UNIVERSITY OF CALIFORNIA SAN DIEGO

The Repurposing of Pharmaceutical Agents to Improve Treatment Outcomes in Antibiotic-Resistant Bacterial Infections

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

By

Josh Sun

Committee in charge:

Professor Victor Nizet, Chair
Professor Peter Ernst
Professor Tracy Handel
Professor Sanford Shattil
Professor Stephon Spector

2018

This Dissertation of Josh Sun is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2018

DEDICATION

To my mom and dad, thank you for giving me a younger brother and for teaching me to stand on my own two feet.

To my friends and extended family, thank you for accepting me, for laughing at my lame jokes, and for believing in me – even when you don't understand what it is that I am doing. To KN, thank you for providing me with the competitive nature to always push to be the best I can be. Winners understand what it's like to lose it all. Rest in peace.

To Victor, thank you for your endless mentorship, support, and for giving me the best lab experience a graduate student can ask for.

EPIGRAPH

"I was born by the river
In a little tent
And just like the river I've been
Running ever since"

Sam Cooke

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Victor Nizet has, by far, been the absolute best mentor that I have ever had. Every advice that Victor provides me with is advice that I take to heart. He provided me with the opportunity to spark my inquisitive and investigative nature and I am incredibly grateful and very lucky to have him as a life-long mentor, colleague, and friend. His contribution to the scientific and medical community is boundless and resonates with both his work ethic and his dynamic attraction for truth and knowledge. He dedicates his time to his students and trainees, and because of his academic dedication, my experience at UCSD would not have been the same without him. His motivation to inspire his students to excel, is what provided with me with the fundamental framework to succeed. My only hope is that I did not let him down as a student, and that the effort that I committed to his research projects affords him and his colleagues the recognition that they deserve of the highest regard.

To Victor's lab, particularly those that mentored me every day, I want to thank you for being incredibly patient with me and for putting in the honest effort in teaching me what it takes to conduct science in a very applicable and collaborative manner. The amount of commitment and ambition you all have for your own personal achievements, pushes me to identify what my personal career goals are and the necessary discipline that is required to achieve them.

To all my family and friends, especially Nathan Sun, Alvin Do, Lindsay Ditmars, Jess Chao, Chris Sun, Simon Du, and Miguel Rebollar, the amount of support you have for me is incredible. You may not understand what I am exactly doing with my life, but you've accepted and supported me nonetheless, and for that, I want to thank you for letting me be me.

To San Jose State University, thank you for showing me that it isn't the name of the institution that drives a successful student, but it's a matter of providing the inspiration necessary for a student to succeed.

Chapter 1, in full, is a fully revised manuscript currently in preparation: Sun J and Nizet V. Repurposing of Existing FDA-Approved Medications as Adjunctive Therapeutics Against Highly Antibiotic-Resistant Bacterial Pathogens. The dissertation author is the primary investigator and the co-first author of this paper.

Chapter 2, in full, is a fully revised manuscript currently in preparation for submission to *Science Translational Medicine* as: Sun J, Uchiyama S, Olson J, Cornax I, Kyaw M, Aguilar B, Haste N, Sakoulas G, Rose W, Marth J, and Nizet V. Mechanistic analysis of platelet-mediated defense against methicillin-resistant *Staphylococcus aureus* provides avenues for therapeutic drug repurposing. The dissertation author is the primary investigator and the co-first author of this paper.

Chapter 3, in full, is a manuscript submitted to *Cell Reports* as: Sun J, LaRock D, Kimmey J, Olson J, Jiang Z, O'Donoghue A, Nizet V, LaRock CN. Inflammasome-independent activation of IL-1β by *Pseudomonas aeruginosa* protease LasB induces pathological inflammation. The dissertation author is first author of this work.

VITA

Education

2018 Ph.D., Biomedical Sciences
University of California, San Diego, La Jolla, CA

2013 B.S., Chemistry, concentration in Biochemistry
San Jose State University, San Jose, CA

PUBLICATIONS

Sun J, LaRock DL, Kimmey JM, Olson J, Jiang Z, O'Donoghue AJ, Nizet V, LaRock CN. *Pseudomonas aeruginosa* proteolytic activation of IL-1β drives pathological inflammation (*In revision*)

Sun J, Uchiyama S, Olson J, Cornax I, Kyaw M, Aguilar B, Haste N, Sakoulas G, Rose W, Marth J, Nizet V. Mechanistic analysis of platelet-mediated defense against methicillin-resistant *Staphylococcus aureus* provides avenues for therapeutic drug repurposing (*In submission*)

Uchiyama S, **Sun J**, Fukahori K, Wu M, Varki A, Marth JD, Nizet V. Dual actions of *group B Streptococcus* capsular sialic acid provide resistance to platelet-mediated antimicrobial killing. (*In review*)

Haste N, **Sun J**, Hensler M, Salvioni A, AguilarB, Olson J, Sakoulas G, Nizet V. Deploying Diflunisal as an Anti-Virulence Strategy Against *Staphylococcus aureus* (*In preparation*)

KEY PRESENTATIONS AND CONFERENCES

Gordon Research Conference: Cell Biology of Megakaryocytes & Platelets: Platelet P2Y12 inhibition enhances platelet-mediated immune killing of methicillin-resistant *Staphylococcus aureus,* **2017** (*poster*)

Rady's Children Hospital: Pediatric Symposium: Platelet P2Y12 inhibition enhances platelet-mediated immune killing of methicillin-resistant *Staphylococcus aureus.,* **2016** (*poster*)

American Society of Gene & Cell Therapy: Therapeutic macrophages protect against methicillin- resistant *Staphylococcus aureus* infection., 21st Annual Meeting, **2018** (*poster*)

ABSTRACT OF THE DISSERTATION

The Repurposing of Pharmaceutical Agents to Improve Treatment Outcomes in Antibiotic-Resistant Bacterial Infections

by

Josh Sun

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2018

Professor Victor Nizet, Chair

Bacterial infections continue to exert a tremendous burden on the public health throughout the developing and developed world, in communities and hospitals, and in vulnerable populations such as children, the elderly, surgical patients, the immunocompromised, and those with cancer and other chronic diseases. Over-prescription of antibiotics, extensive antibiotic use in agricultural settings, increasingly complex hospitalized patient populations undergoing treatment have fueled the rise of several highly antibiotic-resistant "superbugs", exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant *Pseudomonas aeruginosa*, that vex

physicians and drive worse patient outcomes. The dependency on the historic success of classical antibiotic therapy, and the lack of financial and market interests towards novel drug development, has contributed to the current fear of a "post-antibiotic era".

As a novel approach to combat invasive and often antibiotic-resistance pathogens such as MRSA and *P. aeruginosa*, the goal of my thesis research as a dual degree PharmD-PhD student in the Nizet Lab is to identify and develop adjunctive pharmaceutical strategies that improve patient outcomes. Our research group has concluded that the current definition of antibiotic, which only examines direct bactericidal or inhibitory activities on the pathogen, is too narrow and fails to address the fundamental nature of serious human bacterial infections, which are in fact diseases of the host-pathogen interaction. Deficiencies or inflammatory consequences of the immune response and immune cell function drive pathology and severe clinical manifestations, and leading pathogens express multiple virulence determinants (toxins, immune evasion factors) which manipulate the host response to establish disease.

By considering the way pharmaceutical agents, antibiotics and non-antibiotics alike, work in the full context of the host-pathogen interaction and innate immune response, we might identify additional opportunities to modify the underlying process to favor bacterial clearance or reduced host tissue injury. These include blocking bacterial virulence factor expression, boosting host innate immune cell bactericidal activities, reducing pathological inflammation, or increasing host cell resiliency. My studies have uncovered two such opportunities in which the unexpected "repurposing" of current FDA-approved drugs demonstrate therapeutic benefit in infections caused by antibiotic-resistant pathogens, uncovering fundamental new biological principles of the host-pathogen interaction in the process.

CHAPTER 1

Repurposing of Existing FDA-Approved Medications as Adjunctive Therapeutics Against Highly Antibiotic-Resistant Bacterial Pathogens

Josh Sun[1,2] and Victor Nizet[1,2,3] *

Affiliations:

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, CA

[2]Department of Pediatrics, University of California, San Diego, La Jolla, CA

[3]Senior Author

*To whom correspondence should be addressed: *vnizet@ucsd.edu*

Manuscript in final preparation for submission with an additional epilogue that serves as a segue to the overall thesis

1.1 PREFACE

Prescription writing and medication use has been an integral part of everyday life since ancient times, with detailed evidence of compounding formularies and clay tablets discovered across various ancient civilizations. The efficacy of ancient medical practice however remains ambiguous as much of the understanding regarding its human health impact remains anecdotal. In contrast, the origin of the modern pharmaceutical industry can be traced back to a significant lineage in the 19th century – where small apothecaries, also known as the modern pharmacy, and chemical companies transitioned from compounding prescription medications and textile dye development, respectively, to drug standardization, wholesale manufacturing, and applied medical research. With an increasing demand for quality research throughout the first two World Wars, a plethora of drugs such as synthetic vitamins, recombinant hormones, psychotropics, metabolic agents, vaccines, and in particular antibiotics, have paved the road for the rise of the "golden era" of the pharmaceutical drug industry. Though antibiotics have had such profound socioeconomic success and a significant global health impact, it has unfortunately resulted in a current state of innovative complacency. Pathogenic microbes continue to increasingly evolve drug resistance, thereby providing pragmatic evidence of the looming return of the pre-antibiotic era**.**

1.2 INTRODUCTION

In pre-antibiotic America, 30% of all deaths were attributed to bacterial infections (1). The discovery and development of antibiotics marked the advent of the American pharmaceutical industry and is widely regarded as a major socioeconomic success of modern medicine and the human condition. Once feared diseases, such as tuberculosis and pneumonia, can now be mitigated with antibiotic treatment. Within the past three decades, however, the evolution of antibiotic resistance has witnessed a devastating progression, pressuring scientists and clinicians to scramble for alternative and paradigm-changing approaches to treatment. The present alarming prospect of a post-antibiotic era is attributable to a variety of issues, each of which requiring pressing attention. These include the antibiotic-induced evolutionary pressure that accelerates resistance, the disturbingly over-simplistic method for evaluating antibiotic susceptibility, and the lackluster financial motives that diminish the antibiotic development pipeline.

In 2016, the United States Center for Disease Control and Prevention estimated that over 2 million people are sickened by bacterial infections, which results in 23,000 annual deaths, $20 billion in healthcare costs, and $35 billion in loss of productivity. It is estimated that over 300 million pre-mature deaths, up to $100 trillion loss due to decreased global productivity, and mortality rates to exceed that of cancer, will occur by 2050 if drastic measures against anti-microbial resistance are not undertaken (2). All of these considerations ultimately beg the question – is a continual search for additional classical antibiotic compounds truly the best approach by which we can survive the looming antibiotic resistance crisis? By thinking outside-the-box, is it not feasible to identify non-antibiotic medications that work at the host-pathogen interface to provide an immunological advantage to the patient in clearing a drug-resistant infection? A vast

repertoire of FDA approved drugs is available for direct patient use, however the unconventional, and to some counterintuitive, concept of treating infection without directly targeting the pathogen requires further scientific and mechanistic evidence to achieve a paradigm shift in our treatment approach against difficult infectious disease conditions.

1.2.1. A perpetual battle against drug resistance

The development of antibiotics over 70 years ago was thought to initiate the selective evolutionary pressure to obtain antibiotic resistant genes. This phenomenon was reinforced by the fact that microbes that predate the antibiotic era are significantly more susceptible to antibiotics (3). However, recent metagenomic studies discovered that resistance to antibiotics is not only a natural phenomenon that predates the selective pressure caused by modern clinical antibiotic use, but a larger concentration of antibiotic-resistant genes than what was previously acknowledged was identified (4). Genetic analysis of ancient DNA from 30,000-year-old Beringian permafrost sediments identified a diverse set of resistance genes to β-lactam, glycopeptide, and tetracycline-family class of antibiotics. The dramatic decrease in novel antibiotic classes being discovered (5), coupled with the microbes' intrinsic ability to evolve resistant mechanisms, suggests that classical antibiotic drug discovery is only a temporary solution to an infinitely long battle against resistance.

A powerful force that contributes to the rapid selective evolutionary pressure for antibiotic resistance is the extensive agriculture use (6-8) and the over-prescription of antibiotics by physicians (9, 10). Often in the setting of self-limited or uncomplicated viral infections, excessive antibiotic-use fuels our prescription-dependent, overly hygienic society. Extensive research provides evidence of antibiotic-mediated disruption of the stability and architecture of our normal

microbiota, which plays a critical role in defense against infection, maturation and function of our immune system (11, 12). Opportunistic infections such as *Clostridium difficile* (13) and *Candida albicans* (14) are directly correlated to prior antibiotic use, and strong correlations have been established with repeated antibiotic exposure and increased incidences of obesity (15) and asthma (16).

1.2.2. The Diminishing Market Interest

Antibiotic discovery and development has become a cumbersome and bureaucratic process that involves pharmaceutical manufacturers, government agencies, patent officers, scientific researchers, and trial lawyers. Healthcare consumers, politicians, and patients are exasperated at the strikingly high drug prices for generic antibiotics. Tetracycline, for example, was first marketed in 1967 under the brand name Achromycin V and cost $10 (equivalent to approximately $75 today) for one hundred 500 mg tablets. Though tetracycline is now a generic drug nearly 5 decades later, it now costs north of $900 for only sixty 500 mg tablets. To make sense of the price absurdity, there is the growing understanding that fewer therapies are in current development due to the risk-associated return of investment. Novel antibiotic drug development has a very low Net Present Value (NPV) – a metric that pharmaceutical corporations utilize to determine the best avenues of investment at a given time.

NPV is a risk-adjusted measure of projected revenue of a drug after accounting for initial development costs and future expenses. Essentially, it is used to determine the likelihood of producing a financial return of investment for any given drug. Antibiotics, in particular, suffer from very low NPV for a variety of reasons, which include but are not limited to: antibiotic

regimens have a limited duration of administration, newly developed antibiotics are held in reserve to mitigate the development of drug resistance, the super-saturated antibiotic market, escalated approval requirements, and the lack of clear antibiotic trial guidelines. Such factors pose financial investments in novel antibiotic development too high of a risk with very little potential for profitable reward. This causes many small and large pharmaceutical companies to curtail their antibiotic research and development divisions (17), thereby increasing the price of antibiotics given the current demand to combat drug resistant infections and lack of innovation.

1.2.3. Antibiotic Susceptibility Testing – Out with the old, in with the new

One solution to circumvent the lack of market financial interest is to modernize the method by which we test for antibiotic efficacy. Technological and scientific advances in the middle third of the 20th century brought about the advent of light emitting diodes, the first commercial satellite, as well as the clinical "gold standard" of antibiotic susceptibility testing, known as the Kirby-Bauer antimicrobial disk diffusion procedure – a test that uses conditioned bacteriological medium known as the Müller-Hinton broth (MHB) that was developed by both John Mueller and Jane Hinton of Harvard (18). Over 75 years has since passed, and though the current technological evolution of the 21st century brings the prospects of artificial intelligence and commercialized interplanetary space travel, we still carry a perplexing and highly unusual attraction to the historic "gold standard" of antibiotic testing, despite very concerning limitations.

The classification of antibiotic efficacy is currently confined to the standards by which was established well over 5 decades ago – where MHB was, and still is, the main condition for antibiotic clinical susceptibility testing, despite the growing rationale that the parameters of

determining antibiotic efficacy requires, at minimum, a controlled study under a single-compartment *physiological* model, as opposed to a *bacteriological* model. Conducting antibiotic susceptibility testing in a bacteriological environment skews the antibiotic therapeutic efficacy in such a way that only represents direct bactericidal or growth inhibitory properties on the microorganism. This method, though robust, disregards the fundamental nature of a bacterial infection, which involves the invading microbes' exposure to host physiological factors - serum antibodies, immune infiltrating cells, tissue resident cells, etc. Conducting antibiotic susceptibility tests in eukaryotic media would better mimic host physiological conditions and provide an optimal analysis on antibiotic action.

Reports provide evidence of MHB standard susceptibility testing that overlooks the potential therapeutic activity of antibiotics against "multi-drug" resistant gram-negative pathogens (19, 20). In the study, authors demonstrate that standard MHB antibiotic susceptibility testing, which identified a strain of "multi-drug" resistant gram-negative *Acinetobacter baumannii* or *Stenotrophomonas maltophilia*, in fact showed susceptibility to Azithromycin when susceptibility tests were performed in eukaryotic media. Azithromycin is a commonly prescribed antibiotic that is not typically recommended for treatment against antibiotic-resistant gram-negative infections. Thus, the author's validation and identification of a potential alternative antibiotic for such complex infections demonstrates the disturbingly inefficient methods that MHB provides. Imagine a case where standard *in vitro* MHB laboratory testing finds only a single antibiotic capable of treating a life-threatening drug-resistant infection, and the perception that is received by clinicians in believing that there are no alternative therapeutic options. The risk of therapeutic failure is not uncommon, as many people differ in drug response, and the limitations of our current suboptimal

antibiotic testing, which is very likely to produce *false negative*, results is unacceptable and negligent to the well-being of the patient.

1.3 DRUG REPOSITIONING TO COMBAT ANTIBIOTIC RESISTANCE

The therapeutic efficacy of a newly discovered chemical or biological entity is typically examined under a single, or in a very narrow subset, of disease states with a primary focus to fulfill market and regulatory interests. As a result, drugs that are approved by the United Stated Federal Drug Agency (FDA) become compartmentalized according to a specialized field of medicine, thus dismissing the potential for a single drug to have therapeutic application across a wide spectrum of diseases. The vast repertoire of non-antibiotic FDA approved drugs that are readily available for direct patient use provides the feasibility and efficiency to identify unconventional medications that affords clinical benefit against bacterial infections. The ambiguity of unconventional non-antibiotic treatment, however, requires clinical and scientific research in detailing its therapeutic potential.

Approximately 55% of the American population regularly take prescription medications, both antibiotic and non-antibiotic alike, and over 75% of the population regularly use over-the-counter medications (21-23). The rapid evolution and dissemination of resistance by over-use of antibiotics, is a prime example of a drug-induced genetic alteration that occurs within the microbial community. This is indicative of the potential for non-antibiotic medications to modulate host immunity, which may be a contributable force that dictates a patients' response to the ever-changing heterogenetic microbial landscape. The antibiotic-induced selective pressure for microbes to develop antibiotic-resistance, ultimately begs the question – is the search for novel

classes of antibiotics truly the method by which we overcome the development of antibiotic resistance? Maybe our approach against infectious diseases should move away from discovering new antibiotics, and shift to a more patient-centric approach, where we identify non-antibiotic drugs that have dual host- and pathogen-directed modulatory properties in such a way that affords clinical benefit (**Figure. 1**).

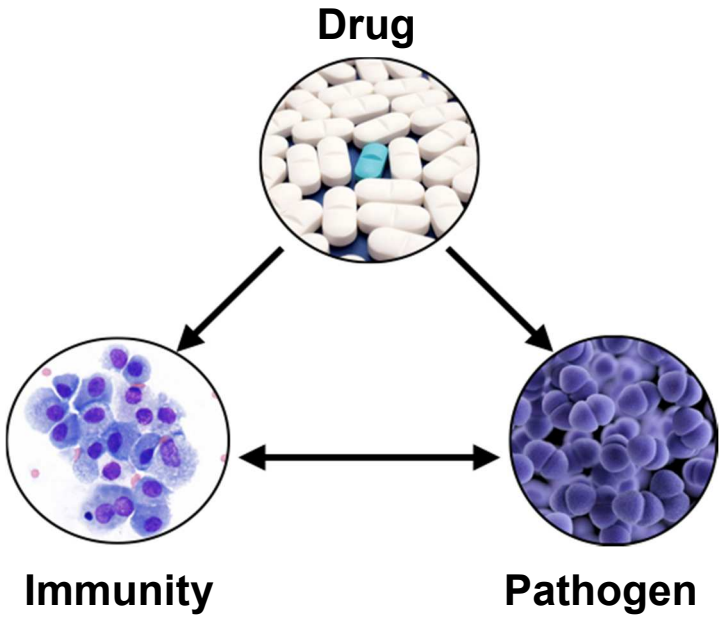


Figure 1.1 Diagram depicting the potential dual action of FDA-approved antibiotic and non-antibiotic drugs. Drugs can directly target the pathogen by inhibition of bacterial growth, killing of the pathogen, or inhibition of pathogen virulence. On the other hand, drugs can modulate patient immunity thereby providing immune-protection, prevention of infection, and preservation of immunity

1.3.1. Overcoming drug resistance

An excellent review by David brown lists FDA-approved drugs, with experimental and clinical evidence, which have the ability to be repositioned as antibiotic-resistant breakers (ARBs) (24). The ESKAPE pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., are of frequent concern given the growing resistance against cephalosporins, fluoroquinolones, and aminoglycoside antibiotics in South Asia, the Middle East, and the Mediterranean. In India and China, 50 – 80% of Enterobacteriaceae (CREs) have been reported to be antibiotic-resistant, pressuring the use of last-resort carbapenem therapy, which is typically reserved for severe cases of infection. However due to its current over-use, bacteria have evolutionarily selected for the development of carbapenem-destroying enzymes, carbapenemases, which reduces the efficacy of carbapenem therapy. Carbapenems belong to a family class of a β-lactam antibiotics, which are cell wall synthesis inhibitors that bind to bacterial transpeptidases (penicillin-binding protein) found on the bacteria's cell surface. Carbapenemases, a β-lactamase, degrades any antibiotic structure that consists of a β-lactam ring. Thus, carbapenemases-expressing bacteria are inherently resistant to penicillin, cephalosporins, and monobactams. In addition, an increasing threat is the dissemination of resistant genes. Through a mechanism coined "horizontal gene transfer", bacteria can share their resistant genes to other bacterial species. *Pseudomonas* spp. for example, has been reported to express a *K. pneumoniae* carbapenemase (25). In an effort to break resistance, β-lactamase inhibitors were developed as "suicide inhibitors" for adjunctive antibiotic therapy, wherein the compound serves solely as a competitive substrate for β-lactamase, thereby mitigating the degradation of the intended antibiotic. Unfortunately, resistance against suicide inhibitors is growing (26, 27), and the discovery for new antibiotic classes, as well as novel β-lactamase

inhibitors are dwindling. To combat this, a variety of FDA-approved drugs that can serve as "antibiotic resistant breakers" (**Table 2**).

A separate study focused on the identification of FDA-approved therapeutics against *Yersinia pestis*, the causative agent of plague (28). Here, the investigators conducted a drug screen of 780 FDA-approved medications and identified 94 drugs with enhanced macrophage resiliency against infection *in vitro*. Of the 94 drugs identified, 3 showed therapeutic efficacy in improving murine survival model, Trifluoperazine, doxapram, and amoxapine. Trifluoperazine in particular, a dopamine antagonist approved for the treatment of psychotic disorders, was not only protective against *Yersinia pestis*, but also demonstrates broad therapeutic efficacy against *Salmonella enterica* and *Clostridium difficile*. Furthermore, experimental data revealed that Trifluoperazine protects human lung fibroblasts from *C. difficile* toxin B intoxication (29), and was found to be directly antibacterial against *Staphylococcus aureus*, *pseudomonas* spp., Shigella spp., and *Salmonella* (30-32). Given Trifluoperazine pathogen- and host-directed effects, further investigation is warranted to elucidate its mechanism. In doing so, biological targets can be identified and exploited as a therapeutic angle. For example. in a study observing Trifluoperazine activity in epithelial cells against *S. typhimurium*, authors described a decrease in intracellular survival that is autophagy mediated (33), thus providing evidence that compounds that induce autophagy during infection may be therapeutic.

Table 1.1. Repurposing drugs as "antibiotic resistant breakers"
(*adapted from Nature Reviews Drug Discovery volume 14, pages 821–832 (2015)*)

FDA-Approved Compounds	Class	Mechanism by approved disease indication	Bacteria (Gram)	Potential "ARB" mechanism
Ciclopirox	Anti-fungal	TBD	–	• Inhibits the synthesis of the LPS • Regulates iron transport • Induces autophagy
Loperamide	Anti-motility	μ-opioid agonist to decrease myenteric plexus activity of large intestine	–	• Facilitates tetracycline uptake • Synergy with cephalosporins
Aspirin	Salicylate	Nonselective COX-1 and -2 inhibition for pain and inflammation, low dose for anti-platelet effects	+	• Reduces resistance to aminoglycosides in *K. pneumoniae* • Enhances the susceptibility of *H. pylori* • Antagonizes LPS signaling • Reduces *S. aureus* virulence
Diclofenac	NSAID	COX-2 > COX-1 inhibition for pain and inflammation	–/+	• Antibacterial against *S. aureus*, *E. coli*, and Mycobacterium spp. • Synergism with streptomycin • Effective V. cholerae, Salmonella spp., Listeria spp., and *M. tuberculosis*, in vivo
Metformin	Anti-diabetic	Inhibition of the mitochondrial respiratory chain, activation of AMPK	–	• Enhances macrophage phagocytosis • Increased survival rate in mice challenged with LPS • Decreased *M. tuberculosis* severity
(+)-Naltrexone and (+)-Naloxone	Opioid Antagonist	μ-opioid > κ-opioid receptor antagonist to reverse alcohol/opioid dependence	–/+	• Inhibition of TLR4 signaling

COX, cyclooxygenase; NSAID, Non-steroidal anti-inflammatory agent; TLR, Toll-like receptor, LPS, Lipopolysaccharide; AMPK, adenosine monophosphate-activated protein kinase

Lastly, tamoxifen is an anti-cancer drug that is indicated for women with ductal carcinoma *in situ*, and prophylaxis and treatment of hormone-positive metastatic breast cancer in both men and women. As a nonsteroidal anti-estrogen drug, tamoxifen competes with estrogen-receptor binding and inhibits the hormone-induced proliferation of cancer cells. Interestingly, tamoxifen improved survival outcomes in a MRSA intra-peritoneal model in a host-directed manner. Tamoxifen was found to induce neutrophil-extracellular trap formation, thus enhancing neutrophil bactericidal activity against Gram-positive and Gram-negative pathogens by ways of intracellular ceramide modulation (34). Further investigation of compounds that modulate ceramide

biosynthesis is thus warranted. In the next section of this chapter, we provide evidence for the need to reposition two astounding FDA-approved drugs with counterintuitive therapeutic application.

1.3.2 Statins

A historical perspective

Akira Endo is a Japanese biochemist whose microbiological research focuses on the relationship between cholesterol biosynthesis and fungi. His investigation on fungal cholesterol biosynthesis resulted in the discovery of three metabolites derived from *Penicillium citrinum* with hypocholesterolemic properties. Upon further analysis, he discovered the hypocholesterolemia drug, Compactin (Pravastatin), which in turn inspired many pharmaceutical companies to begin searching for additional statin therapeutics. Through industry-academic collaborative research, Merck pharmaceuticals birthed the first commercial FDA-approved statin, lovastatin (35).

Clinical and experimental evidence of statin activity against Staphylococcus aureus

Statins are a class of lipid-lowering medications that are designed to selectively inhibit the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), which is responsible for cholesterol biosynthesis. Excessive cholesterol production is directly associated with increased risk cardiovascular disease (CVD), such as myocardial infarction and atherosclerosis, and mortality in patients suffering from CVD. Statin inhibition of HMG-CoA reductase mitigates the pathogenesis of CVD by reducing plasma cholesterol levels. The medical significance of statins, however, extends far beyond that of cholesterol reduction and CVD as numerous studies have suggested that statins not only possess cholesterol-lowering properties, but also possess anti-inflammatory and antithrombotic effects (36-38). An excellent review articulates the clinical,

experimental, and molecular evidence that supports the anti-inflammatory property of statins and its ability to genetically modulate a variety of vascular cell types in the context of atherogenesis and chronic inflammation (39). Experimental observations utilizing *in vitro* cell culture techniques and *in vivo* animal models reinforce the anti-inflammatory role for statins that are observed clinically. For example, statins directly reduce interleukin-6-induced C-reactive protein in hepatocytes at the protein and transcriptional level (40). Though the underlying mechanism behind the pleiotropic effects has yet to be elucidated, it does serve as basis for repositioning statins to modulate host immune function against inflammatory diseases other than CVD, such as in the setting of a blood borne infection and sepsis.

