Transgenic expression of Bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. *Circ Res* 95:734-741.
460. Teijido, O., S. M. Rappaport, A. Chamberlin, S. Y. Noskov, V. M. Aguilera, T. K. Rostovtseva, and S. M. Bezrukov. 2014. Acidification asymmetrically affects voltage-dependent anion channel implicating the involvement of salt bridges. *J Biol Chem* 289:23670-23682.
461. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J Cell Biol* 90:665-669.
462. Maxfield, F. R. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J Cell Biol* 95:676-681.
463. Funk, R. S., and J. P. Krise. 2012. Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: implications for an intracellular distribution-based drug interaction. *Mol Pharm* 9:1384-1395.
464. Logan, R., R. S. Funk, E. Axcell, and J. P. Krise. 2012. Drug-drug interactions involving lysosomes: mechanisms and potential clinical implications. *Expert Opin Drug Metab Toxicol* 8:943-958.
465. Rubartelli, A., F. Cozzolino, M. Talio, and R. Sitia. 1990. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* 9:1503-1510.
466. Jang, C. H., J. H. Choi, M. S. Byun, and D. M. Jue. 2006. Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology (Oxford)* 45:703-710.
467. Porro, G., G. Bertolini, M. A. Bonardi, E. Giovanetti, P. Lento, F. Leoni, D. Modena, G. Pavich, and F. Marcucci. 1998. Diaminic carbonates, a new class of

- anti-inflammatory compounds: their biological characterization and mode of action. *J Pharmacol Exp Ther* 285:193-200.
468. Deininger, M. W., and B. J. Druker. 2003. Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol Rev* 55:401-423.
  469. Wolf, A. M., D. Wolf, H. Rumpold, S. Ludwiczek, B. Enrich, G. Gastl, G. Weiss, and H. Tilg. 2005. The kinase inhibitor imatinib mesylate inhibits TNF- $\alpha$  production in vitro and prevents TNF-dependent acute hepatic inflammation. *Proc Natl Acad Sci U S A* 102:13622-13627.
  470. Tangri, K. K., P. R. Saxena, P. K. Seth, and K. P. Bhargava. 1966. Anti-inflammatory activity of imipramine and congeners. *Biochem Pharmacol* 15:825-831.
  471. Sevilla-Sanchez, D., D. Soy-Muner, and N. Soler-Porcar. 2010. [Usefulness of macrolides as anti-inflammatories in respiratory diseases]. *Arch Bronconeumol* 46:244-254.
  472. Appelqvist, H., P. Waster, K. Kagedal, and K. Ollinger. 2013. The lysosome: from waste bag to potential therapeutic target. *J Mol Cell Biol* 5:214-226.
  473. Rajamaki, K., T. Nordstrom, K. Nurmi, K. E. Akerman, P. T. Kovanen, K. Oorni, and K. K. Eklund. 2013. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. *J Biol Chem* 288:13410-13419.
  474. Ichinohe, T., I. K. Pang, and A. Iwasaki. 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol* 11:404-410.
  475. Wendt, W., X. R. Zhu, H. Lubbert, and C. C. Stichel. 2007. Differential expression of cathepsin X in aging and pathological central nervous system of mice. *Exp Neurol* 204:525-540.
  476. Santamaria, I., G. Velasco, A. M. Pendas, A. Fueyo, and C. Lopez-Otin. 1998. Cathepsin Z, a novel human cysteine proteinase with a short propeptide domain and a unique chromosomal location. *J Biol Chem* 273:16816-16823.
  477. Beauvais, D. M., B. J. Burbach, and A. C. Rapraeger. 2004. The syndecan-1 ectodomain regulates  $\alpha$ v $\beta$ 3 integrin activity in human mammary carcinoma cells. *J Cell Biol* 167:171-181.
  478. Longhurst, C. M., and L. K. Jennings. 1998. Integrin-mediated signal transduction. *Cell Mol Life Sci* 54:514-526.
  479. Shi, C., X. Zhang, Z. Chen, M. K. Robinson, and D. I. Simon. 2001. Leukocyte integrin Mac-1 recruits toll/interleukin-1 receptor superfamily signaling intermediates to modulate NF-kappaB activity. *Circ Res* 89:859-865.
  480. Smiley, S. T., J. A. King, and W. W. Hancock. 2001. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167:2887-2894.
  481. Yan, S. R., K. Sapru, and A. C. Issekutz. 2004. The CD11/CD18 ( $\beta$ 2) integrins modulate neutrophil caspase activation and survival following TNF- $\alpha$  or endotoxin induced transendothelial migration. *Immunol Cell Biol* 82:435-446.
  482. Flick, M. J., X. Du, D. P. Witte, M. Jirouskova, D. A. Soloviev, S. J. Busuttil, E. F. Plow, and J. L. Degen. 2004. Leukocyte engagement of fibrin(ogen) via the