Systemic hyper-inflammation and multiple organ failure are hallmark phenotypes observed in sepsis and has therefore been a potential indication for statin therapy for many years (41). Interventions attempting to mitigate the progression of sepsis should inhibit multiple levels of the inflammatory response, as its pathophysiology is incredibly complex, involving a network of multiple, and potentially converging, cellular pathways that are difficult to delineate and target. Nonetheless, the origination of sepsis is infection-induced, and despite some conflicting reports (42, 43), many clinical and experimental studies have identified that statin therapy proved to be immunomodulatory in this particular disease state. Statins significantly reduced mortality rates in patients suffering from severe sepsis-associated acute respiratory distress syndrome (ARDS) (44). In a separate study, the development of sepsis in patients suffering from chronic kidney disease who are receiving dialysis was identified to be critical risk factors for morbidity and mortality (45), and authors discovered that statin therapy was independently and significantly associated with reduced hospitalization (46). Another clinical study on prior statin use was found to be

significantly associated with reduced rate of severe sepsis and ICU admission (47), indicating that statin therapy could additionally serve as a prophylactic agent. Furthermore, though showing no benefit regarding mortality, the ASEPSIS clinical trial reported that statin therapy significantly reduced the clinical progression and severity of sepsis (48). In our own clinical analysis utilizing a national cohort of patients with *Staphylococcus aureus* bacteremia, the leading cause of sepsis, the continuation of statin therapy is significantly associated with reduced 30-day mortality (49). All of these clinical observations serve as impetus for the rationalization that statins exert both a host- and pathogen-mediated therapeutic effect against this infection-induced dysregulated inflammatory response. *S. aureus* expresses multiple virulence factors that facilitate persistent colonization and evasion of host immunity. Its ability to proliferate within macrophages (50, 51) and rapidly disseminate, makes this pathogen incredibly complex and difficult to treat. Recent experimental studies demonstrate that statins, and their analogs thereof, not only disassembles methicillin-resistant *Staphylococcus aureus* (MRSA) functional membrane microdomains, which in turn perturbs penicillin binding protein 2a (PBP2a) oligomerization and increases bacterial susceptibility to antibiotic therapy, but statins also modulate host macrophage function to enhance the formation of extracellular traps – which directly kills and facilitates the clearance of *S. aureus* (52).

1.3.3. Salicylates

A historical perspective

The therapeutic properties of salicylic acid, the active component of willow bark, spans over 4,000 years of history. Ancient civilizations, such as the Sumerians, the Chinese, and the Greek, utilized extracts from willow bark to treat pain, fever, and inflammation. Though the

medical benefits of salicylic acid had long been recognized, so too had the adverse effects related to its prolonged use of the drug – gastrointestinal distress. It wasn't until the late 1800s, however, when chemists discovered that chemical acetylation of salicylic acid, acetylsalicylic acid, reduced the adverse outcomes that are associated with gastrointestinal irritation, such as nausea, vomiting, bleeding, and ulcers (35). In 1900s, scientists detailed the mechanism by which aspirin exerts its anti-inflammatory properties, by selective and irreversible inhibition of a family of cyclooxygenase (COX) enzymes. COX inhibition reduces the synthesis of pro-inflammatory biological mediators, such as prostaglandins, which among many other biological effects, causes dilation of vascular smooth muscle cells. In addition to its antipyretic, analgesic, and anti-inflammatory properties, studies found clinical benefit afforded to patients taking aspirin to prevent diseases such as, myocardial infarction (53) , stroke (54), and colorectal carcinoma (55). Given its current generic status, its financial affordability, and its indication against a wide spectrum of disease states, truly marks Aspirin as the wonder drug of the century.

Clinical and experimental analysis of aspirin activity against Staphylococcus aureus

At arms to combat the rise of drug-resistant bacterial infections, experimental evidence reveals the ability for salicylic acid to attenuate bacterial virulence by targeting *S. aureus* global regulatory pathways. Authors demonstrate that pre-treatment of bacteria with physiological relevant concentrations of salicylic acid for 18 hours reduced two distinct virulence phenotypes that are relevant to the pathogenesis of experimental infective endocarditis, α-toxin (Hla) and matrix protein binding to fibrinogen and fibronectin. Inhibition of these virulence factors by salicylic acid were associated with inhibition of their respective genes, *fnbA*, *fnbB*, and *hla* (56).

Limitations to the study, however, is that aspirin did not provide therapeutic benefit in a dose-dependent manner *in vivo*. An inverse relationship between aspirin dosage and decreased vegetation size is observed in the experimental infective endocarditis model. Specifically, only doses only ranging from 1 to 10mg/kg/day showed significant reduction in aortic valve vegetation (57, 58). This suggests that aspirin may greater serve to modulate host immunity as opposed to its pathogen-mediated anti-virulence properties. Given aspirins non-selective nature, changes in dosing regimen alters the pharmacokinetics and -dynamics of aspirin activity, thus dictating the required dose to treat a specific disease (59). It has long been realized that salicylate serum concentration correlates with the pharmacological actions and adverse effects observed (**Table 1**).

Table 1.2 Serum Salicylate: Clinical Correlations
Adapted from Lexicomp Drug Reference Handbook 20th Edition

Serum Concentration (ug/mL)	Desired Effects	Adverse Effects/Intoxication
~ 100	Antiplatelet Antipyretic Analgesic	GI intolerance, bleeding, hypersensitivity, hemostatic defects
150 - 300	Anti-inflammatory	GI intolerance, mild salicylism
250 - 400	Treatment of rheumatic fever	GI intolerance, flushing, sweating, thirst, headache, tachycardia
>400 - 500		Respiratory alkalosis, hemorrhage, excitement, confusion, asterixis, pulmonary edema, convulsions, tetany, metabolic acidosis, fever, coma, cardiovascular collapse, renal and respiratory failure

We performed a *S. aureus* bacteremia survival model, as opposed to the infective endocarditis model as described above, to determine whether varying doses of aspirin could alter the outcome of mortality during a bloodborne infection. In similarity, we demonstrate that only low-doses of aspirin, 1.0 and 2.5 mg/kg/day, reduced the progression of mortality in a 10-day observation study (**Fig. 2.)**. Indeed, multiple clinical studies discovered that low-dose aspirin is protective against *S. aureus* embolism and septic shock (60, 61). In particular, a retrospective cohort study evaluating 838 patients with episodes of *S. aureus* bloodstream infection, conducted at a Swiss tertiary referral center, reconfirmed our experimental finding and concluded that low-dose acetylsalicylic acid at the time of infection was significantly associated reduced short-term mortality (62). These observations suggest that the low-dose-specific reduction in mortality may be by host immunomodulatory mechanisms that outweigh the pathogen-mediated virulence inhibition by aspirin treatment, further investigation is however warranted.

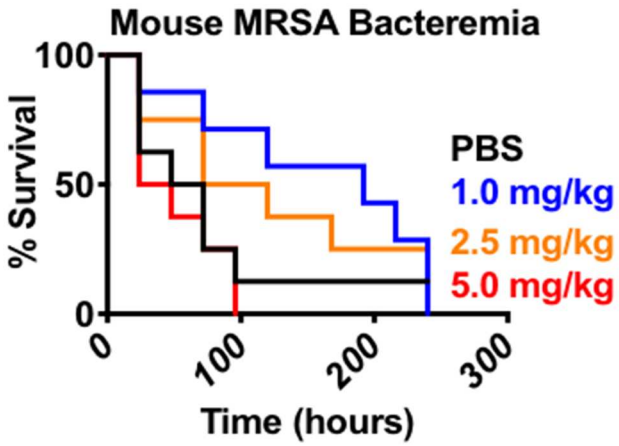


Figure 1.2. Aspirin shows an inverse correlation between dose-response and mortality. 8-10 week-old outbred CD1 mice were infected intravenously and treated with oral doses of acetylsalicylic acid at the time of infection, and every 24 hours after infection for 10 days

Clinical and experimental analysis of Diflunisal activity against Staphylococcus aureus

This sub-section is a modified manuscript currently in preparation titled *"Deploying Diflunisal as an Anti-Virulence Strategy Against Staphylococcus aureus",* by *Haste N., Sun J., et al.* Diflunisal is derivative of salicylic acid with the same mechanism of action – inhibition of prostaglandin synthesis via COX inhibition. Though it was discovered previously that salicylic acid attenuates *S. aureus* virulence by targeting global regulatory pathways, thereby decreasing *Hla* expression, bacterial density, and vegetation in an *in vivo* model of infective endocarditis, Haste et al. did not reconfirm that salicylic acid significantly reduces *S. aureus* virulence in multiple functional assays. Analysis of global functional virulence properties found that Diflunisal, and not salicylic acid, inhibited Hla toxin activity (**Fig. 3A**), red blood cell lysis (**Fig. 3B**), carotenoid pigment formation (**Fig. 3C**), and biofilm formation (**Fig. 3D**), in a dose-dependent manner.

Our cumulative data suggests that both drugs have functionally distinct therapeutic mechanisms that potentially affords benefit during *S. aureus* bacteremia. Given Aspirin's dose-specific therapeutic activity, I postulate that it more favors modulation of host immunity, as opposed to bacterial gene expression, thereby providing the clinically relevant therapeutic benefit observed during *S. aureus* infection. Diflunisal, on the other hand, exerts direct anti-staphylococcal properties in a broad anti-virulence fashion, further research is warranted to determine whether Diflunisal treatment *in vivo* occurs in a pathogen-directed manner. Nonetheless, separate studies revealed the protective effects of Diflunisal against *Staphylococcus aureus* osteomyelitis (63) and *Hla* through its ability to inhibit *agr* transcriptional regulation (64).

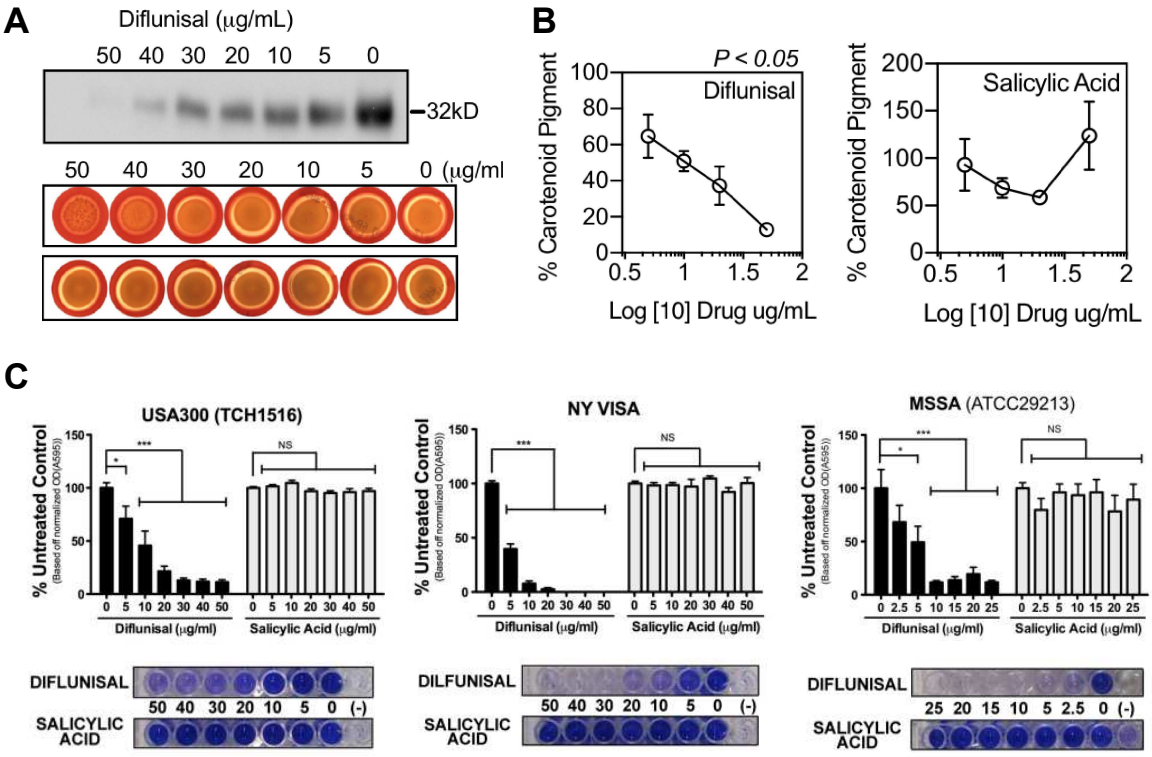


Figure 1.3 Diflunisal exerts global *S. aureus* virulence inhibition. (**A**) Overnight cultures of *S. aureus* pretreated with Diflunisal (DIF) or Salicylic acid (SA) and analyzed for hemolysin expression and activity. (**B**) Golden pigment production assayed in *S. aureus* pre-treated with varying doses of Diflunisal or Salicylic acid (**C**) *S. aureus* pre-treated with varying doses of Diflunisal or Salicylic acid and biofilm production assayed by crystal staining methodology. * $P < 0.05$, *** $P < 0.0001$. Statistical significance determined by students two-tailed t-test. All experiments performed at least 3 independent times.

.

1.4 CONCLUDING THOUGHTS

Though our compartmentalized method of thinking regarding drugs-diseases is imperative to safely monitor toxicity and efficacy, it unfortunately limits its therapeutic potential. It's highly plausible, in fact countless evidence would suggest, that common drugs that are designed to treat a certain type of disease can be used prophylactically or therapeutically against a wide spectrum of diseases. The solution to combat the rise of antibiotic resistance, and the looming era of a post-antibiotic era, may clearly be readily available for patient use.

1.5 SPECIFIC AIMS

AIM 1: Mechanistic analysis of platelet-mediated defense against methicillin-resistant *Staphylococcus aureus* bacteremia

a. Determine the physiological impact of platelets during MRSA infection

b. Identify bacteria-derived virulence factors that modulates platelet function

c. Determine the impact of platelet P2Y12 and platelet sialidase inhibition during experimental MRSA bacteremia *in vivo* and *in vitro*

d. Examine Ashwell-Morell receptor inhibitors as experimental therapy against MRSA bacteremia

AIM 2: Repurposing metalloprotease inhibitors to limit inflammatory damage in *Pseudomonas aeruginosa* pneumonia

a. Determine how IL-1β drives degradation of the lung architecture during *P. aeruginosa* respiratory infection

b. Identify the sources of functional IL-1β generation during *P. aeruginosa* pneumonia (host vs. pathogen)

c. Identify the sequence site of functional proteolytic processing of IL-1β by *P. aeruginosa*

d. Examine the therapeutic repurposing of experimental metalloprotease inhibitors during *P. aeruginosa* respiratory infection

1.6 MATERIAL AND METHODS

Reagents, Bacterial and Cell Lines (*adapted from Haste, et al.)*

Diflunisal, Salicylic acid, and acetylsalicylic acid (aspirin) were obtained from Sigma Chemical Company (St. Louis, MO). Bacterial strains used in this paper included: USA300 MRSA strain TCH1516 (ATCC, Manassas VA), MRSA serotype ST-59 (clinical isolate from G. Sakoulas, UC San Diego), *S. aureus* vancomycin-intermediate strain (NY VISA, a clinical isolate from G. Sakoulas, UC San Diego), MSSA 29213 (ATCC, Manassas VA), MSSA Newman strain, *S. aureus* strains RN6607 and RN9120 (from G. Sakoulas, UC San Diego).

MRSA bacteremia animal model

TCH1516 (MRSA) was grown shaking overnight at 37°C in THB, washed once in 1x PBS, and 1 x 10^8 colony forming units was used to infect 8-10-week-old outbred CD1 mice by intravenous administration and treated with 1.0, 2.5, or 5.0 mg/kg/day acetylsalicylic acid (aspirin) by oral gavage. Survival was observed over a 10-day study. Mice that appeared moribund were euthanized by CO_2 asphyxiation

Western Immunoblots (*adapted from Haste, et al.)*

Bacterial supernatants from 48-hour cultures were collected after centrifugation at 3,200 x g for 10 minutes. Supernatants were filter-sterilized and subsequently concentrated 20-fold using a Vacufuge (Eppendorf, Hauppauge NY). Samples were mixed with 4X sample buffer and 10X reducing agent (Invitrogen, Grand Island NY), boiled for 10 min, loaded onto a 10% Bis-Tris gel (Invitrogen, Grand Island, NY) and run in MOPS running buffer at 120V. The gel was then transferred onto a nitrocellulose membrane using a semi-dry apparatus (Bio-Rad, Hercules CA) at

12V for 45 minutes. The membrane was then blocked for 1 hour at room temperature in 0.05% PBS-Tween + 5% non-fat milk + 2% plasma. The membrane was incubated overnight at 4˚C with anti-a-hemolysin antibody conjugated to horseradish peroxidase (Abcam®, Cambridge MA) at a 1:1,000 dilution in PBS-Tween. The membrane was then washed with PBS-Tween and developed using chemiluminescence.

MRSA hemolysis on blood agar (*adapted from Haste, et al.)*

To test the ability of Diflunisal to inhibit hemolysis on blood agar, 10 mL cultures were prepared containing Diflunisal, salicylic acid or the vehicle control (undiluted vehicle consisted of 0.1M NaHCO3 pH 8 + 25% ETOH) in Todd-Hewitt broth. Briefly, 200 µL of an overnight culture of virulent ST-59 MRSA or WR-69, an MSSA blood isolate that produces only a-toxin, was added to 10 mL of media with or without drug or vehicle in 15 mL round-bottom Falcon tubes (Becton-Dickinson, Franklin-Lakes, NJ). Diflunisal or salicylic acid was added to each tube at 50, 40, 30, 20, 10, 5 or 0 µg/mL. After 24 hours in a shaking incubator at 37°C, the cultures were removed, mixed thoroughly, and 10 ml droplets were plated on 5% sheep blood in tryptic soy agar (Hardy Diagnostics, Santa Maria, CA). The pre-incubated cultures were assayed for their ability to form zones of hemolysis after incubation at 37°C for 18 – 24 h. Following incubation, photos of each plate were taken immediately to document the hemolysis.

S. aureus Golden Pigment Assays (*adapted from Haste, et al.)*

Golden pigment production was assayed in Diflunisal-treated *S. aureus*. In brief, *S. aureus* cultures were prepared as described for the growth curve assays. After 24 hours, the cultures were

pelleted and washed 2x in PBS. The pellets were extracted with 200 μL methanol and the supernatants analyzed by spectrophotometry (A_{450}) for relative levels of staphyloxanthin.

S. aureus Biofilm Formation (*adapted from Haste, et al.)*

In vitro biofilm production by *S. aureus* was assessed by crystal violet staining. Diflunisal was added to a 96 well flat-bottom plate (Corning Incorporated Life Sciences, Tewksbury, MA) containing THY/0.5% glucose media. *S. aureus* was added at ~ 2 x 10^6 CFU/mL, and the plate was incubated for 24 hours at 37°C under stationary conditions. The supernatant was removed and the wells were gently washed 3x in MilliQ water and dried overnight. Biofilm was then stained by addition of 0.2 mL of 0.2% aqueous crystal violet and incubation for 15 minutes. Excess stain was rinsed 4x in MilliQ water, and the stain remaining in the wells was extracted with 0.2 mL of an 80:20 mixture of ethanol:acetone and read at A_{595}.

1.7 ACKNOWLEDGEMENTS

Chapter 1, in full, is a fully revised manuscript currently in preparation: Sun J and Nizet V. Repurposing of Existing FDA-Approved Medications as Adjunctive Therapeutics Against Highly Antibiotic-Resistant Bacterial Pathogens. The dissertation author is the primary investigator and the co-first author of this paper.

1.8 REFERENCES

1. Fair, R.J. and Y. Tor, *Antibiotics and bacterial resistance in the 21st century.* Perspect Medicin Chem, 2014. **6**: p. 25-64.

2. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* The Review on Antimicrobial Resistance, 2014. **20**.

3. Hughes, V.M. and N. Datta, *Conjugative plasmids in bacteria of the 'pre-antibiotic' era.* Nature, 1983. **302**: p. 725.

4. D'Costa, V.M., C.E. King, L. Kalan, M. Morar, W.W.L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G.B. Golding, H.N. Poinar, and G.D. Wright, *Antibiotic resistance is ancient.* Nature, 2011. **477**: p. 457.

5. Coates, A.R., G. Halls, and Y. Hu, *Novel classes of antibiotics or more of the same?* British journal of pharmacology, 2011. **163**(1): p. 184-194.

6. Allen, H.K., J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies, and J. Handelsman, *Call of the wild: antibiotic resistance genes in natural environments.* Nature Reviews Microbiology, 2010. **8**(4): p. 251.

7. Witte, W., *Medical consequences of antibiotic use in agriculture*. 1998, American Association for the Advancement of Science.

8. Shea, K.M., *Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health?* Pediatrics, 2003. **112**(1 Pt 2): p. 253-8.

9. Simmons, H.E. and P.D. Stolley, *This is medical progress? Trends and consequences of antibiotic use in the united states.* JAMA, 1974. **227**(9): p. 1023-1028.

10. Ventola, C.L., *The antibiotic resistance crisis: part 1: causes and threats.* Pharmacy and Therapeutics, 2015. **40**(4): p. 277.
11. Blaser, M., *Antibiotic overuse: stop the killing of beneficial bacteria.* Nature, 2011. **476**(7361): p. 393.

12. Cho, I., S. Yamanishi, L. Cox, B.A. Methé, J. Zavadil, K. Li, Z. Gao, D. Mahana, K. Raju, and I. Teitler, *Antibiotics in early life alter the murine colonic microbiome and adiposity.* Nature, 2012. **488**(7413): p. 621.

13. Theriot, C.M., M.J. Koenigsknecht, P.E. Carlson Jr, G.E. Hatton, A.M. Nelson, B. Li, G.B. Huffnagle, J.Z. Li, and V.B. Young, *Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection.* Nature communications, 2014. **5**: p. 3114.

14. Ben-Ami, R., K. Olshtain-Pops, M. Krieger, I. Oren, J. Bishara, M. Dan, Y. Wiener-Well, M. Weinberger, O. Zimhony, and M. Chowers, *Antibiotic exposure as a risk factor for fluconazole-resistant Candida bloodstream infection.* Antimicrobial agents and chemotherapy, 2012. **56**(5): p. 2518-2523.

15. Cox, L.M. and M.J. Blaser, *Antibiotics in early life and obesity.* Nature Reviews Endocrinology, 2015. **11**(3): p. 182.

16. Russell, S.L., M.J. Gold, M. Hartmann, B.P. Willing, L. Thorson, M. Wlodarska, N. Gill, M.R. Blanchet, W.W. Mohn, and K.M. McNagny, *Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma.* EMBO reports, 2012. **13**(5): p. 440-447.

17. Power, E., *Impact of antibiotic restrictions: the pharmaceutical perspective.* Clinical Microbiology and Infection, 2006. **12**(s5): p. 25-34.

18. Bauer, A.W., W.M. Kirby, J.C. Sherris, and M. Turck, *Antibiotic susceptibility testing by a standardized single disk method.* Am J Clin Pathol, 1966. **45**(4): p. 493-6.

19. Kumaraswamy, M., L. Lin, J. Olson, C.F. Sun, P. Nonejuie, R. Corriden, S. Dohrmann, S.R. Ali, D. Amaro, M. Rohde, J. Pogliano, G. Sakoulas, and V. Nizet, *Standard susceptibility testing overlooks potent azithromycin activity and cationic peptide synergy against MDR Stenotrophomonas maltophilia.* J Antimicrob Chemother, 2016. **71**(5): p. 1264-9.

20. Lin, L., P. Nonejuie, J. Munguia, A. Hollands, J. Olson, Q. Dam, M. Kumaraswamy, H. Rivera, Jr., R. Corriden, M. Rohde, M.E. Hensler, M.D. Burkart, J. Pogliano, G. Sakoulas, and V. Nizet, *Azithromycin Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens.* EBioMedicine, 2015. **2**(7): p. 690-8.

21. Gu, Q., C.F. Dillon, and V.L. Burt, *Prescription drug use continues to increase: U.S. prescription drug data for 2007-2008.* NCHS Data Brief, 2010(42): p. 1-8.

22. Roe, C.M., A.M. McNamara, and B.R. Motheral, *Use of chronic medications among a large, commercially-insured US population.* Pharmacoepidemiol Drug Saf, 2002. **11**(4): p. 301-9.

23. Zhong, W., H. Maradit-Kremers, J.L. St Sauver, B.P. Yawn, J.O. Ebbert, V.L. Roger, D.J. Jacobson, M.E. McGree, S.M. Brue, and W.A. Rocca, *Age and sex patterns of drug prescribing in a defined American population.* Mayo Clin Proc, 2013. **88**(7): p. 697-707.

24. Solomon, S.L. and K.B. Oliver, *Antibiotic resistance threats in the United States: stepping back from the brink.* American family physician, 2014. **89**(12): p. 938.

25. Brown, D., *Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void?* Nature Reviews Drug Discovery, 2015. **14**: p. 821.

26. Oteo, J., J. Campos, E. Lazaro, O. Cuevas, S. Garcia-Cobos, M. Perez-Vazquez, and F.J. de Abajo, *Increased amoxicillin-clavulanic acid resistance in Escherichia coli blood isolates, Spain.* Emerg Infect Dis, 2008. **14**(8): p. 1259-62.

27. Drawz, S.M. and R.A. Bonomo, *Three decades of beta-lactamase inhibitors.* Clin Microbiol Rev, 2010. **23**(1): p. 160-201.

28. Andersson, J.A., E.C. Fitts, M.L. Kirtley, D. Ponnusamy, A.G. Peniche, S.M. Dann, V.L. Motin, S. Chauhan, J.A. Rosenzweig, J. Sha, and A.K. Chopra, *New Role for FDA-Approved Drugs in Combating Antibiotic-Resistant Bacteria.* Antimicrob Agents Chemother, 2016. **60**(6): p. 3717-29.

29. Caspar, M., I. Florin, and M. Thelestam, *Calcium and calmodulin in cellular intoxication with Clostridium difficile toxin B.* J Cell Physiol, 1987. **132**(1): p. 168-72.

30. Mazumder, R., K. Ganguly, S.G. Dastidar, and A.N. Chakrabarty, *Trifluoperazine: a broad spectrum bactericide especially active on staphylococci and vibrios.* Int J Antimicrob Agents, 2001. **18**(4): p. 403-6.

31. Advani, M.J., I. Siddiqui, P. Sharma, and H. Reddy, *Activity of trifluoperazine against replicating, non-replicating and drug resistant M. tuberculosis.* PLoS One, 2012. **7**(8): p. e44245.

32. Hendricks, O., T.S. Butterworth, and J.E. Kristiansen, *The in-vitro antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on Pseudomonas aeruginosa and Staphylococcus aureus.* Int J Antimicrob Agents, 2003. **22**(3): p. 262-4.

33. Conway, K.L., P. Kuballa, J.H. Song, K.K. Patel, A.B. Castoreno, O.H. Yilmaz, H.B. Jijon, M. Zhang, L.N. Aldrich, E.J. Villablanca, J.M. Peloquin, G. Goel, I.A. Lee, E. Mizoguchi, H.N. Shi, A.K. Bhan, S.Y. Shaw, S.L. Schreiber, H.W. Virgin, A.F. Shamji, T.S. Stappenbeck, H.C. Reinecker, and R.J. Xavier, *Atg16l1 is required for autophagy in intestinal epithelial cells and protection of mice from Salmonella infection.* Gastroenterology, 2013. **145**(6): p. 1347-57.

34. Corriden, R., A. Hollands, J. Olson, J. Derieux, J. Lopez, J.T. Chang, D.J. Gonzalez, and V. Nizet, *Tamoxifen augments the innate immune function of neutrophils through modulation of intracellular ceramide.* Nat Commun, 2015. **6**: p. 8369.

35. Endo, A., *A historical perspective on the discovery of statins.* Proc Jpn Acad Ser B Phys Biol Sci, 2010. **86**(5): p. 484-93.

36. Albert, M.A., E. Danielson, N. Rifai, P. Ridker, and P.I. for the, *Effect of statin therapy on c-reactive protein levels: The pravastatin inflammation/crp evaluation (prince): a randomized trial and cohort study.* JAMA, 2001. **286**(1): p. 64-70.

37. Ray, J.G., M. Mamdani, R.T. Tsuyuki, D.R. Anderson, E.L. Yeo, and A. Laupacis, *Use of statins and the subsequent development of deep vein thrombosis.* Archives of Internal Medicine, 2001. **161**(11): p. 1405-1410.

38. Rodriguez, A.L., B.M. Wojcik, S.K. Wrobleski, D.D. Myers, Jr., T.W. Wakefield, and J.A. Diaz, *Statins, inflammation and deep vein thrombosis: a systematic review.* J Thromb Thrombolysis, 2012. **33**(4): p. 371-82.

39. Jain, M.K. and P.M. Ridker, *Anti-Inflammatory Effects of Statins: Clinical Evidence and Basic Mechanisms.* Nature Reviews Drug Discovery, 2005. **4**: p. 977.

40. Arnaud, C., F. Burger, S. Steffens, N.R. Veillard, T.H. Nguyen, D. Trono, and F. Mach, *Statins reduce interleukin-6-induced C-reactive protein in human hepatocytes: new evidence for direct antiinflammatory effects of statins.* Arterioscler Thromb Vasc Biol, 2005. **25**(6): p. 1231-6.

41. Dobesh, P.P. and K.M. Olsen, *Statins role in the prevention and treatment of sepsis.* Pharmacol Res, 2014. **88**: p. 31-40.

42. Quinn, M., C. Moody, B. Tunnicliffe, Z. Khan, M. Manji, S. Gudibande, N. Murphy, T. Whitehouse, C. Snelson, and T. Veenith, *Systematic review of statins in sepsis: There is no evidence of dose response.* Indian J Crit Care Med, 2016. **20**(9): p. 534-41.

43. Kruger, P.S., M.L. Harward, M.A. Jones, C.J. Joyce, K.M. Kostner, M.S. Roberts, and B. Venkatesh, *Continuation of statin therapy in patients with presumed infection: a randomized controlled trial.* Am J Respir Crit Care Med, 2011. **183**(6): p. 774-81.

44. Mansur, A., M. Steinau, A.F. Popov, M. Ghadimi, T. Beissbarth, M. Bauer, and J. Hinz, *Impact of statin therapy on mortality in patients with sepsis-associated acute respiratory distress syndrome (ARDS) depends on ARDS severity: a prospective observational cohort study.* BMC Medicine, 2015. **13**(1): p. 128.

45. Sarnak, M.J. and B.L. Jaber, *Mortality caused by sepsis in patients with end-stage renal disease compared with the general population.* Kidney Int, 2000. **58**(4): p. 1758-64.

46. Gupta, R., L.C. Plantinga, N.E. Fink, and et al., *Statin use and hospitalization for sepsis in patients with chronic kidney disease.* JAMA, 2007. **297**(13): p. 1455-1464.

47. Almog, Y., A. Shefer, V. Novack, N. Maimon, L. Barski, M. Eizinger, M. Friger, L. Zeller, and A. Danon, *Prior statin therapy is associated with a decreased rate of severe sepsis.* Circulation, 2004. **110**(7): p. 880-5.

48. Patel, J.M., C. Snaith, D.R. Thickett, L. Linhartova, T. Melody, P. Hawkey, A.H. Barnett, A. Jones, T. Hong, M.W. Cooke, G.D. Perkins, and F. Gao, *Randomized double-blind placebo-controlled trial of 40 mg/day of atorvastatin in reducing the severity of sepsis in ward patients (ASEPSIS Trial).* Crit Care, 2012. **16**(6): p. R231.

49. Caffrey, A.R., T.T. Timbrook, E. Noh, G. Sakoulas, S.M. Opal, V. Nizet, and K.L. LaPlante, *Evidence To Support Continuation of Statin Therapy in Patients with Staphylococcus aureus Bacteremia.* Antimicrob Agents Chemother, 2017. **61**(3).

50. Koziel, J., A. Maciag-Gudowska, T. Mikolajczyk, M. Bzowska, D.E. Sturdevant, A.R. Whitney, L.N. Shaw, F.R. DeLeo, and J. Potempa, *Phagocytosis of Staphylococcus aureus by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors.* PLoS One, 2009. **4**(4): p. e5210.

51. Jubrail, J., P. Morris, M.A. Bewley, S. Stoneham, S.A. Johnston, S.J. Foster, A.A. Peden, R.C. Read, H.M. Marriott, and D.H. Dockrell, *Inability to sustain intraphagolysosomal killing of Staphylococcus aureus predisposes to bacterial persistence in macrophages.* Cell Microbiol, 2016. **18**(1): p. 80-96.

52. Chow, O.A., M. von Köckritz-Blickwede, A.T. Bright, M.E. Hensler, A.S. Zinkernagel, A.L. Cogen, R.L. Gallo, M. Monestier, Y. Wang, C.K. Glass, and V. Nizet, *Statins enhance formation of phagocyte extracellular traps.* Cell Host Microbe, 2010. **8**(5): p. 445-54.

53. Elwood, P.C., A.L. Cochrane, M.L. Burr, P.M. Sweetnam, G. Williams, E. Welsby, S.J. Hughes, and R. Renton, *A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction.* Br Med J, 1974. **1**(5905): p. 436-40.

54. Hart, R.G., J.L. Halperin, R. McBride, O. Benavente, M. Man-Son-Hing, and R.A. Kronmal, *Aspirin for the primary prevention of stroke and other major vascular events: meta-analysis and hypotheses.* Arch Neurol, 2000. **57**(3): p. 326-32.

55. Nishihara, R., P. Lochhead, A. Kuchiba, S. Jung, M. Yamauchi, X. Liao, Y. Imamura, Z.R. Qian, T. Morikawa, M. Wang, D. Spiegelman, E. Cho, E. Giovannucci, C.S. Fuchs, A.T. Chan, and S. Ogino, *Aspirin use and risk of colorectal cancer according to BRAF mutation status.* Jama, 2013. **309**(24): p. 2563-71.

56. Kupferwasser, L.I., M.R. Yeaman, C.C. Nast, D. Kupferwasser, Y.Q. Xiong, M. Palma, A.L. Cheung, and A.S. Bayer, *Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in Staphylococcus aureus.* J Clin Invest, 2003. **112**(2): p. 222-33.

57. Nicolau, D.P., C.D. Freeman, C.H. Nightingale, R. Quintiliani, C.J. Coe, E.G. Maderazo, and B.W. Cooper, *Reduction of bacterial titers by low-dose aspirin in experimental aortic valve endocarditis.* Infect Immun, 1993. **61**(4): p. 1593-5.

58. Nicolau, D.P., M.N. Marangos, C.H. Nightingale, and R. Quintiliani, *Influence of aspirin on development and treatment of experimental Staphylococcus aureus endocarditis.* Antimicrob Agents Chemother, 1995. **39**(8): p. 1748-51.

59. Patrono, C., *Aspirin as an antiplatelet drug.* N Engl J Med, 1994. **330**(18): p. 1287-94.

60. Pepin, J., V. Tremblay, D. Bechard, F. Rodier, C. Walker, D. Dufresne, A. Lafontaine, N. Li, C. Lacroix, and L. Lanthier, *Chronic antiplatelet therapy and mortality among patients with infective endocarditis.* Clin Microbiol Infect, 2009. **15**(2): p. 193-9.

61. Eisen, D.P., D. Reid, and E.S. McBryde, *Acetyl salicylic acid usage and mortality in critically ill patients with the systemic inflammatory response syndrome and sepsis.* Crit Care Med, 2012. **40**(6): p. 1761-7.

62. Osthoff, M., J.A. Sidler, B. Lakatos, R. Frei, M. Dangel, M. Weisser, M. Battegay, and A.F. Widmer, *Low-Dose Acetylsalicylic Acid Treatment and Impact on Short-Term Mortality in Staphylococcus aureus Bloodstream Infection: A Propensity Score–Matched Cohort Study.* Critical care medicine, 2016. **44**(4): p. 773-781.

63. Hendrix, A.S., T.J. Spoonmore, A.D. Wilde, N.E. Putnam, N.D. Hammer, D.J. Snyder, S.A. Guelcher, E.P. Skaar, and J.E. Cassat, *Repurposing the nonsteroidal anti-inflammatory drug diflunisal as an osteoprotective, antivirulence therapy for Staphylococcus aureus osteomyelitis.* Antimicrobial agents and chemotherapy, 2016. **60**(9): p. 5322-5330.

64. Khodaverdian, V., M. Pesho, B. Truitt, L. Bollinger, P. Patel, S. Nithianantham, G. Yu, E. Delaney, E. Jankowsky, and M. Shoham, *Discovery of antivirulence agents against methicillin-resistant Staphylococcus aureus.* Antimicrobial agents and chemotherapy, 2013. **57**(8): p. 3645-3652.

1.9 EPILOGUE

The purpose of this dissertation is to identify mechanisms by which FDA-approved drugs modulate host innate-immunity or bacterial virulence. In doing so, we aim to (1) uncover fundamental biological processes that occur that the host-pathogen interface and (2) provide a method of drug repurposing to prevent the progression of, or therapeutically treat, the underlying factors that contribute to the pathogenesis of complex bacterial infections. Using pharmacological probes, we have identified a method by which we prevent *S. aureus*-induced thrombocytopenia, thus affording enhanced innate-immunity and preventing virulence-evoked pathological dysregulation of platelet function during infection. Additionally, we identified a method of repurposing a common antibiotic, with intrinsic metalloproteolytic inhibitory properties, as a means of an anti-inflammatory agent against difficult-to-treat pseudomonas pulmonary infection.

CHAPTER 2

Mechanistic analysis of platelet-mediated defense against methicillin-resistant *Staphylococcus aureus* provides avenues for therapeutic drug repurposing

Josh Sun[1,2*], Satoshi Uchiyama[1*], Joshua Olson[1], Ingrid Cornax[1], May Kyaw[1], Bernice Aguilar[1], Nina Haste[1,2], George Sakoulas[1], Warren Rose[3], Jamey Marth[4,5], Victor Nizet[1,2 +]

[1] Department of Pediatrics, University of California, San Diego, La Jolla, CA

[2] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA

[3] University of Wisconsin Madison

[4] Sanford Burnham Prebys

[5]University of California, Santa Barbara

*Authors contributed equally to this work

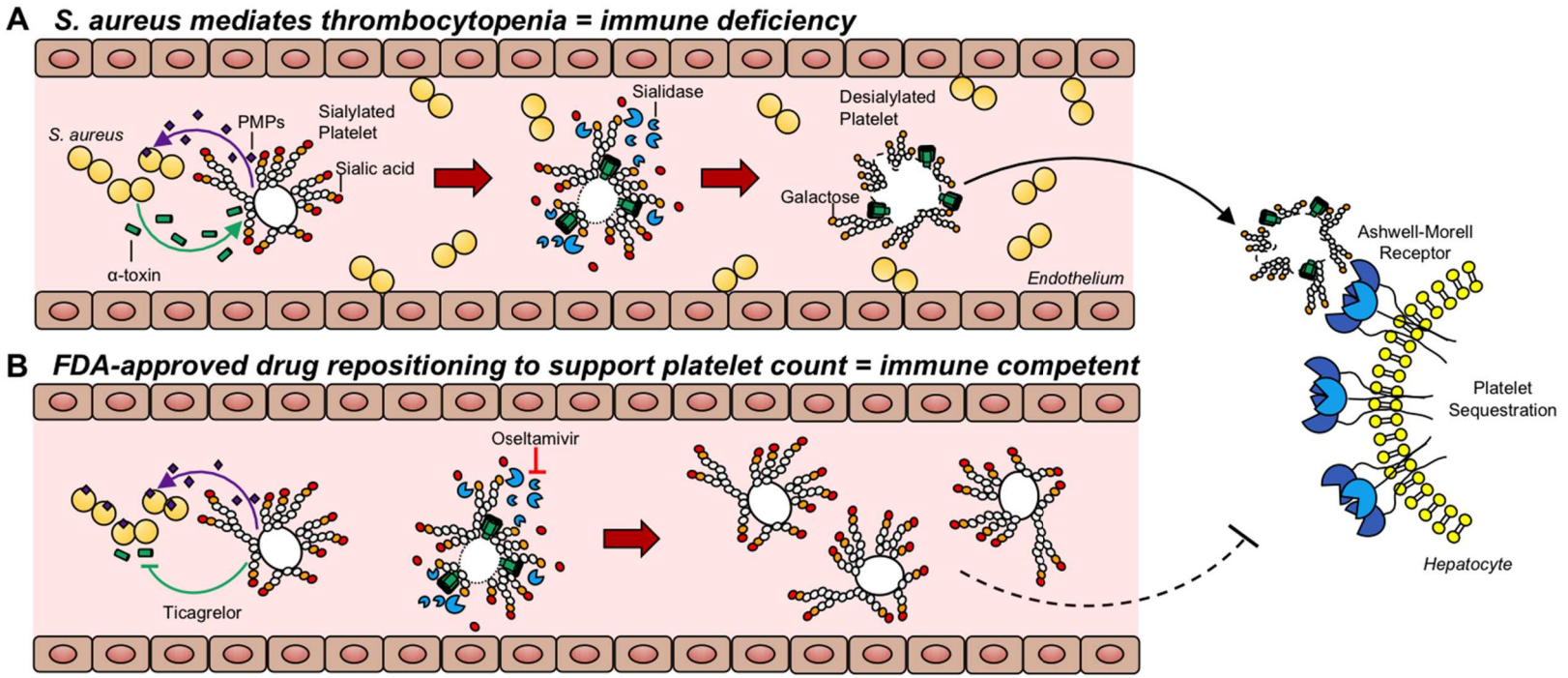
[+] Correspondence: Victor Nizet (vnizet@ucsd.edu)

Manuscript in final preparation for submission

2.1 PREFACE

This chapter addresses a key question regarding the host response against *Staphylococcus aureus* infection: why is thrombocytopenia observed during *S. aureus* bacteremia? We provide a detailed overview of our efforts to uncover the underlying mechanisms by which thrombocytopenia occurs during *S. aureus* bacteremia. Utilizing clinical data analysis, pharmacological probes, and *in vitro* and *in vivo* models of infection, we uncover fundamental biological principles that occur at the platelet-pathogen interface, expanding our definition of platelets, as not just fragmentary mediators of hemostasis, but are critical immune cells that serve to directly contribute to host protection against *S. aureus* bacteremia.

Figure 2.0 Graphical Abstract (A) *S. aureus* α-toxin drives thrombocytopenia by initiating the release of platelet-derived sialidase, an enzyme that serves to cleave sialic acids and expose galactose residues on the cell surface. Galactose is a ligand for the hepatic endocytic Ashwell-Morell receptor, and because platelets are critical immune cells that release platelet-derived microbicidal proteins to kill *S. aureus*, loss of platelets is indicative of innate-immune deficiency. (**B**) FDA-approved drugs such as P2Y12 platelet inhibitor, Ticagrelor, and sialidase inhibitor, Oseltamivir, can be repurposed to mitigate the *S. aureus*-induced platelet clearance. Supporting platelet count during infection provides immune resiliency, thus returning platelet immunity to normalcy.


A S. aureus mediates thrombocytopenia = immune deficiency
S. aureus
PMPs
Sialylated Platelet
Sialic acid
α-toxin
Sialidase
Desialylated Platelet
Galactose
Endothelium
Ashwell-Morell Receptor
Platelet Sequestration
Hepatocyte
B FDA-approved drug repositioning to support platelet count = immune competent
Oseltamivir
Ticagrelor

2.2 ABSTRACT

Staphylococcus aureus is a leading cause of bacteremia, sepsis and infective endocarditis, with management increasingly complicated by high level resistance to methicillin (MRSA) and other frontline antibiotics. Current knowledge gaps regarding the immunological and pathological complexity of *S. aureus* bloodstream infection contribute to alarmingly high morbidity and mortality rates. Recent evidence, including clinical data presented herein, indicate that circulating platelets play an underappreciated yet critical role in bloodstream clearance of the pathogen. We hypothesized that to resist bloodstream clearance, *S. aureus* may dysregulate platelet homeostasis through direct cytotoxic action and/or by accelerating the kinetics of platelet removal from the circulation. *S. aureus* expression of pore-forming α-toxin was inversely correlated to platelet counts among clinical bacteremia isolates and in a murine intravenous infection model. The toxin also induces platelet sialidase expression and shedding of surface sialic acids, which may enhance their hepatic clearance by the Ashwell-Morell receptor (AMR). Ticagrelor (Brillinta™), a commonly-prescribed P2Y12 inhibitor antiplatelet drug used to reduce risk of recurrent stroke or myocardial infarction, blocks α-toxin-mediated platelet injury as assessed by lactate dehydrogenase release and transmission electron microscopy. Ticagrelor treatment blocked *S. aureus* induced thrombocytopenia, preserves platelet immunity, and affords therapeutic benefit in a murine model of lethal *S. aureus* infection. Likewise, genetic deletion of AMR or treatment with glycoprotein inhibitors of the AMR receptor, stabilizes platelet counts and enhances resistance to *S. aureus* bloodstream infection. FDA-approved sialidase inhibitor oseltamivir (Tamiflu™) provided similar therapeutic benefit. The discovery of this novel "toxin-platelet-AMR" regulatory pathway provides a proof-of-concept for repositioning of common FDA-approved drugs ticagrelor and oseltamivir against *S. aureus* blood infection.

2.3 INTRODUCTION

Hematopoietic cells are endowed with a variety of mechanisms to control microbial pathogens, and a shortage of such critical immune cells during *S. aureus* blood-borne infection is widely regarded as a consequence of excessive cellular consumption or a dysregulation of cellular senescence and clearance. Immunological deficits increase the risk of developing life-threatening bacterial infections, thus understanding the mechanistic progression of immunodeficiency during *S. aureus* bacteremia is of crucial importance to identify therapeutic methods of mitigating this debilitating disease.

The past three decades has witnessed an emerging awareness of the involvement of platelets to host immunity against bacterial pathogens. Not only are they mechano-scavengers that bundle bacteria in the blood (1), but they actively patrol the hepatic vasculature and interact with resident Kupffer cells in the liver sinusoids where they facilitate the clearance of bacteria (2). Platelets possess intricate immunomodulatory features and are equipped with toll-like receptors, pattern-recognition receptors, as well as complement receptors that allow for rapid recognition, degranulation, and killing of pathogenic microbes (3). Following recognition of pathogen-associated molecular patterns (PAMPs), platelets secrete inflammatory cytokines to orchestrate a robust multifaceted anti-bacterial immune response (4). Environmental cues from neighboring cells via cell surface receptors, as well as production of chemokines and cytokines, program platelets to efficiently cater to the needs of the local environment. Platelets secrete thrombin-induced platelet microbicidal protein (tPMP), antimicrobial peptide human beta-defensin-1 (hBD-1) and enhances macrophage function to effectively clear *S. aureus* (5-7). Despite the well-evolved host immune response, successful bloodborne pathogens, such as *S. aureus*, express multiple virulence factors that aid in the pathogens persistence within systemic circulation. In particular, *S.*

aureus pore-forming α-toxin (*Hla*) is a secreted cytotoxin that not only causes eukaryotic cellular injury, but is known to disrupt key cellular signaling pathways that govern inflammation, cell-cell interaction, and tissue repair (8).

In this work, we demonstrate that platelets are critical immune cells that are essential for host immunity against *S. aureus* infection and provide compelling evidence that *S. aureus* α-toxin facilitates a dysregulated host immune response, wherein α-toxin induces platelet cytotoxicity, loss of platelet sialic acid composition, and aberrant platelet clearance by the hepatic Ashwell receptor, thus leading to an innate-immune deficient host. Insights into this novel "toxin-platelet-AMR" regulatory pathway provides unique avenues of drug repositioning at the host-pathogen interface.

2.4 RESULTS

Platelets are an essential component of blood immunity against Staphylococcus aureus bacteremia

The average normal human platelet count ranges from 150,000/mm3 to 450,000/mm3 blood. Thrombocytopenia is a condition classified by abnormally low number of circulating platelets. Interestingly, thrombocytopenia is also a profound independent risk factor for mortality in patients suffering from *Staphylococcus aureus* bacteremia (9). In our own clinical analysis, where 49 consecutive bacteremia patients with blood cultures growing MSSA or MRSA were prospectively identified from July 2010 through August 2012 at the University of Wisconsin Hospital (a 493-bed academic medical center in Madison), we observed a strong association with thrombocytopenia (platelet count <100,000/mm^3) and low white blood cell count, compared to other clinical lab parameters (**Fig. 1A**). Surprisingly, the loss of circulating platelets (PLTS < 100,000/mm^3), and not circulating white blood cells (WBC <20,000/mm^3, WBC <10,000/mm^3,

WBC <5000/mm^3), is predictive of mortality in patients with *Staphylococcus aureus* bacteremia. (**Fig. 1B**). These clinical correlations suggest that circulating platelets, and not white blood cells, plays the dominant role in innate-immune clearance of *S. aureus* during infection. To test this, we first determined whether clinical presentations of thrombocytopenia can be recapitulated in our *in vivo* model of *S. aureus* bacteremia. Indeed, mice intravenously infected with MRSA presented with a significant drop in platelet count compared to that of non-infected mice (**Fig. 1C**). To determine if thrombocytopenia is indeed indicative of immune deficiency, we examined the bactericidal capabilities of platelet-depleted blood. Mice depleted of platelets not only presented with deficient blood microbicidal activity against *S. aureus ex vivo* (**Fig. 1D**), but are also incapable of clearing disseminated bacteria *in vivo* (**Fig. 1E**). This surprising finding led us to postulate that thrombocytopenia could potentially be mediated by a pathogen-specific virulence factor that facilitates clearance of platelets to establish an immune deficient host. Upon further examination using isolated human platelets, we demonstrate that *S. aureus* reduces platelet viability (**Fig. 1F**).

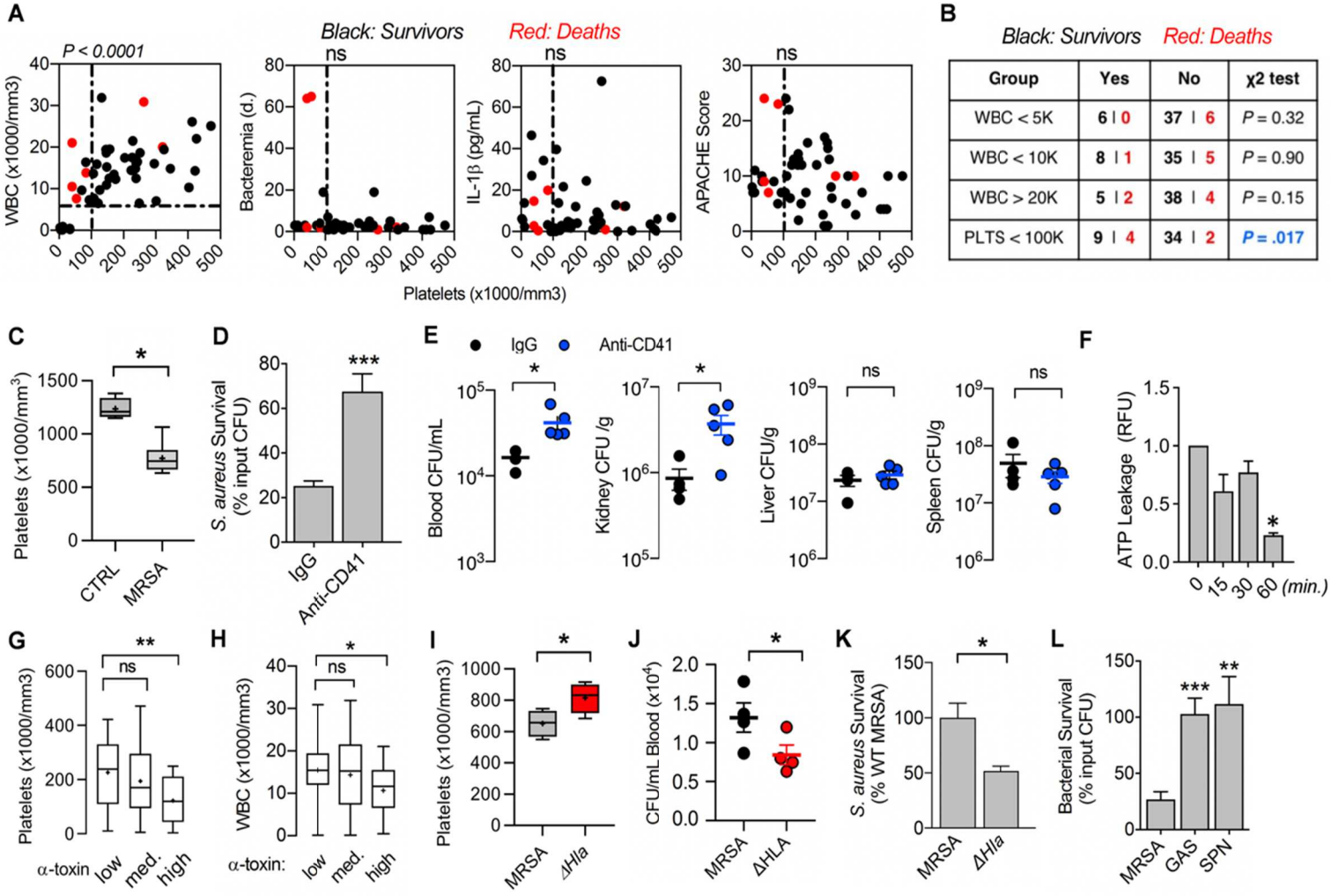
S. aureus α-toxin (Hla) drives aberrant platelet reduction to evade platelet-mediated microbicidal activity

S. aureus α-toxin (*Hla*) is a pore-forming calcium-mediated cytotoxin that is widely regarded to induce platelet cytotoxicity (10) and modulate platelet tissue repair function (11) upon binding its proteinaceous receptor A-disintegrin metalloprotease-10 (ADAM-10) (12). Thus, we identified *S. aureus* α-toxin as the virulence factor responsible for the reduction in platelet count observed in our bacteremia model. We next sought to analyzed the 49 clinical isolates for α-toxin expression by western immunoblot (**Fig. S1**) to determine if we could observe any association between levels of toxin expression and patient platelet count. A significant association between

groups (low, med., high) revealed that isolates with high α-toxin expression, is significantly associated with lower platelet counts (**Fig. 1G**). A significant association was additionally observed between high levels of α-toxin expression and a reduction in white blood cell count (WBC) (**Fig. 1H**), although only a decrease in circulating platelets and not circulating WBC significantly correlates with mortality (**Fig. 1C**).

To examine the action of α-toxin in our *in vivo* models of infection, we performed a targeted mutagenesis of α-toxin (*Hla*) by precise marker-less allelic replacement of USA300 MRSA (TCH1516Δ*Hla*) (Fig. **S1**) and intravenously infected mice with wild-type MRSA or its isogenic Δ*Hla* mutant. Interestingly, an inverse relationship was observed between platelet count and bacterial burden. Mice infected with wild-type MRSA had lower platelet count compared to its isogenic Δ*Hla* mutant (**Fig. 1I**), and inversely presented with higher bacterial burden in the blood (**Fig. 1J**). Given that platelets are myeloid-derived granulocytes, we examined the susceptibility of the two strains and discovered that Δ*Hla* is more susceptible to isolated human platelet killing than the wild-type MRSA strain (**Fig. 1K**). Furthermore, this is observed to be a pathogen-specific phenotype, as human platelets do not kill other leading gram-positive pathogens, such as *group A Streptococcus* and *Streptococcus pneumoniae* (**Fig. 1J**). Cumulatively, the above data provided us with the rationalization that there is an intricate balance of power between circulating platelets attempting to eradicate *S. aureus*, and the bacterium attempting to evade platelet antimicrobial activity by deploying α-toxin as a means to dysregulate platelet immunity. This basis served as impetus for the identification of FDA-approved therapeutics that could tip the intricate balance of power in favor of the host.