- integrin receptor  $\alpha$ 5 $\beta$ 2/Mac-1 is critical for host inflammatory response in vivo. *J Clin Invest* 113:1596-1606.
483. Menezes, G. B., W. Y. Lee, H. Zhou, C. C. Waterhouse, D. C. Cara, and P. Kubes. 2009. Selective down-regulation of neutrophil Mac-1 in endotoxemic hepatic microcirculation via IL-10. *J Immunol* 183:7557-7568.
  484. Issekutz, T. B. 1995. In vivo blood monocyte migration to acute inflammatory reactions, IL-1  $\alpha$ , TNF- $\alpha$ , IFN- $\gamma$ , and C5a utilizes LFA-1, Mac-1, and VLA-4. The relative importance of each integrin. *J Immunol* 154:6533-6540.
  485. Jun, H. K., S. H. Lee, H. R. Lee, and B. K. Choi. 2012. Integrin  $\alpha$ 5 $\beta$ 1 activates the NLRP3 inflammasome by direct interaction with a bacterial surface protein. *Immunity* 36:755-768.
  486. Schornberg, K. L., C. J. Shoemaker, D. Dube, M. Y. Abshire, S. E. Delos, A. H. Bouton, and J. M. White. 2009.  $\alpha$ 5 $\beta$ 1-integrin controls ebolavirus entry by regulating endosomal cathepsins. *Proc Natl Acad Sci U S A* 106:8003-8008.
  487. Pareja, M. E., and M. I. Colombo. 2013. Autophagic clearance of bacterial pathogens: molecular recognition of intracellular microorganisms. *Front Cell Infect Microbiol* 3:54.
  488. Zhou, Y., P. Vedantham, K. Lu, J. Agudelo, R. Carrion, Jr., J. W. Nunneley, D. Barnard, S. Pohlmann, J. H. McKerrow, A. R. Renslo, and G. Simmons. 2015. Protease inhibitors targeting coronavirus and filovirus entry. *Antiviral Res* 116:76-84.
  489. Harder, J., L. Franchi, R. Munoz-Planillo, J. H. Park, T. Reimer, and G. Nunez. 2009. Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF- $\kappa$ B activation but proceeds independently of TLR signaling and P2X7 receptor. *J Immunol* 183:5823-5829.
  490. Mishra, B. B., P. Moura-Alves, A. Sonawane, N. Hacohen, G. Griffiths, L. F. Moita, and E. Anes. 2010. Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol* 12:1046-1063.
  491. Koo, I. C., C. Wang, S. Raghavan, J. H. Morisaki, J. S. Cox, and E. J. Brown. 2008. ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. *Cell Microbiol* 10:1866-1878.
  492. Willingham, S. B., D. T. Bergstralh, W. O'Connor, A. C. Morrison, D. J. Taxman, J. A. Duncan, S. Barnoy, M. M. Venkatesan, R. A. Flavell, M. Deshmukh, H. M. Hoffman, and J. P. Ting. 2007. Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2:147-159.
  493. Rosenberger, C. M., and B. B. Finlay. 2003. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. *Nat Rev Mol Cell Biol* 4:385-396.
  494. Hagar, J. A., and E. A. Miao. 2014. Detection of cytosolic bacteria by inflammatory caspases. *Curr Opin Microbiol* 17:61-66.
  495. Ray, K., B. Marteyn, P. J. Sansonetti, and C. M. Tang. 2009. Life on the inside: the intracellular lifestyle of cytosolic bacteria. *Nat Rev Microbiol* 7:333-340.



- 496. Maslinska, D. 2003. [Apoptosis: physiological cell death and its role in pathogenesis of diseases]. *Neurol Neurochir Pol* 37:315-326.
- 497. Keyel, P. A., R. Roth, W. M. Yokoyama, J. E. Heuser, and R. D. Salter. 2013. Reduction of streptolysin O (SLO) pore-forming activity enhances inflammasome activation. *Toxins (Basel)* 5:1105-1118.
- 498. Hijiya, N., M. L. Metzger, S. Pounds, J. E. Schmidt, B. I. Razzouk, J. E. Rubnitz, S. C. Howard, C. A. Nunez, C. H. Pui, and R. C. Ribeiro. 2005. Severe cardiopulmonary complications consistent with systemic inflammatory response syndrome caused by leukemia cell lysis in childhood acute myelomonocytic or monocytic leukemia. *Pediatr Blood Cancer* 44:63-69.