Figure 2.1. Platelets are an essential component of blood immunity against *Staphylococcus aureus* bacteremia and α-toxin (Δ*Hla*) dysregulates platelet-mediated bacterial clearance (**A**) Correlation of circulating platelet counts with leukocyte counts and duration of bacteremia in 49 consecutive patients with *S. aureus* bacteremia from a tertiary medical center; Spearman's rank correlation coefficient compared variables. (**B**) Mortality in this patient cohort associated with different leukocyte and platelet count cutoffs; Chi-square without Yates correction, $P < 0.05$ considered significant. (**C**) Reduction in platelet count 2 h post intravenous infection of mice with MRSA ($n = 8$) compared to non-infected littermate control ($n = 4$). Experiment reproduced twice and data pooled; data represented as mean ± SEM. (**D**) *Ex vivo* killing of MRSA (2 h co-incubation) by blood collected from mice 16 h after treatment with anti-CD41 antibody ($n = 9$) or IgG control ($n = 12$). (**E**) Mice treated with platelet-depleting anti-CD41 antibody ($n = 5$) or IgG control ($n = 4$) for 16 h prior to intravenous MRSA infection. Organs harvested and bacterial colony forming units (CFU) enumerated 2 h post-infection in triplicate for each sample. (**F**) Washed human platelets were exposed to MRSA at an MOI of 0.1, and cell viability assessed by a measurement of metabolically active ATP (**G**) Assessment of α-toxin production by the infecting isolate in 49 consecutive patients with *S. aureus* bacteremia and its association with patient platelet counts and (**H**) white blood cell counts. (**I**) Wild-type MRSA ($n = 4$) or isogenic Δ*Hla* ($n = 4$) intravenously challenged outbred CD-1 mice. Blood was harvested by cardiac puncture and CBC obtained 4 hours post-infection. (**J**) Wild-type MRSA ($n = 4$) or isogenic Δ*Hla* ($n = 4$) intravenously challenged outbred CD-1 mice. Blood was harvested by cardiac puncture and enumeration of colony forming units (CFU) 4 hours post-intravenous infection. (**K**) *Ex vivo* killing of methicillin-resistant *S. aureus* (MRSA) by freshly isolated human platelets (2 h co-incubation) compared to human Gram-positive bacterial pathogens group A *Streptococcus* (GAS) and *Streptococcus pneumoniae* (SPN) ($n = 9$). (**L**) Human whole blood killing of MRSA over 2 h. Represented by a reduction in colony-forming units (CFU) compared to wild-type (WT) MRSA ($n = 5$). For *ex vivo* platelet assays (**F**) and (**K**), experiments performed in triplicate and repeated three independent times. Where applicable, results are represented as mean ± SEM and statistical significance was determined by unpaired two-tailed Student's T-test. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. WBC, white blood cell; d., days; APACHE, Acute Physiology and Chronic Health Evaluation; ns, not significant.


A
Black: Survivors
Red: Deaths
P < 0.0001
ns
ns
ns
WBC (x1000/mm3)
Bacteremia (d.)
IL-1β (pg/mL)
APACHE Score
Platelets (x1000/mm3)
B
Black: Survivors Red: Deaths
Group | Yes | No | χ2 test
WBC < 5K | 6 | 0 | 37 | 6 | P = 0.32
WBC < 10K | 8 | 1 | 35 | 5 | P = 0.90
WBC > 20K | 5 | 2 | 38 | 4 | P = 0.15
PLTS < 100K | 9 | 4 | 34 | 2 | P = .017
C
Platelets (x1000/mm3)
CTRL
MRSA
D
S. aureus Survival (% input CFU)
IgG
Anti-CD41
E
IgG
Anti-CD41
Blood CFU/mL
Kidney CFU /g
Liver CFU/g
Spleen CFU/g
ns
F
ATP Leakage (RFU)
0 15 30 60 (min.)
G
Platelets (x1000/mm3)
α-toxin low med. high
H
WBC (x1000/mm3)
α-toxin: low med. high
I
Platelets (x1000/mm3)
MRSA
ΔHla
J
CFU/mL Blood (x10^4)
MRSA
ΔHLA
K
S. aureus Survival (% WT MRSA)
MRSA
ΔHla
L
Bacterial Survival (% input CFU)
MRSA
GAS
SPN

FDA-approved P2Y12 inhibitor Ticagrelor subverts α-toxin-mediated platelet cytotoxicity and protects against bacteremia-associated mortality

The toxin-evoked reduction in platelet viability (**Fig. 1J**) provided us with the rationale that platelet inhibitors could provide cellular resiliency against α-toxin activity. Multiple clinical reports have demonstrated reduced mortality rates and length of intensive care unit (ICU) stay in septic patients taking antiplatelet drugs, such as low-dose acetylsalicylic acid (ASA) or Clopidogrel (Plavix^{TM}), which are widely prescribed medications for patients at risk of developing proatherogenic disorders, such as stroke or myocardial infarction (**Table 2.1**).

Table 2.1. Investigative clinical outcomes of anti-platelet therapy in bacteremia and bacteremia-associated complications

Antiplatelet Drug	Study	Investigation	Outcome	Limitations	Citations
Low-dose ASA	A Propensity Score–Matched Cohort Study	30-day all cause mortality analysis of *S. aureus* and *E.coli* bacteremia	Reduced Mortality	Analysis not designed to address which pathways are influenced by and mediate the beneficial effects of low-dose ASA	Oshtoff et al., 2016
Plavix; Low-dose ASA	Retrospective	Association of prehospital APT and mortality in critically ill patients	Reduced Mortality	Including non-sepsis critical patient	Winning et al., 2009
Plavix	Retrospective	Need of treatment on an ICU and the length of stay in the hospital.	Reduced need of treatment and ICU stay	No reliable information on confounding factors: pneumococcal/influenza vaccinations	Winning et al., 2010
Low-dose ASA	Retrospective	Association of ASA administration at time of SIRS/sepsis and mortality	Reduced Mortality	Treatment bias of ASA at time of enrollment	Eisen et al., 2008
ASA	Retrospective	Risk of major embolism	Reduced Mortality	Chronic antiplatelet therapy was not associated with a significantly lower risk of major embolism	Pepin et al., 2009

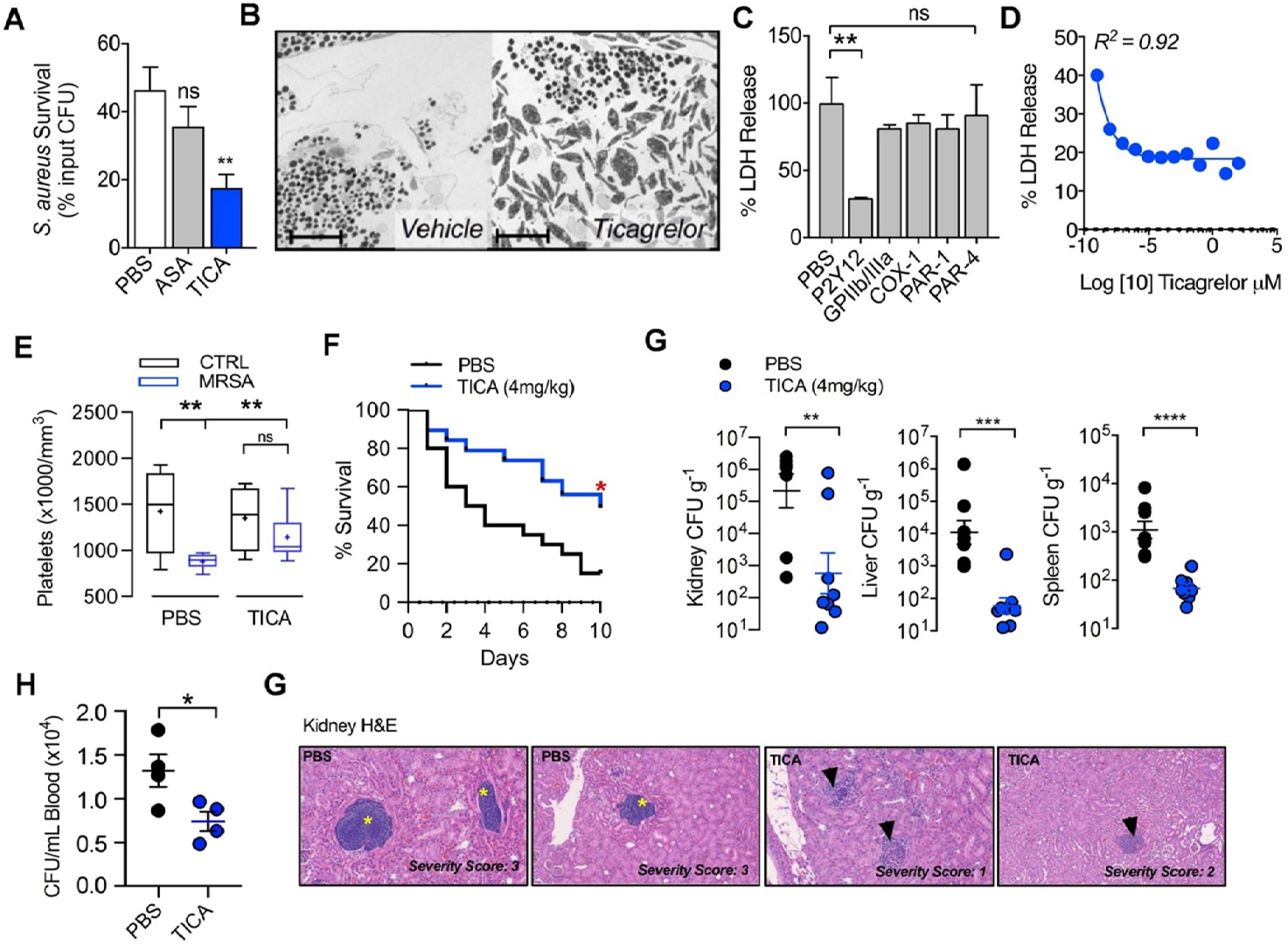
ASA: Acetylsalicylic acid | *S. aureus*: *Staphylococcus aureus* | *E. coli*: *Escherichia coli* | *ICU*: Intensive Care Unit | *SIRS*: systemic inflammatory response syndrome

Thus, we hypothesized that inhibition of platelet cytotoxicity could provide immune resiliency against *S. aureus* α-toxin. Human platelets treated with or without ASA or a metabolically active P2Y12 inhibitor, Ticagrelor (Brilinta^{TM}), revealed that Ticagrelor (TICA) significantly increased platelet microbicidal activity against MRSA (**Fig. 2A**). A descriptive analysis on human platelets treated with or without TICA by transmission electron microscopy (TEM) revealed that platelets maintained their cellular integrity when exposed to the bacterium (**Fig. 2B**). Various classes of platelet inhibitors were then used to analyze their ability to inhibit *S. aureus* α-toxin cytotoxicity. Interestingly, P2Y12 inhibition by ticagrelor, significantly reduced α-toxin-induced ADAM10- activity (**Fig. S2**), mobilization of intracellular Ca^{+2} (**Fig. S2**), thus inhibiting platelet cytotoxicity when compared to other classes of antiplatelet drugs (**Fig. 2C**) in a dose-dependent manner (**Fig. 2D**).

Inhibition of toxin activity suggests that ticagrelor should mitigate the toxin-evoked platelet reduction, as previously observed *in vivo*. (**Fig. 1I).** Indeed, mice treated with ticagrelor presented with an increased number of circulating platelets compared to the non-treated littermate control during infection (**Fig. 2E**), thus improving survival in a 10-day mortality study (**Fig. 2F**), and significantly reducing the bacterial burden in the kidneys, liver, spleen (**Fig. 2G**), and blood (**Fig. 2H**). The heart, kidney, liver, and spleen in each group was examined by a board-certified veterinarian pathologist. The most striking treatment-related differences were observed in the kidneys (**Fig. 2G**) and the heart (**Fig. S2**). A 4-10-fold decrease in the number of bacterial colonies was identified in the renal glomeruli, renal tubules, and within blood vessels of the Ticagrelor-treated mice compared to PBS control, corroborating the bacterial CFU quantification data. Additionally, the bacterial colonies in the vehicle-control group were generally larger and more

densely packed with bacteria, whereas the Ticagrelor-treated mice were frequently disrupted by immune infiltrate, a characteristic rarely observed in the PBS control.

Figure 2.2. Antiplatelet P2Y12 inhibitor Ticagrelor protects against *S. aureus* bacteremia by blunting toxin cytotoxic action. (**A**) Effect of 10 μM Aspirin and 10 μM TICA (15 min pretreatment *ex vivo*) on human platelet killing of MRSA for 2 h (n = 9). Experiments were performed in triplicate and repeated three independent times. (**B**) Representative transmission electron microscopy image of platelets pre-treated with or without 10 μM TICA, and exposed to MRSA at an MOI: 0.1 for 2 hours. (**C**) P2Y12 inhibitor (TICA) pretreatment blocks human platelet cytotoxicity by 5 μg/ml purified α-toxin as measured by LDH release (n = 3) in a (**D**) dose-dependent manner. Inhibitors: P2Y12 (Ticagrelor), GPIIb/IIIa (Eptifibatide), COX-1 (SC560), PAR-1 (Vorapaxar), and PAR-4 (ML-354). (**E**) Blood harvested by cardiac puncture from outbred CD-1 mice treated with 10 μM Ticagrelor (n = 9) or PBS (n = 9) control and intravenously infected with MRSA. Platelets enumerated 4-hour post infection. (**F**) Mortality curves of outbred CD-1 mice pretreated with vehicle (PBS) or 4 mg/kg Ticagrelor beginning 24 h prior to intravenous MRSA infection and q 12 h thereafter over a 10-day observation period (n = 20). Independent experiments repeated twice and data pooled. (**G**) Enumeration of bacterial colony forming unit (CFU) burden at 72 h in organs of mice pretreated with vehicle (PBS) or 4 mg/kg Ticagrelor 12 h prior to intravenous MRSA and q 12 h thereafter; (n= 8). (**H**) Blood enumeration of bacterial colony forming unit (CFU) burden at 4 h in mice pretreated with vehicle (PBS) or 4 mg/kg Ticagrelor 12 h prior to intravenous MRSA. (**I**) Hematoxylin and eosin stain (H&E) of representative histological kidney sections from mice pre-treated with PBS vehicle or 4 mg/kg Ticagrelor 12h prior to MRSA infection and q 12 h thereafter for 72 hours; (n = 8). Yellow stars denote formation of dense bacterial abscesses and black arrows represent immune infiltrate. All histological sections are representative photos of at least 6 samples per two independent experiments. Where applicable, results are represented as mean ± SEM and statistical significance was determined by unpaired two-tailed Student's T-test. For survival curves, statistical significance determined by Log-rank Mantel-Cox test; $*P < 0.05$. For floating bar graphs, + denotes the mean, whiskers represent min. to max, and floating box represents 25th to 75th percentile. Unless otherwise stated, $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.


A
S. aureus Survival (% input CFU)
ns
**
PBS
ASA
TICA
B
Vehicle
Ticagrelor
C
% LDH Release
ns
**
PBS
P2Y12
GPIIb/IIIa
COX-1
PAR-1
PAR-4
D
R² = 0.92
% LDH Release
Log [10] Ticagrelor μM
E
CTRL
MRSA
Platelets (x1000/mm³)
PBS
TICA
F
PBS
TICA (4mg/kg)
% Survival
Days
G
PBS
TICA (4mg/kg)
Kidney CFU g⁻¹
Liver CFU g⁻¹
Spleen CFU g⁻¹
H
CFU/mL Blood (x10⁴)
PBS
TICA
G
Kidney H&E
PBS
Severity Score: 3
PBS
Severity Score: 3
TICA
Severity Score: 1
TICA
Severity Score: 2

S. aureus α-toxin activates endogenous platelet sialidase activity

Platelet senescence and clearance is tightly regulated by multiple mechanisms, however the highly conserved hepatic transmembrane heterodimeric glycoprotein Ashwell-Morell receptor (AMR) has been discovered to be directly responsible for clearing platelets with reduced α2,3-linked sialic acids (13). Interestingly, it was reported that during *S. pneumoniae* sepsis, the AMR mitigates the bacterial neuraminidase shedding of platelet sialic acid and subsequent coagulopathy, thus improving infection outcome (14). However, unlike *S. pneumoniae*, *S. aureus* lacks a bacterial neuraminidase (sialidase) (**Fig 3A**), thus suggesting that changes in platelet glycan composition could be by stimulation of endogenous platelet sialidase. Though sialic acid loss is attributed to a family-class of neuraminidase enzymes, platelets in particular express Neuminidase-1 (15, 16) and we postulated that α-toxin could stimulate the release of such enzymes. Certainly, α-toxin not only induced platelet cytotoxicity (**Fig. 2B and C**), but also stimulates endogenous platelet sialidase activity (**Fig. 3B and C**). The ability for ticagrelor to prevent α-toxin -induced cellular damage *in vitro*, indicated that ticagrelor could also prevent the toxin-evoked activation of platelet sialidase. To test this, isolated human platelets were pre-treated with ticagrelor and exposed to recombinant α-toxin. We observed significant inhibition of platelet sialidase activity (**Fig. 3D**), which provided us with the rationale that inhibition of platelet sialidase, upstream of the hepatic platelet-AMR interface, is a potential method for mitigating the aberrant reduction in platelet count during *S. aureus* bacteremia.

FDA-approved neuraminidase inhibitor oseltamivir subverts α-toxin-mediated platelet desialylation and protects against S. aureus bacteremia

FDA-approved oseltamivir (Tamiflu^{TM}) was initially designed as a viral-specific neuraminidase inhibitor, however both experimental evidence (17) and clinical evidence (18-20), would suggest that oseltamivir is a non-selective neuraminidase inhibitor. We examined if oseltamivir can be repositioned as a potential treatment opportunity during *S. aureus* bacteremia and utilized a known human NEU1-selective neuraminidase inhibitor, C9-butyl-amide-2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) as a comparable control. As predicted, mice treated with either oseltamivir or DANA significantly improved survival outcomes (**Fig. 3E**) and a significant increase in platelet count was additionally observed in the oseltamivir treated group compared to non-treated group (**Fig. 3F**). We next sought to determine if oseltamivir inhibits human sialidase activity *in vivo* and utilized *Asgr2*$^{-/-}$ mice to control for changes in circulating platelets observed during infection. Indeed, oseltamivir inhibits both platelet desialylation *in vivo* (**Fig. 3G**) and platelet sialidase activity *ex vivo* (**Fig. 3H**). Collectively, the above data demonstrates that the toxin-evoked platelet desialylation, coupled with platelet cytotoxicity, ultimately drives the aberrant reduction in platelet count (**Fig. 1C**). In support of this, the inhibition of α-toxin activity (**Fig 2C and D**) or the subsequent activation of platelet sialidase (**Fig. 3D and H**), improves survival outcome during *S. aureus* bacteremia (**Fig. 2F and 3E**). To further prove that preventing excessive platelet clearance is indeed therapeutic during *S. aureus* bacteremia, we investigated potential inhibitors of the hepatic AMR, an event that occurs downstream of the platelet-pathogen interface.

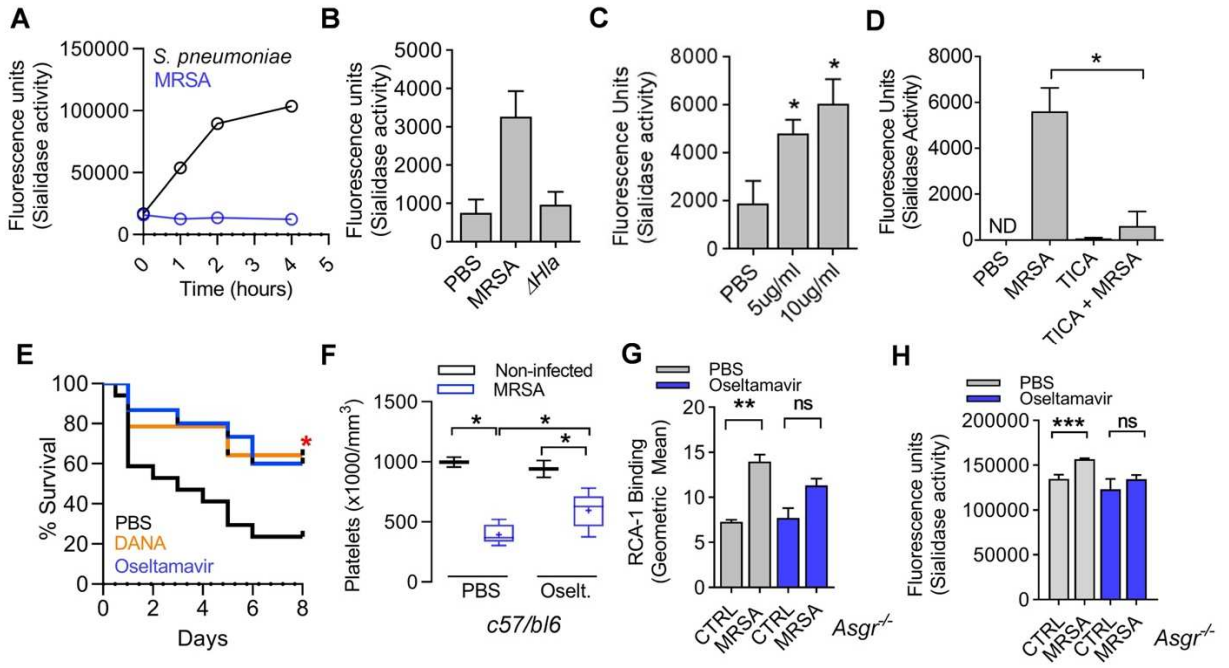


Figure 2.3. *S. aureus* α-toxin rapidly activates endogenous platelet sialidase activity and oseltamivir subverts platelet desialylation. (**A**) MRSA and *S. pneumoniae* sialidase activity assessed for over 4 hours. (**B**) Sialidase activity examined on washed human platelets exposed to wild-type MRSA or its isogenic Δ*Hla* for 1 hour or (**C**) sialidase activity examined on washed human platelets exposed to 5 µg/mL and 10 µg /mL recombinant α-toxin for 30 minutes. (**D**) Sialidase assay performed on washed human platelets treated with or without 10 µM Ticagrelor and exposed to wild-type MRSA for 1 hour. (**E**) 8-day mortality study conducted on C57/Bl6 mice treated with DANA (n = 16), Oseltamivir (n = 16), or PBS control (n = 16). (**F**) C57/Bl6 mice treated with Oseltamivir (n = 6) or PBS control (n = 5) and infected with wild-type MRSA by intraperitoneal injection. Blood harvested 24 hours after infection and platelet counts collected. (**G**) Platelets isolated from Asgr2-/- mice treated with or without Oseltamivir and infected with MRSA were assessed for RCA-1 lectin binding and (**H**) sialidase activity. Where applicable, statistical significance was determined by unpaired Two-tailed Student's T-test. For floating bar graphs, + denotes the mean, whiskers represent min. to max, and floating box represents 25th to 75th percentile. Unless otherwise stated, *$P < 0.05$, **$P < 0.005$. PBS, phosphate buffered saline; ns, not significant. ND, not detectable

Inhibition of the hepatic Ashwell-Morrell receptor (AMR) mitigates S. aureus α-toxin-induced thrombocytopenia

An interesting study demonstrated that the hepatic AMR mitigates lethality during *S. pneumoniae* sepsis and pre-activation of the AMR induces host defense against this infection (21). However, based on the cumulative data demonstrated above, we reasoned that mice either lacking functional AMR, or treated with an AMR inhibitor, should mitigate thrombocytopenia during *S. aureus* bacteremia and ultimately improve survival outcome. Indeed, AMR null mice (*Asgr2*$^{-/-}$) (**Fig. 4A**) and c57/bl6 mice treated with a competitive glycoprotein inhibitor of the hepatic AMR, asialofetuin (**Fig. 4B**), improves survival outcome in a 10-day observation study by supporting platelet count during infection (**Fig. 4C and D**), and therefore reducing bacterial burden in the kidneys, liver, and spleen of mice (**Fig. 4E**). To prove that α-toxin is indeed driving excessive platelet clearance, we infected wild-type c57/bl6 and *Asgr2*$^{-/-}$ mice with either MRSA or its isogenic Δ*Hla* mutant and obtained a platelet count (**Fig. 4F**). Conclusively, no reduction in platelet count was observed in mice lacking the hepatic AMR, thus demonstrating that the endocytic receptor clears toxin-evoked desialylated platelets and drives thrombocytopenia. This pathogen-specific modulation of platelet-AMR pathway during infection led us to rationalize that *S. pneumoniae* must benefit from secretion of proatherogenic and antimicrobial molecules, thereby progressing coagulopathy. Given its resistant nature to platelet-derived antimicrobial killing (**Fig. S3**), the host attempts to mitigate this disease by AMR-mediated clearance of senescent platelets. *S. aureus* however, seems to benefit from removing platelets from circulation due its susceptibility to platelet-derived antimicrobial killing (**Fig. S3**).

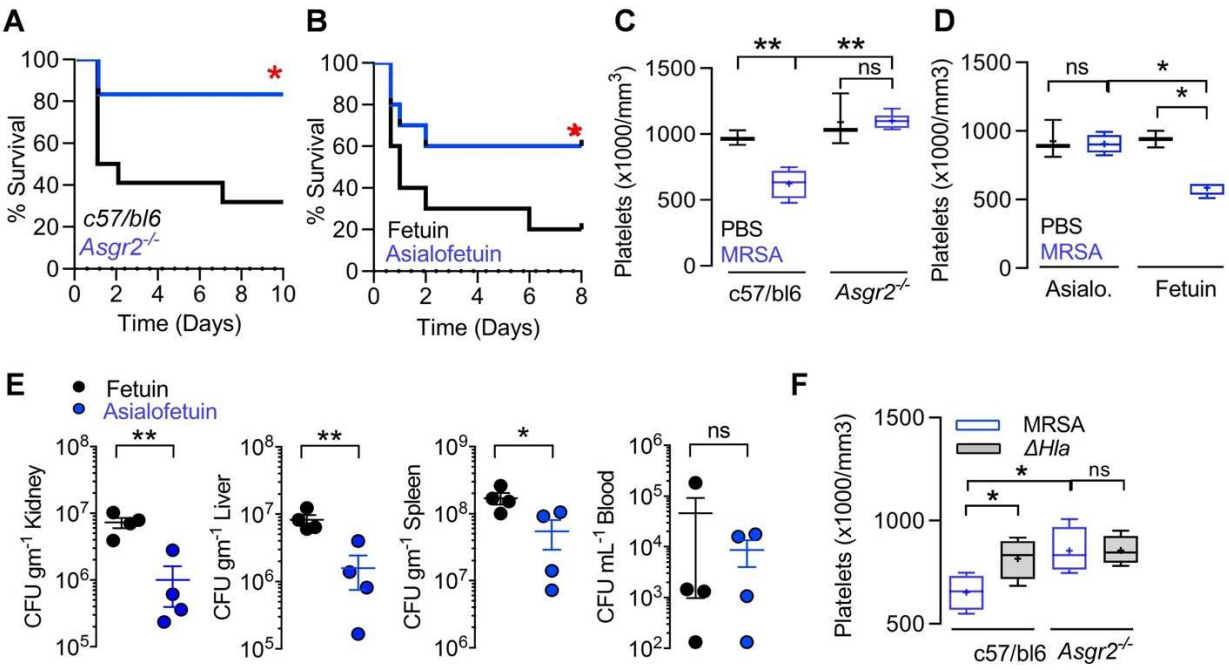


Figure 2.4. Inhibition of the hepatic Ashwell-Morrell receptor (AMR) mitigates toxin-induced thrombocytopenia (**A**) 10-day mortality study with C57/Bl6 (n = 22) and Asgr2$^{-/-}$ mice (n = 16) challenged by intraperitoneal injection with MRSA. Study performed two independent times and data pooled. (**B**) 8-day mortality study with C57/Bl6 treated with fetuin (n = 10) or asialofetuin (n = 10) and challenged by intraperitoneal injection with MRSA. (**C**) C57/Bl6 (n = 4) and *Asgr2*$^{-/-}$ (n = 6) mice challenged by intraperitoneal injection with MRSA, blood harvested by cardiac puncture, and platelet count enumerated. (**D**) C57/Bl6 mice treated with asialofetuin (n = 4) or fetuin (n = 4) and challenged by intraperitoneal injection with MRSA, platelet count enumerated, and (**E**) kidneys, liver, spleen, and blood harvested 24 hours post infection for bacterial colony forming unit enumeration. (**F**) C57/Bl6 and *Asgr2*$^{-/-}$ mice challenged with wild-type MRSA its isogenic Δ*Hla*. 4 hours post-infection, blood was harvested by cardiac puncture for enumeration of platelet count. All data represented as mean ± SEM. Where applicable, statistical significance was determined by unpaired Two-tailed Student's T-test. For survival curves, statistical significance determined by Log-rank Mantel-Cox test; *P < 0.05. For floating bar graphs, + denotes the mean, whiskers represent min. to max, and floating box represents 25th to 75th percentile. Unless otherwise stated, *P < 0.05, **P < 0.005.; PBS, phosphate buffered saline; ns, not significant.

2.5 DISCUSSION

Staphylococcus aureus remains the leading cause of bacteremia with higher morbidity and mortality rates compared to bacteremia caused by any other pathogen. Treatment against this disease is incredibly complex, as the pathogenesis of *S. aureus* bacteremia includes multi-factorial dynamics that aide in the pathogens persistence against host immunity and standard antibiotic treatment. Life-threatening complications of *S. aureus* bacteremia, such as metastatic infections, infective endocarditis, and disseminated intravascular coagulation, continue to vex physicians and drive worsened patient outcomes. Thrombocytopenia, platelet count <150,000/mm^3 blood, is a common phenotype observed during bacteremia and is the most predictive independent risk factor for bacteremia-associated mortality, especially in cases of neonatal sepsis and critically ill septic patients in the intensive care unit (22, 23). The underlying cause of thrombocytopenia is multifactorial and ambiguous, however our mechanistic analysis of platelet-mediated defense, provides a pathophysiology of thrombocytopenia observed during *S. aureus* bacteremia. We demonstrate that in order for *S. aureus* to evade platelet microbicidal activity, the pathogen deploys α-toxin to induce platelet cytotoxicity, stimulate the release of endogenous platelet sialidase, thereby dysregulating the platelet clearance mechanism by the hepatic Ashwell-Morell receptor. Pharmacologically targeting multiple levels of this "toxin-platelet-AMR" pathway provides therapeutic evidence by which we can mitigate the progression of this immunocompromised state and protect against *S. aureus* bacteremia.

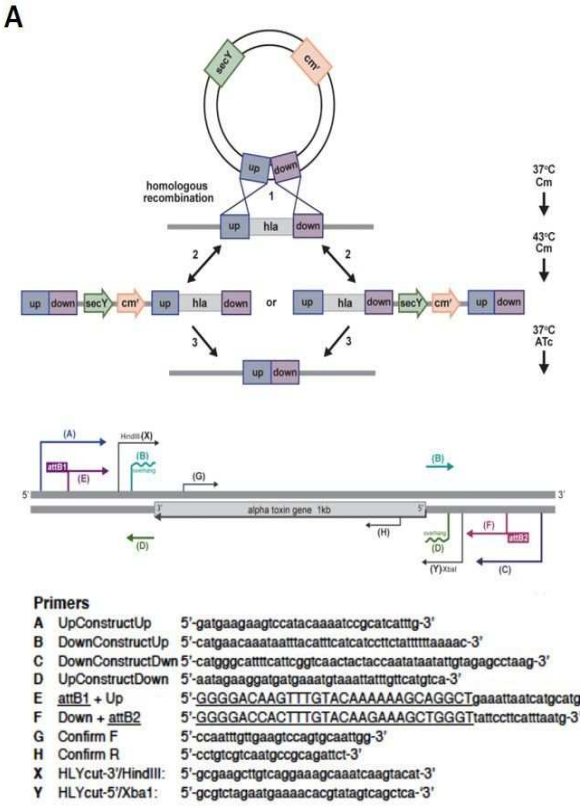
There are a number of limitations to our current study. Our *in vivo* models only describe the ability for ticagrelor or oseltamivir to inhibit the toxin-platelet-AMR pathway to thrombocytopenia, it does not, however, address its potential anti-inflammatory effects, or whether treatment could recover platelet count after diagnosis of thrombocytopenia. Furthermore, the

underlying cause thrombocytopenia is a pathogen-specific observation, thus complicating pharmacologic intervention given that the identification of the infecting isolate requires time. It is also paradoxically reported that P_2Y12 inhibition inhibits platelet anti-staphylococcal activity. Therefore, suggesting that P_2Y12 receptor stimulation is necessary for platelet-mediated *S. aureus* killing (24). However, the release of PMPs is not P_2Y12 receptor-specific. For example, secretion of anti-staphylococcal molecules can occur by thrombin-mediated enzymatic activation of cell-surface protease-activating receptor 1 (PAR-1) (6). Further investigation to explain the discrepancy in our results is warranted. Additionally, although oseltamivir's mechanism of action is by inhibition of viral neuraminidase, its activity against mammalian neuraminidase is still a subject of discussion. A study demonstrated that oseltamivir had limited inhibitory properties against human neuraminidases (25), however clinical observations demonstrate oseltamivir's ability to recover platelet count in patients with idiopathic thrombocytopenic purpura (20, 26), and an influenza-independent increase in platelet count in immune thrombocytopenia (27). Given that there are no approved inhibitors of the hepatic AMR, it would be of crucial interest to determine if transfusion of platelets pre-treated with oseltamivir or ticagrelor, would bypass hepatic sequestration, improve the longevity of circulating platelets, thereby providing immune resiliency and reducing mortality in thrombocytopenic *S. aureus* infected patients.

Our work contributes to the greater understanding of the complexity that revolves around the pathophysiology of infection-induced thrombocytopenia. Currently, thrombocytopenia is quickly regarded as a benign clinical consequence of bone marrow suppression and unless classical symptoms of platelet disorders are presented, such as excessive bleeding or bruising, medical intervention is typically disregarded over fear of initiating a cascade of coagulopathy. However, the human body produces approximately 10 billion platelets per day, and given our findings, we

propose that the standardized symptoms of thrombocytopenia should not be confined to their compartmentalized hemostatic properties but should include a state of immune deficiency. Conclusively, we provide avenues for therapeutic drug repositioning to mitigate this "toxin-platelet-AMR" dysregulation of host innate-immunity, in hopes address a pressing unmet clinical need.

2.6 SUPPLEMENTAL



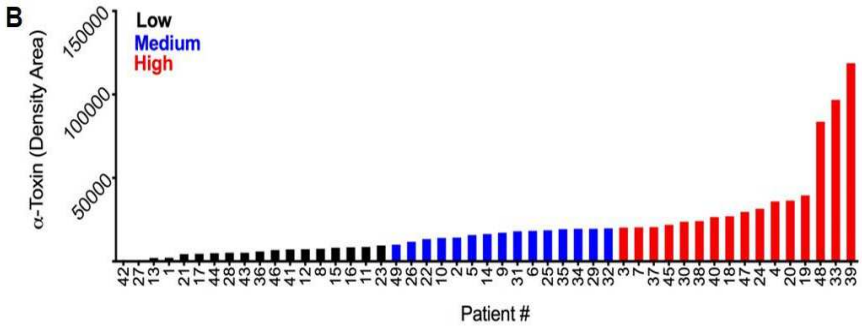
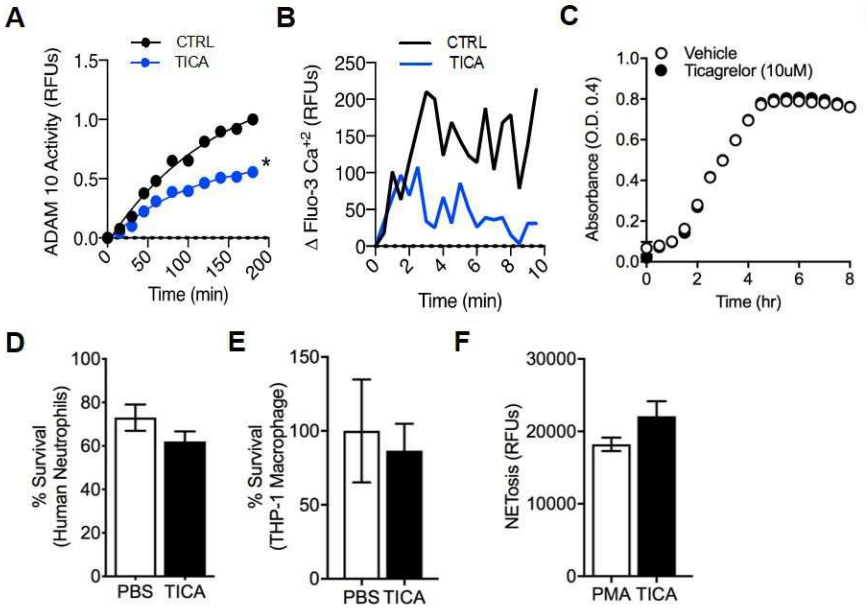


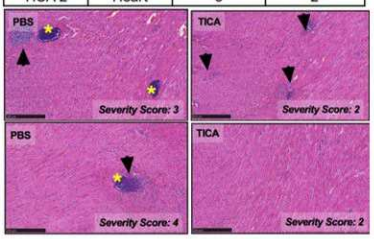
Figure S2.1 Primer design, generation of isogenic Δ*Hla*, and grouping of α-toxin expression from patient isolates (**A**) Targeted mutagenesis of *Hla* was conducted by precise, markerless allelic replacement of USA300 TCH1516 *Hla* by PCR-based methods (**B**) Western immunoblot band analysis on 49 patient clinical isolates grouped according to density area. Groups: low <10,000 (*black*); med. <20,000 (*blue*); high >20,000 (*red*). Densitometry performed using ImageJ software.

Figure S2.2 Evaluating Ticagrelor inhibition of α-toxin ADAM-10 activity, off-target effects, and histopathological scoring (**A**) TICA treatment of human platelets reduces proteolytic cleavage of ADAM10-specific fluorogenic substrate. Data is representative of three independent experiments and statistical significance determined by least squares ordinary fit, $*P < 0.5$. (**B**) Measurement of intracellular calcium in human platelets loaded with 2 μM Fluo-3 dye and stimulated with 5ug/mL recombinant α-toxin; calcium influx is measured every 30 seconds by fluorescence and normalized to baseline (non-stimulated platelet control). Data is representative of three independent experiments. (**C**) Growth curve analysis of pre-treated MRSA with TICA 10uM. Absorbance measured at an optical density (O.D.) of 0.4 measured over the course of 8 hours. (**D**) Quantification of MRSA colony-forming units (CFUs) in a purified human neutrophil killing assay. Human neutrophils and (**E**) THP-1-derived macrophages treated with vehicle control or 10 μM Ticagrelor for 30 minutes prior to 1 h exposure to MRSA. (**F**) Isolated human neutrophils pre-treated with vehicle or 10 μM Ticagrelor and subsequently exposed to PMA for quantification of neutrophil extracellular trap (NET) production. (**G**) Hematoxylin and eosin stain (H&E) of representative histological heart, liver, and spleen sections from mice pre-treated with vehicle or 4 mg/kg Ticagrelor 12h prior to MRSA infection and q 12 h thereafter for 72 hours; ($n = 2$). Yellow stars denote dense bacterial colonies. In the Ticagrelor-treated mice, the bacterial colonies were smaller, less frequent, and often surrounded by an inflammatory infiltrate (black arrow) comprising neutrophils with fewer macrophages. Where applicable, all data represented as mean ± SEM and are representative of at least three independent experiments. Statistical significance determined by unpaired Two-tailed Student's t test. $*P < 0.05$

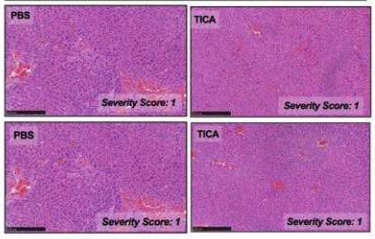


G

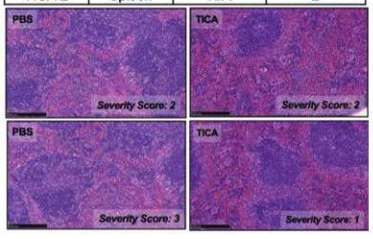
Mouse	Organ	Colony Count	Severity Score
PBS 1	Heart	35	3
PBS 2	Heart	32	4
TICA 1	Heart	6	2
TICA 2	Heart	3	2



Mouse	Organ	Colony Count	Severity Score
PBS 1	Liver	N/A	1
PBS 2	Liver	N/A	1
TICA 1	Liver	N/A	1
TICA 2	Liver	N/A	1



Mouse	Organ	Colony Count	Severity Score
PBS 1	Spleen	N/A	2
PBS 2	Spleen	N/A	3
TICA 1	Spleen	N/A	2
TICA 2	Spleen	N/A	2



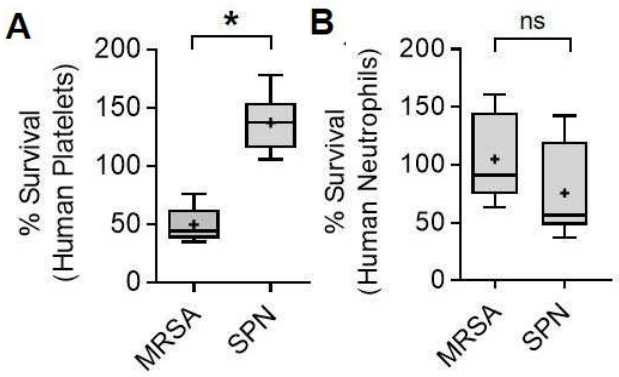


Figure S2.3 Evaluating platelet vs. neutrophil microbicidal specificity against *S. aureus* and *S. pneumoniae.* (**A**) Washed isolated human platelets exposed to MRSA or SPN at a MOI of 0.01 for 2 hours. Samples were sonicated, serial diluted, and plated onto THA plates for enumeration of bacterial colony forming unit (CFU) (**B**) Washed isolated human neutrophils exposed to MRSA or SPN at a MOI of 0.01 for 2 hours. Samples were sonicated, serial diluted, and plated onto THA plates for enumeration of bacterial colony forming unit (CFU). All data represented as mean ± SEM and are representative of at least 3 independent experiments. Statistical significance determined by unpaired Two-tailed Student's t test. **P* < 0.05

2.7 MATERIALS AND METHODS

Ethics Statement

Animal and human studies were conducted in accord with protocols approved by the University of California, San Diego (UCSD) Institutional Animal Care and Use Committee and Institutional Review Board.

Patient Isolates

All laboratory tests were performed by investigators blinded to patient information. Consecutive patients from previously published study (28) with blood cultures growing methicillin susceptible (MSSA) or methicillin-resistant *S. aureus* (MRSA) from April 2009 through March 2010 at the University of Wisconsin Hospital (a 493-bed academic medical center in Madison, WI) were analyzed for α-toxin expression by western immunoblot and densitometry band analysis by Image J. Levels of α-toxin expression was grouped in the following order: low: >10,000; medium: 10,000 – 20,000; high: >20,000. Patient demographics, blood work, and infection source were collected at time of administration. As previously described, bacteremia isolate source was classified into 3 groups: (1) non-catheter primary endovascular, encompassing both endocarditis and unknown endovascular sources; (2) secondary to another primary non-endovascular focus of infection (e.g. lung, soft tissue, bone/joint); or (3) catheter source. Patient serum samples and bacterial isolates obtained at the onset of presentation and stored at –80°C until analysis.

Bacterial strains and plasmids

Community acquired Methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA300 (TCH1516) and its isogenic *Hla* mutant, was used in the study. Targeted mutagenesis of *Hla* was conducted by precise, markerless allelic replacement of USA300 TCH1516 *Hla* (Locus tag

USA300HOU_1099, NC_010079.1 (1170314.1171273, complement)) by PCR-based methods (**Fig. S1**). Strains were routinely grown in Todd Hewitt broth (THB) and propagated shaking at 37°C to mid-log phase (optimal density at 600 nm (OD_{600}) = 0.4), unless otherwise stated. Bacteria was then collected by centrifugation at 4000 RPM for 10 minutes, washed once in 1X PBS, and resuspended in 1x PBS.

Human primary cell isolation

For platelet isolation, human venous blood was drawn using a 20 G needle from healthy human donors using acid-citrate-dextrose buffer (ACD; Sigma) as an anticoagulant (1:6 v/v), unless otherwise stated. To obtain platelet-rich plasma (PRP), blood was centrifuged at 1000 RPM for 10 minutes with no brake. To avoid contaminations with other cell types, only the upper two thirds of the platelet-rich plasma fractions were used. PRP was centrifuged at 1500 RPM for 10 minutes. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature. Blood was drawn according to a protocol approved by the local ethics committee.

For neutrophil isolation, human venous blood was drawn using a 20 G needle from healthy human donors using heparin as an anticoagulant. Neutrophils were isolated using Polymorph Prep (Axis-Shield, Dundee, Scotland) according to the manufacturer's protocol.

Platelet depletion studies

Endotoxin and azide-free 1 mg/kg anti-CD41 antibody (clone MWReg30, Biolegend) or 1 mg/kg isotype control rat IgG1 (clone RTK2071, Biolegend) was injected intraperitoneally (i.p.). 1 x 10^8 MRSA was administered by tail vein injection (i.v.) 16 hours post-antibody treatment. 4

hours post-MRSA infection, mice were euthanized by CO_2, blood was collected by cardiac puncture with a 25G needle attached to a syringe containing 100 mL ACD buffer, and a complete blood count (CBC) was obtained. Blood was serial diluted and plated onto Todd Hewitt agar (THA) plates for CFU enumeration. Liver, spleen and kidneys were harvested, homogenized, and plated in serial dilutions onto THA plates for CFU enumeration.

For ex vivo platelet-depleted studies, endotoxin and azide-free 1 mg/kg anti-CD41 antibody (clone MWReg30, Biolegend) or 1 mg/kg isotype control rat IgG1 (clone RTK2071, Biolegend) was injected intraperitoneally (i.p.) 16 hours post antibody injection, blood was collected by cardiac puncture with a 25G needle attached to a syringe containing 100 mL ACD buffer. Platelet depleted blood was infected *ex vivo* with 1 x 10^6 MRSA and incubated at 37C rotating for 1 hour.

Mice Studies

*For MRSA vs. Δ*Hla* Platelet Count,* wild-type MRSA and isogenic Δ*Hla* cultures were grown shaking overnight at 37°C in THB, washed once in 1x PBS, and 1 x 10^8 colony forming units (CFU) were injected intravenously (i.v.) into outbred 8- to 10-week-old CD1 mice (Charles Rivers Laboratories). Platelet count and CFU burden enumerated 4 hours post infection.

For Asgr2$^{-/-}$ and AMR inhibitor studies, 8- to 12-week-old Asgr2 knockout (*Asgr2$^{-/-}$*) mice on a C57/Bl6 (Jackson Laboratories) genetic background were generated as described before (29). MRSA wild-type cultures grown overnight shaking at 37°C in THB and washed once in 1x PBS. 1 x 10^8 colony forming units (CFU) were injected intraperitoneally (i.p.) unless otherwise specified in the figure legends. Mice mortality was observed over the course of 10 days. For AMR inhibitor

studies, C57/Bl6 mice were treated with 25mg/mL Asialofetuin or Fetuin and infected by intraperitoneal injection of 1 x 10^8 CFU MRSA. Mortality was observed over the course of 8 days. For both studies, mice that appeared moribund were euthanized by CO_2 asphyxiation and platelet counts were enumerated 4 hours post infection. For AMR inhibitor CFU enumeration, mice were euthanized 24 hours post-infection, organs harvested, and dilution-plated onto THA plates.

For neuraminidase inhibitor studies, 8- to 10-week-old wild-type C57/Bl6 mice were treated with Oseltamivir (5 mg/kg) in 100 μL PBS and NEU1-selective neuraminidase inhibitor, C9-butyl-amide-2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) (2 mg/kg) at the time of- and 3 hours-post intraperitoneal infection with 1 x 10^8 CFU MRSA. Platelets were enumerated 4 hours post-infection and assayed for sialidase activity with the use of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU; Sigma). *For ex vivo sialidase studies*, murine platelet rich plasma (PRP) was isolated by cardiac puncture with a 25 G needle attached to a syringe containing 100 μL ACD and centrifuged at 100 x *g* for 10 min with no brake. Following isolation, 25 μL of PRP was collected and added to white 96-well plate (Costar) with 25 μL RPMI. With 125 μM 4MU. Plate was incubated at 37° C + 5% CO_2 for 30 minutes followed by an addition of 1M Na_2CO_3. Fluorescence was measured at excitation 530nm and emission 585nm.

For Ticagrelor studies, MRSA cultures were grown shaking overnight at 37°C in THB, washed once in 1x PBS, and 1 x 10^8 colony forming units (CFU) were injected intravenously (i.v.) into outbred 8- to 10-week-old CD1 (Charles River Laboratories, Wilmington, MA, USA) mice. Where indicated, Ticagrelor (4 mg/kg) or vehicle (water) was delivered by oral gavage 24 hours prior- and every 24 hours post-infection over a course of 10 days. Mice that appeared moribund were euthanized by CO_2 asphyxiation. *For quantification of CFU burden and histological*

preparation, mice were treated with Ticagrelor (4 mg/kg) or vehicle (water) 12 hours prior- and every 24 hours post-intravenous injection of 1 x 10^8 CFU MRSA. At 12 hours post-infection, two mice from each group (vehicle control and Ticagrelor-treated) were euthanized by CO_2 asphyxiation and the kidneys, spleen, heart, and liver were harvested and fixed 10% neutral-buffered formalin for 24 hours and then routinely processed and paraffin-embedded for histological analysis. Five-micron thick hematoxylin and eosin-stained sections of each tissue were examined by a veterinary pathologist agnostic to treatment group. Distinct bacterial colonies visible at 4X magnification were counted in three longitudinal sections of heart and six longitudinal sections of kidney. Lesions related to bacterial infection were described and graded (minimal-1, mild-2, moderate-3, or severe-4) based on degree of tissue damage. At 72 hours post infection, remaining surviving mice were euthanized by CO_2 asphyxiation, blood was collected by cardiac puncture, and organs were excised. Blood and organ homogenate (MagNA Lyser instrument (Roche Diagnostics Corporation, Indianapolis, IN) were serially diluted in molecular grade H_2O and plated onto Todd Hewitt agar (THA) for enumeration of bacterial CFU. Study was performed in three independent times and data from one representative experiment are shown. *For platelet quantification,* mice were treated with Ticagrelor (4 mg/kg) or vehicle (water) every 12 hours for 72 hours prior to intravenous (i.v.) injection of 1 x 10^8 CFU MRSA. 4 hours post-infection, blood was collected by cardiac puncture with a 25G needle attached to a syringe containing 100 mL ACD buffer, transferred into EDTA tubes and a complete blood count (CBC) was obtained.

Platelet Cytotoxicity

For platelet LDH assay, human platelets were pre-treated with 10μM Ticagrelor (Sigma) or vehicle control and incubated at 37° C + 5% CO_2 rotating. 20 minutes post-treatment, platelets

were exposed to 5ug/mL recombinant α-toxin (H9395 Sigma**)** for 30 minutes at 37 C + 5% CO2 standing. Samples were spun down at 500G for 5 minutes, supernatants were collected, and evaluated for release of lactate dehydrogenase (Promega).

For platelet ATP viability, human platelets were isolated from healthy human donors and incubated at 37C + 5% CO2 rotating. Platelets were exposed to 5ug/mL recombinant α-toxin and measured for platelet viability after 30 minutes at 37 C + 5% CO2 standing (Promega, CellTiter-Glo® Luminescent Cell Viability Assay)

Human Platelet Neuraminidase (Sialidase) Assay

Washed 3 x 10^7 human platelets was assayed for sialidase activity with the use of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU; Sigma) using a modified protocol as described (Condorelli et al., 2017) . Cells were added to a 96-well plate (Costar), treated with or without drugs for 15 minutes, and subsequently exposed to 3 x 10^7 CFU MRSA (MOI: 1) for 1 hour. After incubation at 37° C + 5% CO_2, 125 μM 4MU and 1M Na_2CO_3 was added to each sample and fluorescence determined at excitation 530nm and emission 585nm. Background fluorescence was measured following the same procedure as described, but with presence of 1mM DANA

Human Platelet ADAM10 Protease Assay

Human platelets were isolated from healthy donors using hirudin as an anticoagulant. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature. Platelet metalloprotease activity on 2 × 10^7 human platelets was

quantified utilizing an ADAM10-specific fluorogenic peptide substrate in RPMI (without phenol red). Human platelets were pre-treated with 10μM Ticagrelor (Sigma) or vehicle control and incubated at 37C rotating. 20 minutes post-treatment, platelets were exposed to 5 mg/mL recombinant alpha-toxin and a fluorogenic ADAM10 specific substrate (PEPMCA001, Biozyme) at 37 C + 5% CO2 standing. Fluorescence was measured every 15 minutes for 2.5 hours. Excitation and emission wavelengths are 325 and 393 nm respectively.

Calcium Assay (Fluo-3 Assay)

Human donor blood was collected using hirudin as an anticoagulant. Platelet-rich plasma (PRP) isolated and incubated with 2mM Fluo-3 AM (Thermo Fisher Scientific) at 37 C + 5% CO2 rotating for 20 minutes. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature. Human platelets were pre-treated with 10μM Ticagrelor (Sigma) or vehicle control and after 15 minutes, platelets were exposed to 5mg/mL recombinant alpha-toxin. Fluorescence was measured at 505 nm excitation and 530 nm emission every 30 seconds.

Transmission Electron Microscopy

Isolated human platelets were pre-treated with 10μM Ticagrelor (Sigma) with appropriate concentrations of vehicle control at 37°C + 5% CO2. After 20 minutes, platelets were infected with MRSA at an MOI = 0.01 for 2 hours at 37°C + 5% CO2. After incubation, samples were fixed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 h, post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 h, and stained in block in 2% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50–60

nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4k HS digital camera (FEI). Images were taken from multiple random fields at 1200 ×, 2900 ×, 23,000 ×; gross morphology was analyzed in a blinded fashion.

Growth Curve Assay

Sterile non-pyrogenic tubes containing Todd Hewitt broth treated with Ticagrelor (10 μM) or untreated were inoculated with sufficient amounts of overnight bacterial cultures to achieve an optical density (600 nm) of 0.1. Tubes were incubated in a shaking 37 °C incubator, and absorbance measurements were taken every 30 min (600nm) for 8 h using a Spectronic 20D+ spectrophotometer (Thermo Scientific, Waltham, MA, USA)

Bactericidal Assays

For human platelet killing assays, isolated human platelets were pre-treated with 10μM Ticagrelor (Sigma) with appropriate concentrations of vehicle control at 37°C + 5% CO2. After 20 minutes, platelets were infected with MRSA at an MOI = 0.01 for 2 hours at 37°C + 5% CO2. After incubation, infected platelets were sonicated (Fisher Sonic Dismembrator 550) for 3 seconds, serially diluted, and plated on Todd Hewitt agar plates. Percent killing by platelets was determined by dividing the number of surviving colony forming units (CFU) by the number of bacteria added from inoculum.

For human neutrophil killing assay, freshly isolated human neutrophils in serum-free RPMI were added to 96-well plates at a density of 5×10^4 cells per well and treated with Ticagrelor (10 μM) or vehicle control for 1 h at 37 °C + 5% CO_2. Neutrophils were infected with MRSA at

an MOI = 0.01 for 2 hours at 37°C + 5% CO2. After incubation, cells were lysed with 0.025% Triton X-100, serial diluted, and plated on Todd Hewitt agar plates. Percent killing by platelets was determined by dividing the number of surviving colony forming units (CFU) by the number of bacteria added from inoculum.

For THP-1 macrophage (Mφ) *killing assay,* human THP-1 monocytes cell line was provided and authenticated by ATCC and stored at the UCSD cell culture facility. Cells were cultured in RPMI (with phenol red) medium supplemented with 10% fetal bovine serum (FBS). Human THP-1 Mφ were differentiated for 48 hours with 25 nM PMA (Sigma) with a subsequent 24 hours rest period in RPMI + 10% FBS in a 96 well plate. On day of infection, cells were washed once with 1X PBS, treated with Ticagrelor (10 μM) or untreated control for 1 hour, and infected with MRSA at an MOI of 1:100. After incubation, cells were lysed with 0.025% Triton X-100, serial diluted, and plated on Todd Hewitt agar plates. Percent killing by platelets was determined by dividing the number of surviving colony forming units (CFU) by the number of bacteria added from inoculum.

For human whole blood killing assays, whole blood was drawn from healthy donors using ACD as an anticoagulant (1:6). Blood was pre-treated with varying concentrations of Ticagrelor or appropriate vehicle control at 37°C + 5% CO2 rotating. After pretreatment, blood was infected with MRSA (1:10) and incubated for 1 hour at 37°C + 5% CO2 rotating. After incubation, samples were sonicated (Fisher Sonic Dismembrator 550) for 3 seconds, serial diluted, and plated on THA plates. Percent killing by platelets was determined by dividing the number of surviving colony forming units (CFU) by the number of bacteria added from inoculum.

Induction and quantification of NETs

To induce extracellular trap production, neutrophils were seeded in 96-well plates at a density of 5 x 10^4 cells per well in RPMI (without phenol red). Cells were incubated with Ticagrelor (10 uM) or untreated control at 37 °C with 5% CO for 1 hour before addition of NET-inducing phorbol 12-myristate 13-acetate (PMA) for 2 h at 37 °C with 5% CO2. Where applicable, 25 nM PMA was included as a positive control. Extracellular DNA content was quantified using a Quant-IT PicoGreen dsDNA Assay kit (Life Technologies, Carlsbad, CA) .

Statistics

Statistical significance was calculated by unpaired Student t-test using GraphPad Prism unless otherwise stated.

2.8 ACKNOWLEDGEMNTS

Chapter 2, in full, is a fully revised manuscript currently in preparation for submission to *Science Translational Medicine* as: Sun J, Uchiyama S, Olson J, Cornax I, Kyaw M, Aguilar B, Haste N, Sakoulas G, Rose W, Marth J, and Nizet V. Mechanistic analysis of platelet-mediated defense against methicillin-resistant *Staphylococcus aureus* provides avenues for therapeutic drug repurposing. The dissertation author is the primary investigator and the co-first author of this paper. We thank Jason Munguia (UCSD) for technical assistance and Ajit Varki (UCSD) for helpful advice and discussions.

2.8 REFERENCES

1. Gaertner, F., Z. Ahmad, G. Rosenberger, S. Fan, L. Nicolai, B. Busch, G. Yavuz, M. Luckner, H. Ishikawa-Ankerhold, R. Hennel, A. Benechet, M. Lorenz, S. Chandraratne, I. Schubert, S. Helmer, B. Striednig, K. Stark, M. Janko, R.T. Bottcher, A. Verschoor, C. Leon, C. Gachet, T. Gudermann, Y.S.M. Mederos, Z. Pincus, M. Iannacone, R. Haas, G. Wanner, K. Lauber, M. Sixt, and S. Massberg, *Migrating Platelets Are Mechano-scavengers that Collect and Bundle Bacteria.* Cell, 2017. **171**(6): p. 1368-1382.e23.

2. Wong, C.H., C.N. Jenne, B. Petri, N.L. Chrobok, and P. Kubes, *Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance.* Nat Immunol, 2013. **14**(8): p. 785-92.

3. Yeaman, M.R., *Platelets: at the nexus of antimicrobial defence.* Nat Rev Microbiol, 2014. **12**(6): p. 426-37.

4. Cognasse, F., K.A. Nguyen, P. Damien, A. McNicol, B. Pozzetto, H. Hamzeh-Cognasse, and O. Garraud, *The Inflammatory Role of Platelets via Their TLRs and Siglec Receptors.* Front Immunol, 2015. **6**: p. 83.

5. Yeaman, M.R., D.C. Norman, and A.S. Bayer, *Staphylococcus aureus susceptibility to thrombin-induced platelet microbicidal protein is independent of platelet adherence and aggregation in vitro.* Infect Immun, 1992. **60**(6): p. 2368-74.

6. Ali, R.A., L.M. Wuescher, K.R. Dona, and R.G. Worth, *Platelets Mediate Host Defense against Staphylococcus aureus through Direct Bactericidal Activity and by Enhancing Macrophage Activities.* J Immunol, 2017. **198**(1): p. 344-351.

7. Kraemer, B.F., R.A. Campbell, H. Schwertz, M.J. Cody, Z. Franks, N.D. Tolley, W.H. Kahr, S. Lindemann, P. Seizer, C.C. Yost, G.A. Zimmerman, and A.S. Weyrich, *Novel anti-bacterial activities of beta-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation.* PLoS Pathog, 2011. **7**(11): p. e1002355.

8. Berube, B.J. and J. Bubeck Wardenburg, *Staphylococcus aureus alpha-toxin: nearly a century of intrigue.* Toxins (Basel), 2013. **5**(6): p. 1140-66.

9. Gafter-Gvili, A., N. Mansur, A. Bivas, N. Zemer-Wassercug, J. Bishara, L. Leibovici, and M. Paul, *Thrombocytopenia in Staphylococcus aureus bacteremia: risk factors and prognostic importance.* Mayo Clin Proc, 2011. **86**(5): p. 389-96.

10. Bhakdi, S., M. Muhly, U. Mannhardt, F. Hugo, K. Klapettek, C. Mueller-Eckhardt, and L. Roka, *Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets.* J Exp Med, 1988. **168**(2): p. 527-42.

11. Powers, M.E., R.E. Becker, A. Sailer, J.R. Turner, and J. Bubeck Wardenburg, *Synergistic Action of Staphylococcus aureus alpha-Toxin on Platelets and Myeloid Lineage Cells Contributes to Lethal Sepsis.* Cell Host Microbe, 2015. **17**(6): p. 775-87.

12. Inoshima, I., N. Inoshima, G.A. Wilke, M.E. Powers, K.M. Frank, Y. Wang, and J. Bubeck Wardenburg, *A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice.* Nat Med, 2011. **17**(10): p. 1310-4.

13. Hoffmeister, K.M. and H. Falet, *Platelet clearance by the hepatic Ashwell-Morrell receptor: mechanisms and biological significance.* Thromb Res, 2016. **141 Suppl 2**: p. S68-72.

14. Grewal, P.K., S. Uchiyama, D. Ditto, N. Varki, D.T. Le, V. Nizet, and J.D. Marth, *The Ashwell receptor mitigates the lethal coagulopathy of sepsis.* Nat Med, 2008. **14**(6): p. 648-55.

15. Zhang, X.H., Q.M. Wang, J.M. Zhang, F.E. Feng, F.R. Wang, H. Chen, Y.Y. Zhang, Y.H. Chen, W. Han, L.P. Xu, K.Y. Liu, and X.J. Huang, *Desialylation is associated with apoptosis and phagocytosis of platelets in patients with prolonged isolated thrombocytopenia after allo-HSCT.* J Hematol Oncol, 2015. **8**: p. 116.

16. Jansen, A.J., E.C. Josefsson, V. Rumjantseva, Q.P. Liu, H. Falet, W. Bergmeier, S.M. Cifuni, R. Sackstein, U.H. von Andrian, D.D. Wagner, J.H. Hartwig, and K.M. Hoffmeister, *Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice.* Blood, 2012. **119**(5): p. 1263-73.

17. Li, J., D.E. van der Wal, G. Zhu, M. Xu, I. Yougbare, L. Ma, B. Vadasz, N. Carrim, R. Grozovsky, M. Ruan, L. Zhu, Q. Zeng, L. Tao, Z.M. Zhai, J. Peng, M. Hou, V. Leytin, J. Freedman, K.M. Hoffmeister, and H. Ni, *Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia.* Nat Commun, 2015. **6**: p. 7737.

18. Li, M.F., X.L. Li, K.L. Fan, Y.Y. Yu, J. Gong, S.Y. Geng, Y.F. Liang, L. Huang, J.H. Qiu, X.H. Tian, W.T. Wang, X.L. Zhang, Q.X. Yu, Y.F. Zhang, P. Lin, L.N. Wang, X. Li, M. Hou, L.Y. Liu, and J. Peng, *Platelet desialylation is a novel mechanism and a therapeutic target in thrombocytopenia during sepsis: an open-label, multicenter, randomized controlled trial.* J Hematol Oncol, 2017. **10**(1): p. 104.

19. Shao, L., Y. Wu, H. Zhou, P. Qin, H. Ni, J. Peng, and M. Hou, *Successful treatment with oseltamivir phosphate in a patient with chronic immune thrombocytopenia positive for anti-GPIb/IX autoantibody.* Platelets, 2015. **26**(5): p. 495-7.

20. Bigot, P., M. Auffret, S. Gautier, M. Weinborn, N.K. Ettahar, and P. Coupe, *Unexpected platelets elevation in a patient with idiopathic thrombocytopenia treated with oseltamivir for influenza infection.* Fundam Clin Pharmacol, 2016. **30**(5): p. 483-5.

21. Grewal, P.K., P.V. Aziz, S. Uchiyama, G.R. Rubio, R.D. Lardone, D. Le, N.M. Varki, V. Nizet, and J.D. Marth, *Inducing host protection in pneumococcal sepsis by preactivation of the Ashwell-Morell receptor.* Proc Natl Acad Sci U S A, 2013. **110**(50): p. 20218-23.

22. Ree, I.M.C., S.F. Fustolo-Gunnink, V. Bekker, K.J. Fijnvandraat, S.J. Steggerda, and E. Lopriore, *Thrombocytopenia in neonatal sepsis: Incidence, severity and risk factors.* PLoS One, 2017. **12**(10): p. e0185581.

23. Claushuis, T.A., L.A. van Vught, B.P. Scicluna, M.A. Wiewel, P.M. Klein Klouwenberg, A.J. Hoogendijk, D.S. Ong, O.L. Cremer, J. Horn, M. Franitza, M.R. Toliat, P. Nurnberg, A.H. Zwinderman, M.J. Bonten, M.J. Schultz, and T. van der Poll, *Thrombocytopenia is associated with a dysregulated host response in critically ill sepsis patients.* Blood, 2016. **127**(24): p. 3062-72.

24. Trier, D.A., K.D. Gank, D. Kupferwasser, N.Y. Yount, W.J. French, A.D. Michelson, L.I. Kupferwasser, Y.Q. Xiong, A.S. Bayer, and M.R. Yeaman, *Platelet antistaphylococcal responses occur through P2X1 and P2Y12 receptor-induced activation and kinocidin release.* Infect Immun, 2008. **76**(12): p. 5706-13.

25. Hata, K., K. Koseki, K. Yamaguchi, S. Moriya, Y. Suzuki, S. Yingsakmongkon, G. Hirai, M. Sodeoka, M. von Itzstein, and T. Miyagi, *Limited inhibitory effects of oseltamivir and zanamivir on human sialidases.* Antimicrobial agents and chemotherapy, 2008. **52**(10): p. 3484-3491.

26. Alioglu, B., A. Tasar, C. Ozen, B. Selver, and Y. Dallar, *An experience of oseltamivir phosphate (tamiflu) in a pediatric patient with chronic idiopathic thrombocytopenic purpura: a case report.* Pathophysiol Haemost Thromb, 2010. **37**(2-4): p. 55-8.

27. Jansen, A.J., J. Peng, H.G. Zhao, M. Hou, and H. Ni, *Sialidase inhibition to increase platelet counts: A new treatment option for thrombocytopenia.* Am J Hematol, 2015. **90**(5): p. E94-5.

28. Rose, W.E., J.C. Eickhoff, S.K. Shukla, M. Pantrangi, S. Rooijakkers, S.E. Cosgrove, V. Nizet, and G. Sakoulas, *Elevated serum interleukin-10 at time of hospital admission is predictive of mortality in patients with Staphylococcus aureus bacteremia.* J Infect Dis, 2012. **206**(10): p. 1604-11.

29. Ishibashi, S., R.E. Hammer, and J. Herz, *Asialoglycoprotein receptor deficiency in mice lacking the minor receptor subunit.* J Biol Chem, 1994. **269**(45): p. 27803-6.

CHAPTER 3

Inflammasome-independent activation of IL-1β by *Pseudomonas aeruginosa* protease LasB induces pathological inflammation

Josh Sun[1], Doris L. LaRock[2], Jacqueline M. Kimmey[2], Joshua Olson[2], Zhenze Jiang[1], Anthony J. O'Donoghue[1], Victor Nizet[1,2,6], Christopher N. LaRock[2,3,4,5,6*]

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, CA

[2]Department of Pediatrics, UC San Diego, La Jolla, CA

[3]Department of Microbiology and Immunology, Emory School of Medicine, Atlanta GA

[4]Division of Infectious Diseases, Emory School of Medicine, Atlanta GA

[5]Antimicrobial Resistance Center, Emory University, Atlanta GA

[6]Senior Author

*To whom correspondence should be addressed: christopher.larock@emory.edu

Manuscript in Revision at Cell Reports

3.1 PREFACE

The following chapter is a direct revision of a manuscript currently in review. We reveal a non-canonical regulator of IL-1β maturation, wherein a bacterial protease directly matures IL-1β, bypassing the need for canonical host-derived proteolytic activation of this cytokine. This work not only identifies and validates an inflammatory bacterial target, but also provides a rationale for therapeutic drug repurposing.

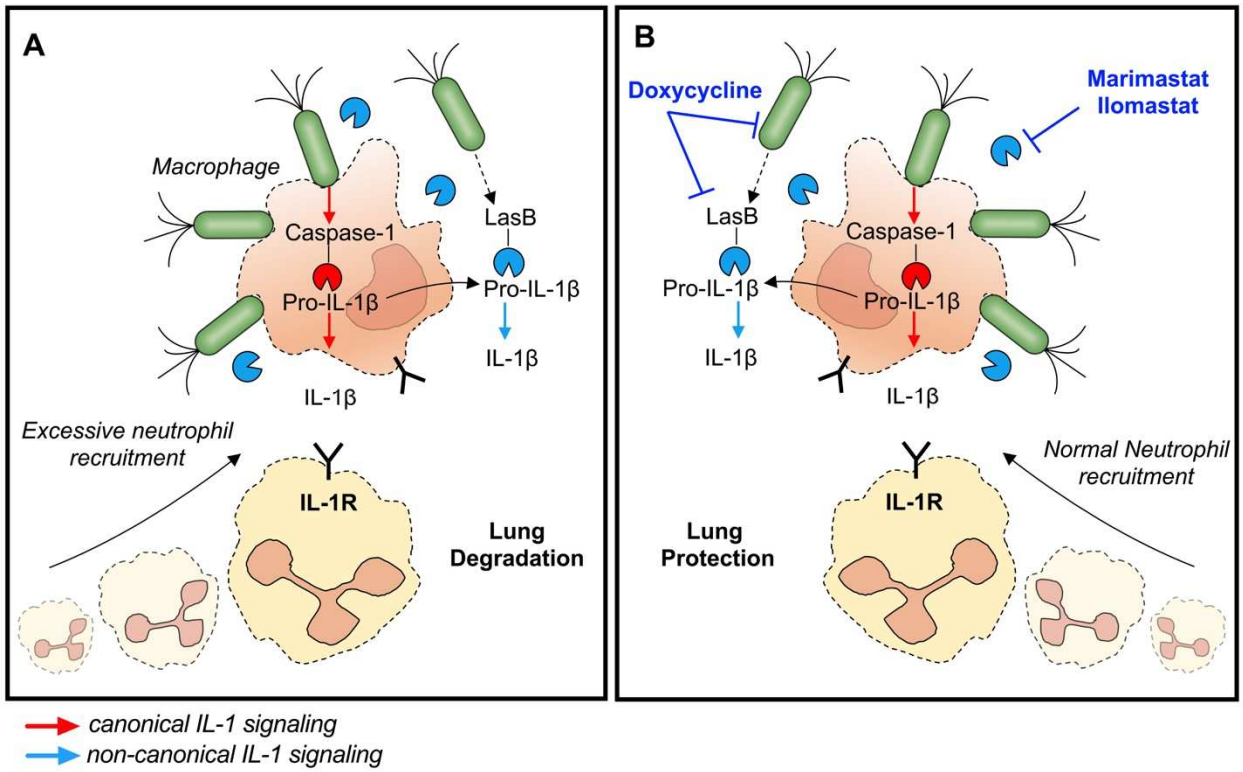


Figure 3.0 Graphical Abstract. (A) Non-canonical activation of IL-1β by pseudomonal LasB drives excessive neutrophil recruitment and degradation of lung architecture. (B) FDA-repurposing of metalloprotease inhibitors (Doxycycline, Marimastat, Ilomastat) mitigates LasB-mediated extra-inflammasomal activation of IL-1β and returns host immunity to normalcy.

3.2 ABSRACT

Pulmonary damage by *Pseudomonas aeruginosa* during cystic fibrosis infections and ventilator-associated pneumonia is mediated both by pathogen virulence factors and host inflammation. Impaired immune function due to tissue damage and inflammation, coupled with pathogen multidrug resistance, complicates management of these deep-seated infections. Preservation of lung function and effective immune clearance may therefore be enhanced by selective control of inflammation. Pathological inflammation during *P. aeruginosa* pneumonia is driven by interleukin-1β (IL-1β), a proinflammatory cytokine canonically regulated by caspase-family inflammasome proteases. We report that plasticity in IL-1β proteolytic activation allows its direct maturation by the pseudomonal protease LasB. Inhibition of LasB limits IL-1β activation, neutrophilic inflammation, and destruction of lung architecture typically observed during pulmonary infection. Discovery of this IL-1β regulatory mechanism provides a distinct target for anti-inflammatory therapeutics, wherein LasB inhibition by repurposed matrix metalloprotease inhibitors and tetracycline-family antibiotics mitigates a major inflammatory driver of pathology in *P. aeruginosa* pulmonary infections.

3.3 INTRODUCTION

Pseudomonas aeruginosa is a prominent cause of severe opportunistic pulmonary infections associated with mechanical ventilation and the genetic disease cystic fibrosis (CF). *P. aeruginosa* infection is often refractory to antibiotic therapy due to multidrug resistance, making it a World Health Organization and U.S. Centers for Disease Control priority pathogen for therapeutic development. *P. aeruginosa* infection destroys lung architecture and function, largely attributable to inflammatory- and neutrophil-mediated degradation of mucin layers and structural proteins of the pulmonary connective tissue (94, 95). Neutrophil chemokines such as IL-1β ((96, 97) and IL-8 (98), the latter itself regulated by IL-1β (99), initiate and maintain this inflammatory cycle. Anti-inflammatory agents can mitigate pathological tissue destruction to help preserve pulmonary function during *P. aeruginosa* pneumonia (100) and CF ((101, 102).

Newly synthesized IL-1β (pro-IL-1β) is inactive and requires proteolytic processing into a mature active form. Canonically, this is carried out by the inflammasome, a macromolecular complex of intracellular pattern recognition receptors and the proteases caspase-1 or caspase-11 ((103). During infection, inflammasomes are formed upon detection of pathogen-associated molecular patterns (PAMPs), including many present in *P. aeruginosa* such as flagellin (FliC), the type III secretion basal body rod (PscI), the type IV pilin (PilA), RhsT, exolysin (ExlA), exotoxin A (ExoA), cyclic 3′-5′ diguanylate (c-di-GMP), and lipopolysaccharide (LPS), which are varyingly detected by NLRC4, NLRP3, or caspase-11 ((104-112). Some pathogens limit their inflammatory potential by inhibiting the inflammasome (113), and *P. aeruginosa* dampens inflammasome activation via the effector ExoU (111). Therefore, despite the multitude of inflammasome-activating signals that *P. aeruginosa* express during infection, caspases, NLRP3, and NLRC4, are not essential for pro-IL-1β maturation in macrophages, epithelial cells, or neutrophils infected with

P. aeruginosa (114, 115). Correspondingly, *P. aeruginosa*-infected caspase-1$^{-/-}$ and caspase-1/-11$^{-/-}$ mice succumb to a destructive neutrophilic pulmonary inflammation from which IL-1 receptor (IL-1R1$^{-/-}$) mice are protected (21, 22). These observations highlight the contribution of IL-1β to *P. aeruginosa* infection but suggest an alternative maturation mechanism to the inflammasome.

The pathological cascade of protease dysregulation and activation seen during lung infections provide a possibility for IL-1β maturation by alternative mechanisms. Caspase-8 (23-25), and the neutrophil granular proteases elastase (NE) and proteinase 3 (PR3) (96, 97, 116), cleave IL-1β under some experimental conditions, though the contribution of these mechanisms to inflammation have not been fully explored. However, bronchial secretions also possess abundant protease activity from microbial sources (95). Here we find that IL-1β is not exclusively matured by host proteases, but that *P. aeruginosa* protease LasB also drives this inflammatory pathway. Targeting this bacterial protease may therefore provide supportive therapy to limit inflammatory pathology in pulmonary infection.

3.4 RESULTS

Caspase-independent IL-1β drives neutrophilic inflammation during P. aeruginosa lung infection

Inflammation drives poor clinical outcomes during *P. aeruginosa* lung infection ((117). C57Bl/6 mice infected intratracheally with *P. aeruginosa* had markedly disrupted airway architecture within 24 h, concurrent with neutrophil infiltration into the lung tissue and bronchoalveolar lavage fluid (BAL) (Figure 1A). We examined the contribution of pro-inflammatory cytokines to this process using the FDA-approved IL-1 receptor (IL-1R1) antagonist anakinra, which inhibits both IL-1β and IL-1α signaling, and interrupts a positive feedback loop

boosting production of the two cytokines. We found anakinra treatment decreased IL-1β and IL-1α, while other important proinflammatory cytokines (KC, IL-6, TNFα) were not significantly impacted (Figure 1B). As observed during human ventilator-associated pneumonia and chronic CF pneumonitis, *P. aeruginosa* persisted in the BAL (Figure 1C) and the lung (Figure 1D) despite significant IL-1R1 signaling-dependent neutrophil infiltration (Figure 1E).

While IL-1α can signal independently, IL-1β is typically released by secretion or cell lysis and requires additional proteolytic maturation, activities that are often sequentially co-regulated by the inflammasome-activated caspases -1 and -11 (103). Release of IL-1α was unaltered in *P. aeruginosa*-infected caspase-1/-11$^{-/-}$ C57Bl/6 mice, but surprisingly, IL-1β release was only moderately attenuated (Figure 1F). Thus in addition to IL-1α, a significant pool of extracellular IL-1β remains and can be active as an IL-1R1 agonist if the inhibitory pro-domain is proteolytically removed. Neutrophil granular proteases can provide such activation ((96, 97, 108, 114), however, since neutrophil recruitment is itself IL-1β-dependent (Figure 1A, 1E), we rationalized that additional proteases could likely initiate the process.

Consistent with our observations *in vivo*, supernatants from bone-marrow derived macrophages (BMM) from caspase-1/-11$^{-/-}$ mice infected with *P. aeruginosa* still significantly activated IL-1R1 reporter cells (Figure 1G). Corroborating these results, caspase-1/11-specific inhibitor YVAD treatment of human cell line types relevant to lung infection (macrophages, THP-1; neutrophils, HL60; type II alveolar epithelial cells, A549) only prodouced a modest IL-1 signaling decrease upon *P. aeruginosa* challenge (Figure 1H). Absolute quantities of IL-1α and IL-1β (pro- and mature- forms) measured by enzyme-linked immunosorbent assay remained

unaltered, and IL-1 signaling was inhibited by monoclonal antibodies specific to IL-1R1 or IL-1β, but not by an antibody to IL-1α. (Figure 1I). Together, these results indicate that *P. aeruginosa* is stimulating IL-1R1 signaling through a pool of mature extracellular IL-1β activated independently of caspase-1/11.

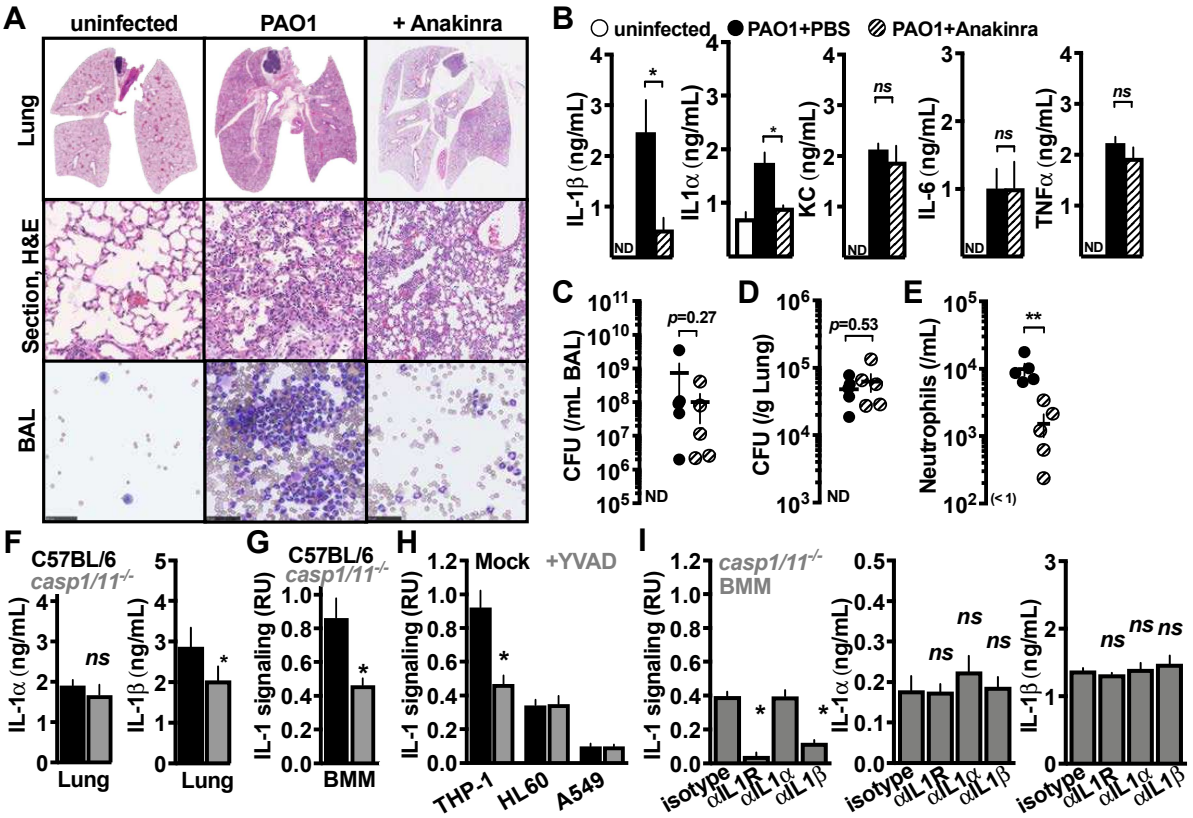


Figure 3.1. Caspase-independent IL-1β drives neutrophilic inflammation during *P. aeruginosa* lung infection C57BL/6 mice intratracheally infected with 10^7 CFU of PAO1 and treated with anakinra (50 ug/kg) or PBS control, compared to uninfected mice. Mice were euthanized after 24 h and (**A**) lung histology sections or cytological smears of bronchoalveolar lavage fluid (BAL) prepared with differential MGG stain, (**B**) BAL cytokines measured by enzyme-linked immunosorbent assay, (**C-D**) bacterial colony forming units (CFU) in BAL or lung homogenate, and (**E**) neutrophils enumerated. (**F**) C57BL/6 or isogeneic caspase-1/11$^{-/-}$ mice intratracheally infected with 10^7 CFU PAO1 24 h, euthanized, and BAL cytokines measured by enzyme-linked immunosorbent assay. (**G**) Relative IL-1 signaling from bone marrow macrophages (BMM) derived from caspase-1/11$^{-/-}$ and C57Bl/6 mice and infected with PAO1. (**H**) Relative IL-1 signaling by human THP-1 macrophages, HL60 neutrophils, or A549 epithelial cells treated with caspase-1 inhibitor (YVAD) or control (Mock) 1 h prior to infection as above. (**I**) Mature IL-1 and enzyme-linked immunosorbent assay measurement of IL-1α and IL-1β present in any form, released from PAO1-infected caspase-1/11$^{-/-}$ BMM with monoclonal antibodies neutralizing IL-1R1, IL-1α, IL-1β, or an isotype control. Infections were at MOI=10 and after 2 h the supernatant collected and mature IL-1 quantified using IL-1R1 reporter cells. Where applicable, data are mean ± SEM and represent at least 3 independent experiments; significance determined by unpaired two-tailed Student's T-test, $*P < 0.05$.

IL-1β is activated by the P. aeruginosa LasB protease

The observed induction of IL-1 signaling through a caspase-1/-11-independent mechanism was conserved across numerous *P. aeruginosa* isolates (Figure 2A). Proteases contributing to IL-1β activation were further evaluated using a panel of small molecule inhibitors specific to each of the major protease catalytic classes. Pharmacological blockade of metalloproteases, but not cysteine proteases (e.g. caspases-1, 11, and 8) or serine proteases (e.g. NE and PR3), abrogated IL-1β signaling in *P. aeruginosa*-infected caspase-1/11$^{-/-}$ BMM (Figure 2B). By comparative analysis of isogenic *P. aeruginosa* metalloprotease mutants (Δ*lasA*, Δ*lasB*, Δ*aprA*), the highly-expressed LasB (118) was the most active protease as measured by casein hydrolysis (Figure 2C), and the major driver of IL-1β signaling (Figure 2D). Complementation with the LasB coding sequence under its native promoter restored the ability of Δ*lasB P. aeruginosa* to induce IL-1β signaling in infected caspase-1/-11$^{-/-}$ BMM (Figure 2E). LasB can direct the maturation of other proteases (119, 120), however, mutants of these proteases still activated IL-1β signaling (Figure 2D) and purified recombinant LasB was sufficient to induce caspase-1/-11-independent IL-1β signaling (Figure 2F). Taken together these data indicate that LasB induces pro-IL-1β maturation independent of caspase-1/-11 during *P. aeruginosa* infection.

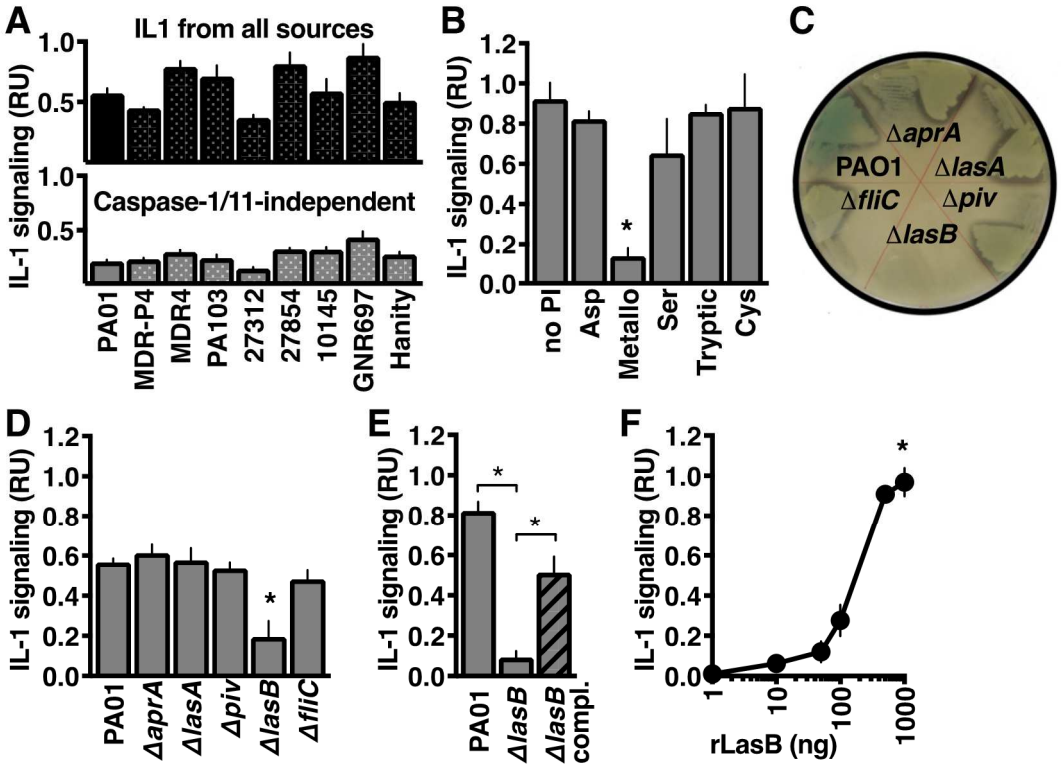


Figure 3.2 IL-1β is activated by the *P. aeruginosa* LasB protease. (**A**) Relative IL-1 signaling by caspase-1/11$^{-/-}$ (grey) or control C57Bl/6 BMMs (black) after 2 h co-incubation of the indicated *Pseudomonas* strains. (**B**) Relative IL-1 signaling by caspase-1/11$^{-/-}$ BMM 2 h post-infection by PAO1 that were previously incubated 1 h with the indicated protease inhibitors classes. (**C**) Visualization of bacterial proteolytic activity by decreased media opacity on LB agarose plates containing casein. (**D**) Relative IL-1 signaling by caspase-1/11$^{-/-}$ BMM 2 h post-infection with isogenic mutant strains of PAO1. (**E**) Relative IL-1 signaling by caspase-1/11$^{-/-}$ BMM 2 h post-infection by PAO1, Δ*lasB*, or plasmid-complemented Δ*lasB*. (**F**) Relative IL-1 signaling by caspase-1/11$^{-/-}$ BMM after 2 h incubation with recombinant LasB. Where applicable, data are mean ± SEM and represent at least 3 independent experiments; significance determined by unpaired two-tailed Student's T-test, **P* < 0.05.

Differentially activated IL-1β maintains activity

Further examination of pro-IL-1β maturation by western immunoblot revealed that LasB generated a 17 kDa form similar in mass to mature IL-1β resulting from caspase-1 cleavage (Figure 3A). We interrogated this proteolysis with a series of internally quenched fluorescent peptides and found that LasB preferentially cleaved within the sequence HDAPVRSLN of pro-IL-1 (Figure 3B). Mass spectroscopy confirmed that LasB cleaved between Ser-121 and Leu-122, generating an IL-1β product five amino acids shorter than that matured by caspase-1 (Figure 3B, Supplemental Figure 1). During infection, the signature of IL-1β-targeted proteolysis (Figure 3B) is consistent with a significant role for LasB-mediated maturation (hydrolysis of HDAPVRSLN) of IL-1β. To examine whether this truncated form was sufficient for IL-1R activation, we generated recombinant IL-1β with amino-terminal truncations replicating these cleavage events. Relative to ^{1Met}IL-1β (full-length, pro-IL-1β), ^{117Ala}IL-1β (matching caspase-1 cleavage) and ^{122Leu}IL-1β (matching LasB cleavage) possessed similar and significant signaling activity (Figure 3C).

To validate cleavage specificity, we generated pro-IL-1β point mutants not cleavable by LasB. A LasB substrate specificity profile was generated using a mass spectrometry-based substrate profiling assay previously validated on other microbial proteases ((121-124). The peptide degradation pattern showed LasB has a distinct preference for cleaving peptide bonds when Ser or Thr are in the P1 position (amino-terminal side of bond) and hydrophobic amino acids such as Phe, Leu, Nle, Tyr, Trp and Ile in the P1′ position (Figure 3D, Supplementary Tables 1-3). This substrate cleavage preference corresponds well to hydrolysis of the Ser-Leu bond of pro-IL-1β (Figure 3B). As bulky hydrophobic amino acids are preferred in the P1′ position of LasB, and

because Gly itself is poorly tolerated, we generated recombinant pro-IL-1β with the native Leu mutated to Gly (IL-1β^{Leu122Gly}). In addition, we mutated Leu to Ala (IL-1β^{Leu122Ala}) in the P1′ position as this amino acid was neutral, neither favored nor disfavored. LasB poorly activated IL-1β^{Leu122Ala} and IL-1β^{Leu122Gly} but activation by caspase-1, which recognizes a distal and non-overlapping site, was maintained (Figure 3E). In summary, the LasB and caspase-1 mechanisms for generating mature IL-1β are distinguishable by substrate specificity (a hydrophobic P1' vs aspartic acid P1 site), enzyme class (metalloprotease vs cysteine protease), and cellular source (microbial vs host).

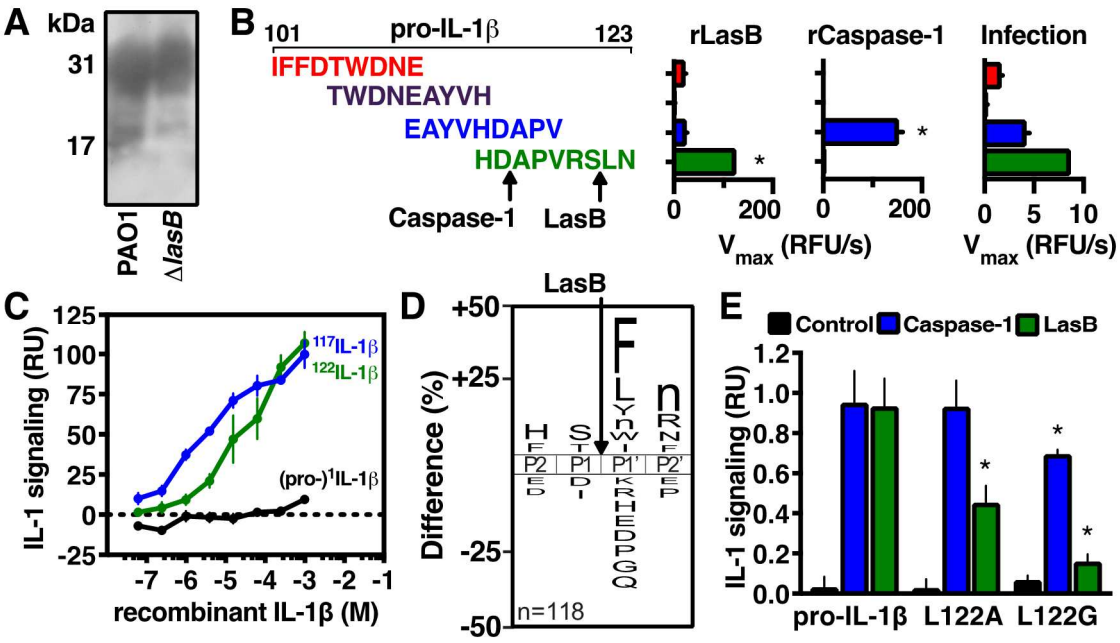


Figure 3.3 Differentially activated IL-1β maintains activity. (**A**) Western blot analysis of IL-1β released by C57Bl/6 BMMs 2 h post-infection with PAO1 or Δ*lasB*. (**B**) Cleavage of internally-quenched fluorescent IL-1β peptide fragments (amino acids 101-121) by recombinant LasB, or caspase-1, or proteases in BAL collected from C57BL/6 mice 24 h post-intratracheal infection with 10^7 CFU of PAO1. (**C**) Relative IL-1 signaling by titrated recombinant pro-IL-1β or the N-terminal truncated ^{117Ala}IL-1β (Caspase-1 product) and ^{122Leu}IL-1β (LasB product). (**D**) IceLogo frequency plot showing amino acids significantly enriched (above X-axis) and de-enriched (below) in the P2 to P2′ positions following incubation of LasB with a mixture of 228 synthetic tetradecapeptides. Cleavage occurs between P1 and P1', lowercase "n" corresponds to norleucine. (**E**) Relative IL-1 signaling by recombinant pro-IL-1β, or P1' site-directed mutants L122A and L122G, incubated 2 h with recombinant Caspase-1 or LasB. Where applicable, data are mean ± SEM and represent at least 3 independent experiments; significance determined by unpaired two-tailed Student's T-test, $*P < 0.05$.

Metalloprotease inhibitors of LasB prevent IL-1β -mediated pathological inflammation

Since IL-1β inhibition protects against lung damage (Figure 1B), and because LasB drives IL-1β maturation (Figure 2D, 3A-3E), we examined whether protease inhibitors active against LasB could limit lung injury. Two investigational hydroxamate-based antineoplastic agents that inhibit matrix metalloproteases, marimastat and ilomastat, inhibited both LasB cleavage of the IL-1β-derived substrate (Figure 4A) and *P. aeruginosa* activation of IL-1β (Figure 4B) with picomolar affinities. During murine infection, marimastat and ilomastat each showed therapeutic effects to decrease IL-1β (Figure 4C), limit neutrophil recruitment (Figure 4D), and reduce pulmonary pathology (Figure 4E). To date, the only FDA-approved metalloprotease inhibitor is Periostat, an oral formulation of the antibiotic doxycycline used at concentrations that inhibits the activity of collagenase (MMP-1), but which are not antimicrobial ((125-127). As with marimastat and ilomastat, doxycycline and the structurally-related antibiotic tetracycline also inhibited both the metalloprotease activity of LasB and its activation of IL-1β (Figure S2A-2E).

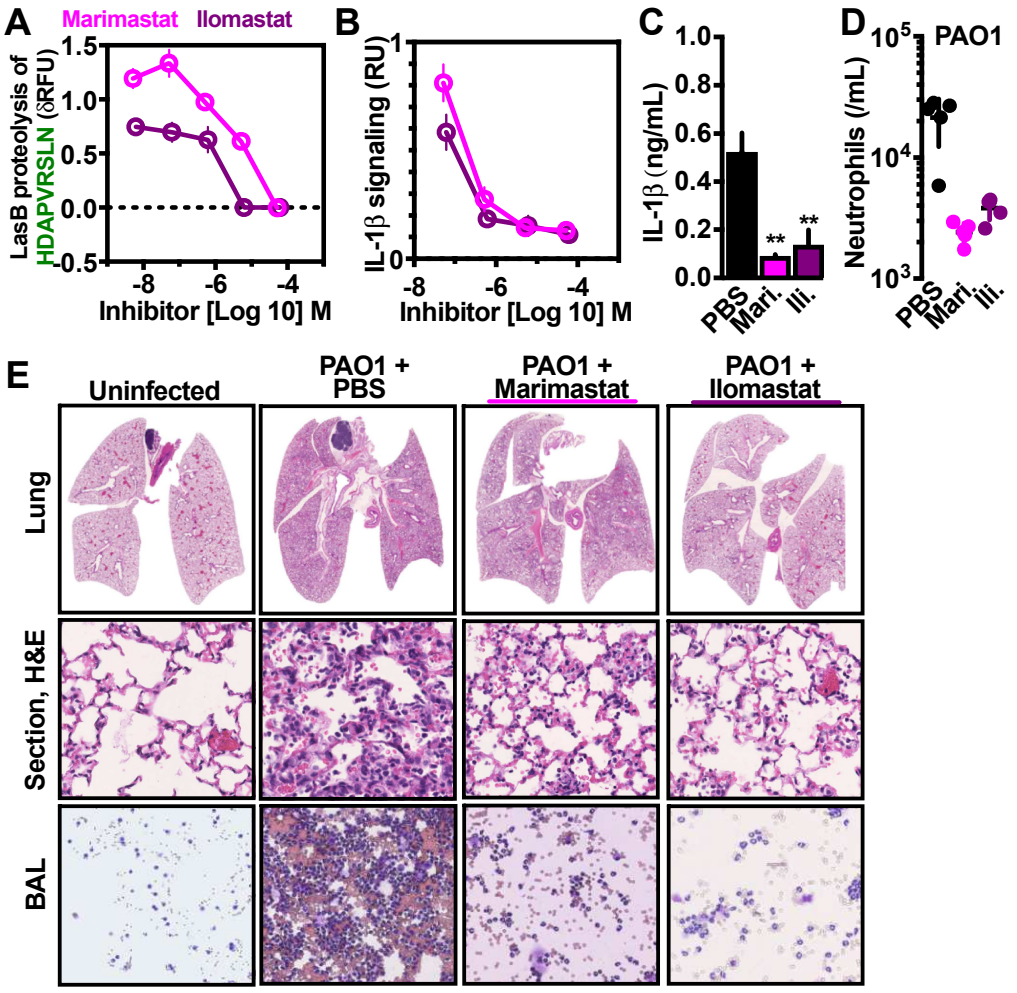


Figure 3.4 Metalloprotease inhibitors of LasB prevent IL-1β-mediated pathological inflammation. Marimastat and Ilomastat inhibition of LasB proteolysis of (**A**) an internally quenched IL-1β fragment HDAPVRSLN or (**B**) IL-1 signaling by THP-1 macrophages 2 h post-infection with PAO1, MOI=10. C57BL/6 mice intratracheally infected with 10^7 CFU PAO1 and treated with 25 ug/kg Ilomastat, 25 ug/kg Marimastat, or PBS control. After 24 h, mouse BAL was harvested and (**C**) IL-1β measured by enzyme-linked immunosorbent assay and (**D**) neutrophils enumerated. (**E**) Representative histology sections cytological smears of bronchoalveolar lavage fluid prepared with differential MGG stain. Where applicable, data are mean ± SEM and represent at least 3 independent experiments; significance determined by unpaired two-tailed Student's T-test, $*P < 0.05$.

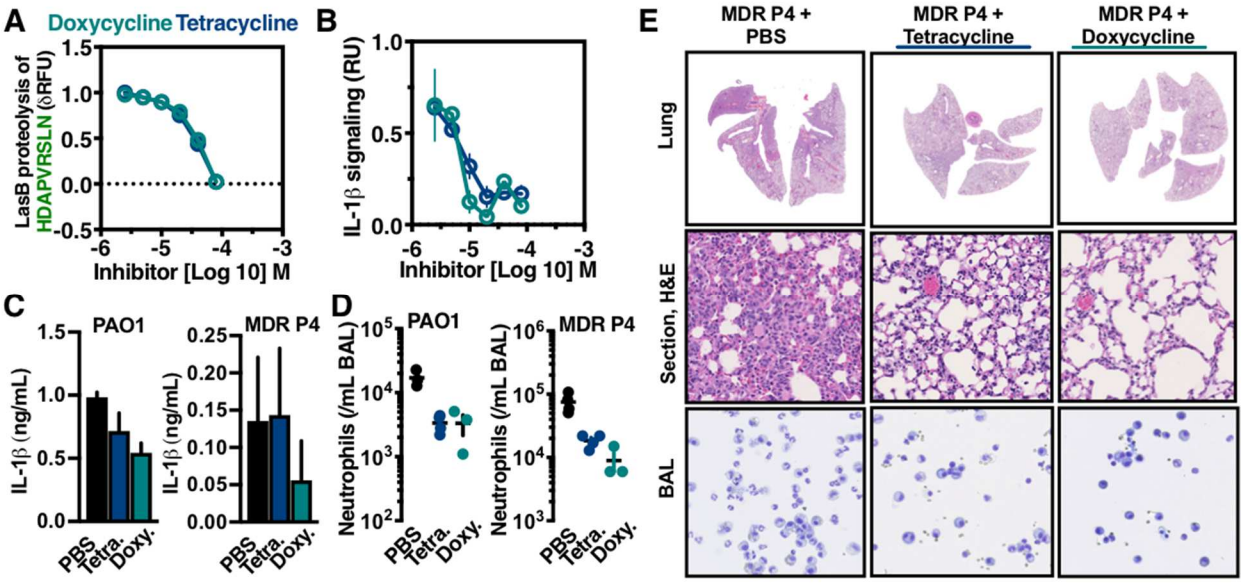


Figure 3.5. FDA approved metalloprotease inhibitors, tetracycline-family antibiotics, inhibit LasB and prevent IL-1β-mediated pathological inflammation. Doxycycline and Tetracycline inhibition of LasB proteolysis of (**A**) an internally quenched IL-1β fragment HDAPVRSLN or (**B**) IL-1 signaling by THP-1 macrophages 2 h post-infection with PAO1, MOI=10. C57BL/6 mice intratracheally infected with 10^7 CFU PAO1 or $5x10^6$ CFU antibiotic-resistant MDR-P4 and intraperitoneally treated with 1 mg/kg doxycycline or 1 mg/kg tetracycline. After 24 h, mouse BAL was harvested and (**C**) IL-1β measured by enzyme-linked immunosorbent assay and (**D**) neutrophils enumerated. (**E**) Representative histology sections and BAL cytological smears of BAL prepared with differential MGG stain. Where applicable, data are mean ± SEM and represent at least 3 independent experiments; significance determined by unpaired two-tailed Student's T-test, $*P < 0.05$.

3.5 DISCUSSION

Opportunistic *P. aeruginosa* lung infections can destroy tissue structure and impair organ function. Our findings reveal a mechanism by which a bacterial protease, LasB, contributes to pathological inflammation by directly activating the neutrophil chemokine IL-1β. LasB is one of the most abundant virulence factors in the lung microenvironment during *P. aeruginosa* infection and can cleave numerous host factors (128), even exerting broadly anti-inflammatory influences through destructive proteolysis of PAMPs such as flagellin (129), and various cytokines including IFN, IL-6, IL-8, MCP-1, TNF, trappin-2 and RANTES ((130-133). Consequently, LasB-deficient bacteria may preferentially induce a KC, IL-6, and IL-8 dominant inflammatory response ((130), whereas we find wild-type *P. aeruginosa* induce a strong IL-1β response.

LasB activates IL-1β through direct proteolytic removal of its inhibitory amino-terminal pro-domain, bypassing the need for host caspases. We recently hypothesized that IL-1β evolved as a sensor of diverse proteases ((134), a model further supported by the present discovery of a *P. aeruginosa* protease with this activity. In lung infection, LasB activation of IL-1β augments neutrophil recruitment and promotes destruction of the pulmonary tissue. IL-1β inhibition protects against this pathology, however, clinical interventions to date have utilized expensive biologics (e.g. IL-1R1 antagonists) associated with increased risk for severe infections (134, 135). The proteolytic activation of IL-1β may be a more tractable pharmacological target, made possible by disambiguation of the molecular networks involved, perhaps allowing the repurposing existing proteases inhibitors.

Inhibitors of caspase-1 are not clinically available, nor would they be predicted to have strong therapeutic utility, given our findings of inflammasome-independent IL-1β maturation via

LasB (Figure 4). Alpha-1-antitrypsin suppresses NE-mediated degradation of the CF lung ((136, 137), potentially also limiting pro-IL-1β maturation by NE (116), as well as LasB, which is also blocked by alpha-1-antitrypsin (138). Metalloprotease inhibitors such as marimastat and ilomastat may likewise prove beneficial by cross-inhibiting matrix metalloproteases contributing to CF pathology (139) alongside LasB (Figure 4). Immediately at hand is doxycycline, an antibiotic that also inhibits matrix remodeling (125-127), proinflammatory mitogen-activated protein kinases (MAPK) (140), reactive nitrogen species generation (141), and COX-2 (142). Doxycycline targeting of LasB (Figure S2) may be an adjunctive benefit toward restoration of lung homeostasis during *P. aeruginosa* infection, even when strains are resistant to the direct action of the antibiotic.

3.6 SUPPLEMENTAL

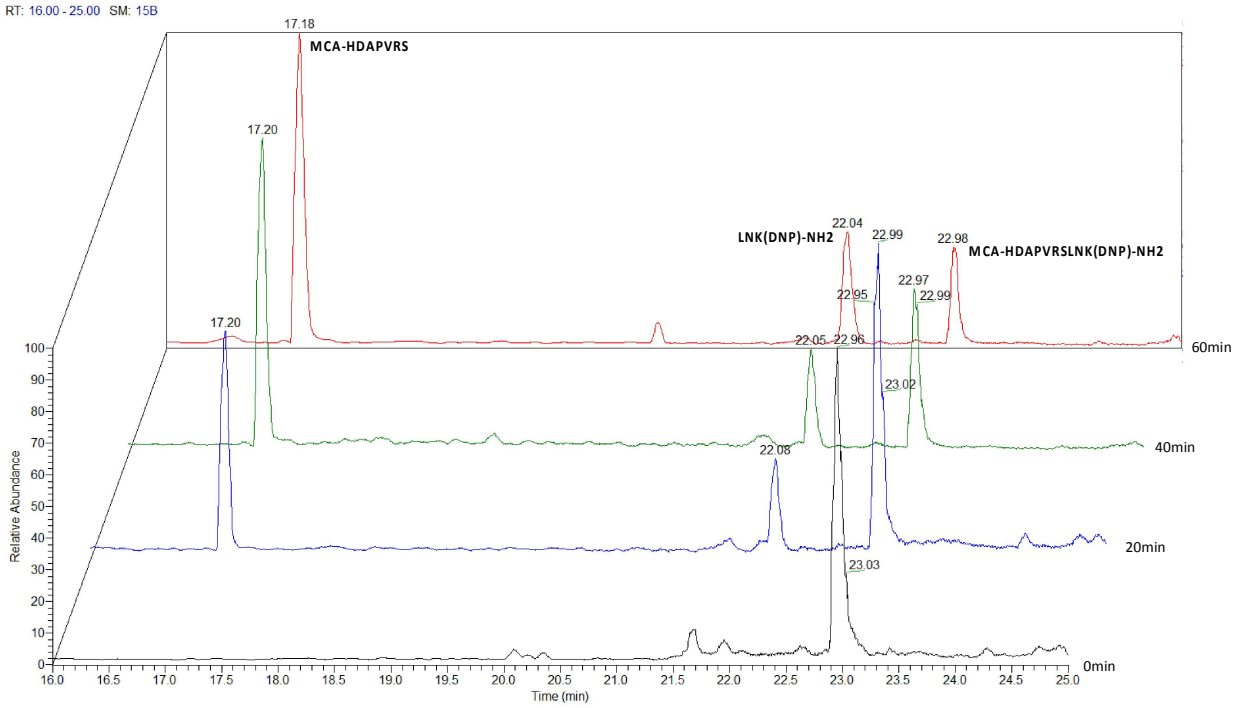


Figure S3.1 Mass spectrum showing the hydrolysis of MCA HDAPVRSLNK(DNP)-NH2 by LasB. The deposited dataset ftp://massive.ucsd.edu/MSV000081623 contains the processed mass spectrometry data used for generating the specificity logo for LasB contained in Fig. 3D.

3.7 MATERIALS AND METHODS

Bacterial Culture

P. aeruginosa PAO1 along with corresponding transposon Tn5 IS50L mutants disrupting the *lasB, fliC, aprA, lasA,* or *piv* (143) were generously provided by Professor Colin Manoil (University of Washington, Seattle, WA); other strains were acquired from ATCC or the UCSD clinical lab. Bacteria were routinely were propagated in Luria broth (LB) medium or on LB agar plates at 37 ºC. For infections, bacterial cultures were grown to late exponential phase (OD_{600} 1.2) then washed and diluted in PBS.

Animal Experiments

The UCSD Institutional Animal Care and Use Committee approved all animal use and procedures. Eight-to-ten week old female C57Bl/6 and isogenic caspase-1/-11$^{-/-}$ mice were anesthetized with ketamine/xylazine intraperitoneally, then 10^7 CFU PAO1 or 5 x 10^6 CFU MDR-P4 inoculated intratracheally in 30 ul of 1x PBS, 25ug/kg Ilomastat, and 25ug/kg Marimastat. For antibiotic treatment, 1 mg/kg Doxycycline or 1 mg/kg Tetracycline were administered intraperitoneally at time of infection. Mice were euthanized by CO_2 asphyxiation, and bronchiolar lavage fluid or lung homogenate were dilution plated onto LB agar plates for enumeration of bacterial CFU, or used for quantification of cytokines or protease activity. White blood cells in the bronchiolar lavage fluid were counted on a hemocytometer, while cytologic examination was performed on cytospin preparations fixed and stained using Hema 3 (Fisher HealthCare™ Hema 3™). Histologic sections were prepared from formalin-fixed and paraffin-embedded lungs, stained with hematoxylin and eosin (H&E). Cytospin and histology slides were imaged on a Hamamatsu Nanozoomer 2.0Ht Slide Scanner.

Bacterial Strain Construction

LasB and the upstream 260 bp regulatory region in PAO1 were cloned into pUC18T-mini-Tn7T-*hph* (144) using Polymerase Incomplete Primer Extension (PIPE) cloning (145) with primers caattcgatcatgcatgagctagctgccacctgcttttct (lasB-F), ccaagcttctcgaggaattccttacaacgcgctcggg (lasB-R), agaaaagcaggtggcagctagctcatgcatgatcgaattg (Tn7-F), and cccgagcgcgttgtaaggaattcctcgagaagcttgg (Tn7-R). Transformants into Top10 cells were selected on LB agar plates containing 100 μg/mL Hygromycin B (Life Technologies). Stable complementation into PAO1 Δ*lasB* with this construct was performed as previously described (144), and transformants selected with 400 μg/mL Hygromycin B.

pET-pro-IL-1β and the purification of pro-IL-1β have been previously described (134). Constructs for the expression of ^{116A}IL-1β, ^{122L}IL-1β, IL-1β^{L122A}, and IL-1β^{L122G} were generated by PIPE cloning from pET-proIL-1β (134) with the corresponding primers sets in the Key Resources table, and proteins were expressed and purified in the same manner as for pro-IL-1β previously (134).

***in vitro* infection models**

Macrophages were generated from femur exudates of wild-type C57Bl/6 (Jackson Laboratories) or caspase-1/11$^{-/-}$ (kindly provided by R. Flavell) mice as previously (134). THP-1, HL60, and A549 cells were propagated as detailed previously (146). One hour before infection, the media was replaced with RPMI lacking phenol red, fetal bovine serum, and antibiotics. Inhibitor treatments were added 1 h before infection and include: 20 μg/mL Anakinra (Amgen), 100 ng/mL rIL-1β (R&D Systems), 5 μM caspase inhibitors zVAD-fmk, YVAD-fmk, DEVD-fmk, and IETD-fmk (R&D Systems), 10 μg/mL complete protease inhibitor cocktail (Roche), 1x

protease inhibitors AEBSF, Antipain, Aprotinin, Bestatin, EDTA, E-64, Phosphoramidon, Pepstatin, and PMSF (G-Biosciences). Except when noted, cells were routinely infected by co-incubation with *P. aeruginosa* at a multiplicity of infection of 10, spun into contact for 3 min at 300 g, and cells or supernatants were harvested for analysis after 2 h.

Cytokine measurements

Relative IL-1 signaling by cells was measured by removing 50 µl supernatants from infected or treated cells onto transgenic IL-1R reporter cells (Invivogen). After 18 h, reporter cell supernatants were analyzed for secreted alkaline phosphatase activity using HEK-Blue Detection reagent (Invivogen). Cytokines were quantified by enzyme-linked immunosorbent assay following the manufacturer's protocol (R&D Systems).

Substrate specificity profiling

10 nM LasB (Elastin Products Co.) was incubated in triplicate with a mixture of 228 synthetic tetradecapeptides (0.5 uM each) in PBS, 2mM DTT as described previously (123). After 15, 60, 240 and 1200 min, aliquots were removed, quenched with 6.4 M GuHCl, immediately frozen at -80°C. Controls were performed with LasB treated with GuHCl prior to peptide exposure. Samples were acidified to < pH 3.0 with 1% formic acid, desalted with C18 LTS tips (Rainin), and injected into a Q Exactive Mass Spectrometer (Thermo) equipped with an Ultimate 3000 HPLC. Peptides separated by reverse phase chromatography on a C18 column (1.7 um bead size, 75 um x 20 cm, heated to 65°C) at a flow rate of 400 nl min^{-1} using a 55-min. linear gradient from 5% B to 30% B, with solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. Survey scans were recorded over a 150–2000 m/z range at 70000 resolutions (AGC

target 1×10^6, 75 ms maximum). MS/MS was performed in data-dependent acquisition mode with HCD fragmentation (30 normalized collision energy) on the 10 most intense precursor ions at 17500 resolutions (AGC target 5×10^4, 120 ms maximum, dynamic exclusion 15 s).

Peak integration and data analysis were performed using Peaks software (Bioinformatics Solutions Inc.). Quantification data are normalized by LOWESS and filtered by 0.3 peptide quality. Missing and zero values are imputed with random numbers in the range of the average of smallest 5% of the data ± sd. Enzymatic progress curves of each unique peptide were obtained by performing nonlinear least-squares fitting on their peak areas in the MS precursor scans using the first-order enzymatic kinetics model: $Y = (\text{plateau}-Y_0)\times(1-\exp(-t \times k_{cat}/K_M \times [E_0])) + Y_0$, where E_0 is the total enzyme concentration. Nonlinear fitting was performed on cleavage products only if the following criteria were met: Peptides were detected in at least 2 of the 3 replicates and the peak intensity of peptides increased by >50,000 and >5-fold over the course of the assay. Proteolytic efficiency was solved from the progress curves by estimating total enzyme concentration and is reported as k_{cat}/K_M and clustered into 8 groups by Jenks optimization method. IceLogo software was used for visualization of amino-acid frequency from P2 to P2′ positions using cleavage sequences in top 3 clusters (118 sequences). Mass spectrometry files can be found at ftp://massive.ucsd.edu/MSV000081623.

Protease Measurements

Internally-quenched peptides 7-Methoxycoumarin- (Mca) labeled on the amino terminus and 2, 4-dinitrophenyl (Dnp) on the carboxy terminus were synthesized with the sequences of IFFDTWDNE, TWDNEAYVH, EAYVHDAPV, and HDAPVRSLN, corresponding to amino acids 103-111, 107-115, 111-119, and 115-123 of the reference human pro-IL-1β sequence

(UniProt: P01584; CPC Scientific). In triplicate, 10 μM peptides were incubated in PBS, 1 mM $CaCl_2$, 0.01% Tween-20, with 5 nM human caspase-1 (Enzo) or LasB (Elastin Products Co.). The reaction was continuously monitored using an EnSpire plate reader (PerkinElmer) with fluorophore excitation at 323 nm and emission at 398 nm and the maximum kinetic velocity calculated as previously (134).

The cleavage site was determined by incubating 10 nM of LasB with 10 uM of HDAPVRSLN. At 20, 40 and 60 min intervals each reaction was quenched with 6.4 M GuHCl and the cleavage products desalted and analyzed by mass spectrometry as described above, except using a 20-min linear gradient from 5% B to 50% B and only selecting top 5 peptides for MS/MS.

Statistical Analysis

Statistical significance was calculated by unpaired Student t test (*, $P < 0.05$; **, $P < 0.005$) using GraphPad Prism, unless otherwise indicated. Data are representative of at least three independent experiments. For iceLogo plots, only amino acids that a significantly ($P < 0.05$) increased or decreased in frequency are shown.

3.8 ACKNOWLEDGMENTS

Chapter 3, in full, is a manuscript submitted to *Cell Reports* as: Sun J, LaRock D, Kimmey J, Olson J, Jiang Z, O'Donoghue A, Nizet V, LaRock CN. Inflammasome-independent activation of IL-1β by *Pseudomonas aeruginosa* protease LasB induces pathological inflammation. The dissertation author is first author of this work. We thank Christopher Lietz (UCSD) for assistance with mass spectrometry and data analysis, the UCSD Histopathology Core facility, the UCSD Neuroscience Microscopy Shared Facility (P30 NS047101), Jason Munguia (UCSD) for technical assistance, and Joanna Goldberg (Emory) for helpful advice and discussions. J.S. received support from NIH/NIGMS T32 GM007752, J.K. from a UC President's Postdoctoral Fellowship, V.N. from NIH/NICHD grant U54 HD090259 and NIH/NHLBI R01 HL125352, and C.L. the A.P. Giannini Foundation and NIH/NIAID K22 AI055396. C.L. has a research agreement with Antabio developing small molecule inhibitors of LasB.

AUTHOR CONTRIBUTIONS

J.S., A.O., V.N., A.O., and C.L. designed experiments and interpreted the data. J.S., D.L., J.K., J.O., Z.J., A.O., and C.L conducted the studies. J.S., V.N., and C.L. wrote the manuscript with the assistance of all of the authors.

3.9 REFERENCES

1. Abdul-Sater, A.A., Tattoli, I., Jin, L., Grajkowski, A., Levi, A., Koller, B.H., Allen, I.C., Beaucage, S.L., Fitzgerald, K.A., and Ting, J.P.Y. *Cyclic-di-GMP and cyclic-di-AMP activate the NLRP3 inflammasome.* EMBO reports. 2013, **14**(10): p. 900-906.

2. Amin, A.R., Attur, M.G., Thakker, G.D., Patel, P.D., Vyas, P.R., Patel, R.N., Patel, I.R., and Abramson, S.B.. *A novel mechanism of action of tetracyclines: effects on nitric oxide synthases.* Proceedings of the National Academy of Sciences. 1996, **93**(24): p. 14014-14019.

3. Anantharajah, A., Buyck, J.M., Faure, E., Glupczynski, Y., Rodriguez-Villalobos, H., De Vos, D., Pirnay, J.P., Bilocq, F., Guery, B., and Tulkens, P.M. *Correlation between cytotoxicity induced by Pseudomonas aeruginosa clinical isolates from acute infections and IL-1β secretion in a model of human THP-1 monocytes.* Pathogens and disease. 2015, **73**(7): p. 049.

4. Basso, P., Ragno, M., Elsen, S., Reboud, E., Golovkine, G., Bouillot, S., Huber, P., Lory, S., Faudry, E., and Attrée, I. *Pseudomonas aeruginosa pore-forming exolysin and Type IV Pili Cooperate to induce host cell lysis.* MBio. 2017, **8**(1): e. 02250-02216.

5. Baxter, B.T., Pearce, W.H., Waltke, E.A., Littooy, F.N., Hallett, J.W., Kent, K.C., Upchurch, G.R., Chaikof, E.L., Mills, J.L., and Fleckten, B. *Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (Phase II) multicenter study.* Journal of vascular surgery. 2002, **36**(1): p. 1-12.

6. Beaufort, N., Corvazier, E., Mlanaoindrou, S., de Bentzmann, S., and Pidard, D. *Disruption of the endothelial barrier by proteases from the bacterial pathogen Pseudomonas aeruginosa: implication of matrilysis and receptor cleavage.* PLoS One. 2013, **8**(9): e. 75708.

7. Beringer, P.M., Owens, H., Nguyen, A., Benitez, D., Rao, A., and D'Argenio, D.Z. *Pharmacokinetics of doxycycline in adults with cystic fibrosis.* Antimicrobial agents and chemotherapy. 2012, **56**(1): p. 70-74.

8. Cabral, V.P., Andrade, C.A.F.d., Passos, S.R.L., Martins, M.d.F.M., and Hökerberg, Y.H.M. *Severe infection in patients with rheumatoid arthritis taking anakinra, rituximab, or abatacept: A systematic review of observational studies.* Revista brasileira de reumatologia. 2016, **56**(6): p. 543-550.

9. Cantin, A.M., and Woods, D.E. *Aerosolized prolastin suppresses bacterial proliferation in a model of chronic Pseudomonas aeruginosa lung infection.* American journal of respiratory and critical care medicine. 1999, **160**(4): p. 1130-1135.

10. Casilag, F., Lorenz, A., Krueger, J., Klawonn, F., Weiss, S., and Häussler, S. *The LasB elastase of Pseudomonas aeruginosa acts in concert with alkaline protease AprA to prevent flagellin-mediated immune recognition.* Infection and immunity. 2016, **84**(1): p. 162-171.

11. Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S., Wieczorek, M., Kroona, H., Leimer, A.H., and Cheronis, J. *Converting enzyme-independent release of tumor necrosis factor α and IL-1β from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3.* Proceedings of the National Academy of Sciences of the United States of America. 1999, **96**: p. 6261-6266.

12. Conese, M., Copreni, E., Di Gioia, S., De Rinaldis, P., and Fumarulo, R. *Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. Journal of Cystic fibrosis.* 2003, **2**(3): p. 129-135.

13. Franchi, L., Stoolman, J., Kanneganti, T.D., Verma, A., Ramphal, R., and Núñez, G. *Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation.* European journal of immunology. 2007, **37**(11): p. 3030-3039.

14. Fritzsching, B., Zhou-Suckow, Z., Trojanek, J.B., Schubert, S.C., Schatterny, J., Hirtz, S., Agrawal, R., Muley, T., Kahn, N., and Sticht, C. *Hypoxic Epithelial Necrosis Triggers Neutrophilic Inflammation via IL-1 Receptor Signaling in Cystic Fibrosis Lung Disease.* American journal of respiratory and critical care medicine. 2015, **191**(8): p. 902-913.

15. Gaggar, A., Hector, A., Bratcher, P.E., Mall, M.A., Griese, M., and Hartl, D. *The role of matrix metalloproteinases in cystic fibrosis lung disease.* European Respiratory Journal. 2011, **38**(3): p. 721-727.

16. Ganesan, S., Rathinam, V.A.K., Bossaller, L., Army, K., Kaiser, W.J., Mocarski, E.S., Dillon, C.P., Green, D.R., Mayadas, T.N., Levitz, S.M., et al. *Caspase-8 modulates dectin-1 and complement receptor 3-driven IL-1beta production in response to beta-glucans and the fungal pathogen, Candida albicans.* J Immunol. 2014, **193**(5): p. 2519-2530.

17. Greten, F.R., Arkan, M.C., Bollrath, J., Hsu, L.-C., Goode, J., Miething, C., Göktuna, S.I., Neuenhahn, M., Fierer, J., and Paxian, S. *NF-κB is a negative regulator of IL-1β secretion as revealed by genetic and pharmacological inhibition of IKKβ*. Cell. 2007, **130**(5): p. 918-931.

18. Hsu, L.-C., Enzler, T., Seita, J., Timmer, A.M., Lee, C.-Y., Lai, T.-Y., Yu, G.-Y., Lai, L.-C., Temkin, V., Sinzig, U., et al. *IL-1 [beta]-driven neutrophilia preserves antibacterial defense in the absence of the kinase IKK [beta]*. Nature immunology. 2011, **12**(2): p. 144-150.

19. Jaffar-Bandjee, M.C., Lazdunski, A., Bally, M., Carrère, J., Chazalette, J.P., and Galabert, C. *Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by Pseudomonas aeruginosa.* Journal of Clinical Microbiology. 1995, **33**(4): p. 924-929.

20. Karmakar, M., Sun, Y., Hise, A.G., Rietsch, A., and Pearlman, E. *Cutting edge: IL-1β processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1.* The Journal of Immunology. 2012, **189**(9): p. 4231-4235.

21. Kessler, E., Safrin, M., Gustin, J.K., and Ohman, D.E. *Elastase and the LasA Protease of Pseudomonas aeruginosa Are Secreted with Their Propeptides.* Journal of Biological Chemistry. 1998, **273**(46): p. 30225-30231.

22. Konstan, M.W., Byard, P.J., Hoppel, C.L., and Davis, P.B. *Effect of high-dose ibuprofen in patients with cystic fibrosis.* New England Journal of Medicine. 1995, **332**(13): p. 848-854.

23. Kung, V.L., Khare, S., Stehlik, C., Bacon, E.M., Hughes, A.J., and Hauser, A.R. *An rhs gene of Pseudomonas aeruginosa encodes a virulence protein that activates the inflammasome.* Proceedings of the National Academy of Sciences. 2012, **109**(4): p. 1275-1280.

24. Labrousse, D., Perret, M., Hayez, D., Da Silva, S., Badiou, C., Couzon, F., Bes, M., Chavanet, P., Lina, G., and Vandenesch, F. *Kineret®/IL-1ra Blocks the IL-1/IL-8 Inflammatory Cascade during Recombinant Panton Valentine Leukocidin-Triggered Pneumonia but Not during S. aureus Infection.* PloS one. 2014, **9**(6): e. 97546.

25. LaFayette, S.L., Houle, D., Beaudoin, T., Wojewodka, G., Radzioch, D., Hoffman, L.R., Burns, J.L., Dandekar, A.A., Smalley, N.E., and Chandler, J.R. *Cystic fibrosis–adapted Pseudomonas aeruginosa quorum sensing lasR mutants cause hyperinflammatory responses*. Science advances. 2015, **1**(6): e1500199.

26. LaRock, C.N., and Cookson, B.T. *The Yersinia Virulence Effector YopM Binds Caspase-1 to Arrest Inflammasome Assembly and Processing*. Cell Host & Microbe. 2012, **12**(6): p. 799-805.

27. LaRock, C.N., and Cookson, B.T. *Burning down the house: cellular actions during pyroptosis.* PLoS pathogens. 2013, **9**(12): e. 1003793.

28. LaRock, C.N., Todd, J., LaRock, D., Olson, J., O'Donoghue, A.J., Robertson, A.A.B., Cooper, M.A., Hoffman, H.M., and Nizet, V. *IL-1β is an innate immune sensor of microbial proteolysis.* Science Immunology. 2016, **1**(2).

29. Li, H., O'Donoghue, A.J., van der Linden, W.A., Xie, S.C., Yoo, E., Foe, I.T., Tilley, L., Craik, C.S., da Fonseca, P.C.A., and Bogyo, M. *Structure and function based design of Plasmodium-selective proteasome inhibitors.* Nature. 2016, **530**(7589): p. 233.

30. Lin, A.E., Beasley, F.C., Keller, N., Hollands, A., Urbano, R., Troemel, E.R., Hoffman, H.M., and Nizet, V. *A Group A Streptococcus ADP-Ribosyltransferase Toxin Stimulates a Protective Interleukin 1β-Dependent Macrophage Immune Response*. mBio. 2015, **6**(2): e. 00133-00115.

31. Lindestam Arlehamn, C.S., and Evans, T.J. *Pseudomonas aeruginosa pilin activates the inflammasome.* Cellular microbiology. 2011, **13**(3): p. 388-401.

32. Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., and Beyaert, R. *Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8.* J Exp Med. 2008, **205**(9): p. 1967-1973.

33. Martin, S.L., Downey, D., Bilton, D., Keogan, M.T., Edgar, J., and Elborn, J.S. *Safety and Efficacy of Recombinant Alpha1-Antitrypsin Therapy in Cystic Fibrosis.* Pediatric pulmonology. 2006, **41**(2): p. 177-183.

34. Matheson, N.R., Potempa, J., and Travis, J. *Interaction of a novel form of Pseudomonas aeruginosa alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. Biological chemistry.* 2006, **387**(7): p. 911-915.

35. Miao, E.A., Ernst, R.K., Dors, M., Mao, D.P., and Aderem, A. *Pseudomonas aeruginosa activates caspase 1 through Ipaf.* Proceedings of the National Academy of Sciences. 2008, **105**(7): p. 2562-2567.

36. Miao, E.A., Mao, D.P., Yudkovsky, N., Bonneau, R., Lorang, C.G., Warren, S.E., Leaf, I.A., and Aderem, A. *Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome.* Proceedings of the National Academy of Sciences of the United States of America. 2010, **107**(7): p. 3076-3080.

37. Nakamura, H., Yoshimura, K., McElvaney, N.G., and Crystal, R.G. *Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line.* Journal of Clinical Investigation. 1992, **89**(5): p. 1478.

38. O'Connor, C.M., Gaffney, K., Keane, J., Southey, A., Byrne, N., O'Mahoney, S., and Fitzgerald, M.X. *~ 1-Proteinase Inhibitor, Elastase Activity, and Lung Disease Severity in Cystic Fibrosis.* American Review of Respiratory Disease. 1993, **148**: p. 1665-1665.

39. O'Donoghue, A.J., Eroy-Reveles, A.A., Knudsen, G.M., Ingram, J., Zhou, M., Statnekov, J.B., Greninger, A.L., Hostetter, D.R., Qu, G., and Maltby, D.A. *Global identification of peptidase specificity by multiplex substrate profiling.* 2012, Nature methods. **9**(11): p. 1095-1100.

40. O'Donoghue, A.J., Knudsen, G.M., Beekman, C., Perry, J.A., Johnson, A.D., DeRisi, J.L., Craik, C.S., and Bennett, R.J. *Destructin-1 is a collagen-degrading endopeptidase secreted by Pseudogymnoascus destructans, the causative agent of white-nose syndrome.* Proceedings of the National Academy of Sciences. 2015, **112**(24): p. 7478-7483.

41. Oh, J., Li, X.H., Kim, S.K., and Lee, J.H. *Post-secretional activation of Protease IV by quorum sensing in Pseudomonas aeruginosa*. 2017, Sci Rep. **7**(1): p. 4416.

42. Parmely, M., Gale, A., Clabaugh, M., Horvat, R., and Zhou, W.W. *Proteolytic inactivation of cytokines by Pseudomonas aeruginosa.* Infection and Immunity. 1990, **58**(9): p. 3009-3014.

43. Patel, R.N., Attur, M.G., Dave, M.N., Patel, I.V., Stuchin, S.A., Abramson, S.B., and Amin, A.R. *A novel mechanism of action of chemically modified tetracyclines: inhibition of COX-2-mediated prostaglandin E2 production.* The Journal of Immunology. 1999, **163**(6): p. 3459-3467.

44. Roncase, E.J., Moon, C., Chatterjee, S., González-Páez, G.E., Craik, C.S., O'Donoghue, A.J., and Wolan, D.W. *Substrate Profiling and High Resolution Co-complex Crystal Structure of a Secreted C11 Protease Conserved across Commensal Bacteria.* ACS Chemical Biology. 2017, **12**(6): p. 1556.

45. Saint-Criq, V., Villeret, B., Bastaert, F., Kheir, S., Hatton, A., Cazes, A., Xing, Z., Sermet-Gaudelus, I., Garcia-Verdugo, I., and Edelman, A. *Pseudomonas aeruginosa LasB protease impairs innate immunity in mice and humans by targeting a lung epithelial cystic fibrosis transmembrane regulator–IL-6–antimicrobial–repair pathway.* Thorax. 2017, p. 210298.

46. Sapadin, A.N., and Fleischmajer, R. *Tetracyclines: nonantibiotic properties and their clinical implications.* Journal of the American Academy of Dermatology. 2006, **54**(2): p. 258-265.

47. Schultz, M.J., Rijneveld, A.W., Florquin, S., Edwards, C.K., Dinarello, C.A., and van der Poll, T. *Role of interleukin-1 in the pulmonary immune response duringPseudomonas aeruginosa pneumonia.* American Journal of Physiology-Lung Cellular and Molecular Physiology. 2002, **282**(2): p. L285-L290.

48. Suter, S., Schaad, U.B., Roux, L., Nydegger, U.E., and Waldvogel, F.A. *Granulocyte neutral proteases and Pseudomonas elastase as possible causes of airway damage in patients with cystic fibrosis.* Journal of Infectious Diseases. 1984, **149**(4): p. 523-531.

49. Sutterwala, F.S., Mijares, L.A., Li, L., Ogura, Y., Kazmierczak, B.I., and Flavell, R.A. *Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome*. The Journal of Experimental Medicine. 2007, **204**(13): p. 3235-3245.

50. Thompson, R.W., and Baxter, B.T. *MMP inhibition in abdominal aortic aneurysms: rationale for a prospective randomized clinical trial.* Annals of the New York Academy of Sciences. 1999, **878**(1): p. 159-178.

51. Twigg, M.S., Brockbank, S., Lowry, P., FitzGerald, S.P., Taggart, C., and Weldon, S. *The role of serine proteases and antiproteases in the cystic fibrosis lung.* Mediators of inflammation. 2015, p. 293053.

52. Van Opdenbosch, N., Van Gorp, H., Verdonckt, M., Saavedra, P.H., de Vasconcelos, N.M., Gonçalves, A., Walle, L.V., Demon, D., Matusiak, M., and Van Hauwermeiren, F. *Caspase-1 Engagement and TLR-Induced c-FLIP Expression Suppress ASC/Caspase-8-Dependent Apoptosis by Inflammasome Sensors NLRP1b and NLRC4.* Cell reports. 2017, **21**(12): p. 3427-3444.

CHAPTER 4

CONCLUSIONS

The current rate by which we discover novel antibiotic classes is unfortunately sluggish and poses a high-risk investment that deters financial interest. Thus, this lack of antibiotic drug discovery and the increasing rate of drug resistance amongst bacterial pathogens is fueling the eminent rise of a "post-antibiotic" era – an era in which minor wounds can result in deadly and incurable infections. In fact, drug resistance has been identified in a variety of bacterial isolates against all available antibiotics. Taking this into consideration, we raise a pressing question – is the discovery of new antibiotics truly the most effective and efficient method by which we combat the rise in drug resistance? If so, are we only providing a temporary solution to an infinitely long evolutionary arms race against the development of antibiotic resistance? Antibiotic drug discovery was indeed the pillar of modern medical success and a major driver of the advancement of the pharmaceutical industry – having saved hundreds of millions of lives and globally increased the average life-expectancy rate. Nonetheless, how do we pay close attention to the historic success of antibiotic efficacy, without compromising the identification of innovative or alternative methods in which we can utilize to fight against this ever-growing problem?

In both chapters, we identified FDA-approved drugs with unconventional therapeutic applications against two very different bacterial infections. In chapter 1, we demonstrated that targeting multiple levels of the *Staphylococcus aureus*-induced dysregulation of the platelet clearance pathway, by ways of preventing aberrant platelet clearance, is protective against mortality during *S. aureus* bacteremia. We identified platelet-inhibitor, Ticagrelor (Brilinta^{TM}), and sialidase inhibitor, Oseltamivir (Tamiflu^{TM}) as ready-to-use adjunctive agents alongside

antibiotic therapy to improve patient outcome. Furthermore, we reinforced our definition of a platelet as a critical immune cell, and not just a cell that initiates blood coagulation.

In chapter 2, we identified and validated an inflammatory target against IL-1β-mediated pathological lung damage during pseudomonas pulmonary infection. Pseudomonas metalloprotease, LasB, is a non-canonical, or inflammasome-independent, activator of IL-1β that drives excessive pulmonary neutrophil recruitment. Inhibition of LasB with FDA-approved metalloprotease inhibitor, Doxycycline, mitigates LasB activity, and returns the host inflammatory response to normalcy and prevents excessive lung damage.

Cumulatively, our work suggests that FDA-approved drugs are currently over-compartmentalized to a specified disease state, thus disregarding the potential therapeutic efficacy against a wide spectrum of disorders. Though a specific and compartmentalized approach towards drug development is critical, especially in regards to the monitoring of drug-related adverse effects, we need to utilize such drugs to its full therapeutic potential.