1. Advanced Profile

Other Required Profile Information

Immigration (status/visa) :	
Current Academic Title :	
Current Academic Position :	
Career Stage :	

Institution Where Work Will Be Done

Institution :	University of Pittsburgh
Department :	
Division :	

Professional Time Usage Details

Administration :	0%
Patient Care :	0%
Research :	0%
Teaching :	0%
Coursework :	0%
Other :	0%
Total :	0%

2. Project Summary

Application Info

Application ID :	18PRE33960033
Funding Component Name :	Association Wide
Program Name :	Summer 2018 Predoctoral Fellowship
Principal Investigator Name :	Tolani Olonisakin
Status :	Submitted to GO
Award Start Date :	07-01-2018
Project Title :	Role of thrombospondin-1 in platelet-mediated protection during Pseudomonas aeruginosa-induced injury
Application Deadline date :	11-01-2017
Award End Date :	06-30-2020

Project Summary Details

Percent of Applicant's total effort devoted to this project :	100
Project Name :	Role of thrombospondin-1 in platelet-mediated protection during Pseudomonas aeruginosa-induced injury

Project Summary

Nosocomial pneumonia remains a primary complication following acute stroke that is associated with increased mortality. Though several pathogens are known to cause post-stroke pneumonia, Pseudomonas aeruginosa is unique among causative agents of post-stroke pneumonia as mortality in nosocomial pneumonia due to P. aeruginosa is distinctively high. Secretion of toxic exoproducts enhances virulence of P. aeruginosa, yet host factors that disarm P. aeruginosa-encoded virulence factors and protect against injury are not fully understood. The proposed research project identifies thrombospondin-1 (TSP-1), a matricellular protein found in the airspaces following severe lung inflammatory injury, as a major constituent of platelet alpha-granules that protects against pathogen-mediated proteolysis in the lung. We have previously shown that TSP-1 is an endogenous protease inhibitor of neutrophil granule proteases and that TSP-1 promotes resolution of experimental lung injury. We thus hypothesize that beyond repairing vascular injury induced by P. aeruginosa, platelets provide protection through the release of TSP-1 to disarm pathogen-encoded protease and curtail lung inflammation. Our work highlights TSP-1, a principal constituent of platelets, as an endogenous host factor that may lessen severity of P. aeruginosa-induced lung injury in critically ill infected stroke patients and suggests that TSP1-targeted therapeutics may prove useful in nosocomial P. aeruginosa infection.

3. Science Classification

Research Classification Type

Research Classification Type:	Basic Research is meant to increase our scientific knowledge base by studying fundamental life processes. This type of research is aimed at increasing our understanding of basic biological, behavioral or disease mechanisms. While Basic Research may ultimately lead to treatments or therapies, the goal of Basic Research studies is to establish a proof of principle. Research that exclusively involves in vitro studies using human tissues not linked to identified individuals falls within Basic Research.
Is the project translational?	Yes
Explanation:	Findings from the study may pave way for therapeutics that reduce severity of post-stroke pneumonia and lower incidence of morbidity and mortality.

Science Classification

Major Science Classification(1)	Sub-Classification(s)
Lung Respiration and Resuscitation - Basic Science	1. ARDS Acute Respiratory Distress Syndromes and Acute Lung Injury
Major Science Classification(2)	**Sub-Classification(s)**
Microbiology and Microbial Pathogenesis - Basic Science	1. Host - Pathogen Interactions 2. Host Response and Inflammation

4. Research Classification

Research Classification 1

CVD/Stroke Classification :	Broadly Stroke-Related

5. Institutional Personnel

Grants Officer 1

Position Title :	
First Name :	Jennifer
Last Name :	Woodward
Phone Number :	
E-mail :	offres@pitt.edu

Fiscal Officer 1

Position Title :	
First Name :	Mark
Last Name :	Stofko
Phone Number :	
E-mail :	fiscalofficer@cfo.pitt.edu

6. Third Party Personnel

Sponsor 1

Degree :	MD
Institution Name :	University of Pittsburgh
Position Title :	Professor of Medicine
First Name :	Janet
Last Name :	Lee
Phone Number :	4126922210
E-mail :	leejs3@upmc.edu

Referent 1

Degree :	
Institution Name :	University of Pittsburgh
Position Title :	Associate Professor of Medicine and Pharmacology
First Name :	Richard
Last Name :	Steinman
Phone Number :	
E-mail :	steinman@pitt.edu

Referent 2

Degree :	
Institution Name :	University of Pittsburgh
Position Title :	Professor of Medicine
First Name :	Sally
Last Name :	Wenzel
Phone Number :	

E-mail :	wenzelse@upmc.edu

Referent 3

Degree :	
Institution Name :	University of Southern California
Position Title :	Associate Professor of Medicine
First Name :	Nuria
Last Name :	Pastor-Soler
Phone Number :	
E-mail :	pastorso@usc.edu

Consultant 1

Degree :	MD
Institution Name :	University of Pittsburgh Medical Center
Position Title :	Associate Professor of Medicine, Cell Biology & Pediatrics
First Name :	Joseph
Last Name :	Pilewski
Phone Number :	
E-mail :	pilewskijm@upmc.edu

Consultant 2

Degree :	MD
Institution Name :	University of Michigan Medical Center
Position Title :	Henry Sewall Professor of Medicine and Chief
First Name :	Theodore
Last Name :	Standiford

Phone Number :	
E-mail :	tstandif@med.umich.edu

7. Lay Summary Form

Research Categories

Research Name :	1. Basic biomedical research 2. Stroke

Lay Summary Form

1. What is the major problem being addressed by this study? :	Pneumonia is an important complication of acute stroke. Patients that develop pneumonia after acute stroke have a higher risk of death and a poorer long-term outcome compared to stroke patients that do not develop pneumonia. Though antibiotics are frequently prescribed to treat pneumonia in stroke patients, some bacteria such as Pseudomonas aeruginosa are highly resistant and many patients that develop Pseudomonas aeruginosa pneumonia do not respond to antibiotic treatment. Our research is aimed at identifying factors that can help patients fight Pseudomonas aeruginosa pneumonia. Rather than target the infectious organism (as is traditionally done through antibiotic use), we are proposing host-targeted therapies that enhance our patients' own natural defense mechanisms in fighting bacteria

2. What specific questions are you asking and how will you attempt to answer them?	We present evidence that thrombospondin-1 (TSP-1), a protein found primarily in platelets, can inhibit a toxic product secreted by Pseudomonas aeruginosa. We have previously shown that this protein TSP-1 enhances resolution of lung injury in mice and tempers neutrophil function during bacterial infection. We will now test how effective this protein is in inhibiting Pseudomonas aeruginosa's toxic exoproduct and attempt to understand how this protein regulates our immune response to Pseudomonas aeruginosa.
3. Overall, what is the potential impact of this work to the mission of the AHA? You might address: What is the long-term biomedical significance of your work, particularly as it pertains to the cardiovascular area? What major therapeutic advance(s) do you anticipate that it will lead to? For instance, new drug(s), a surgical technique/procedure, a diagnostic tool/test, a previously undetected risk factor, etc :	Antibiotic resistance remains a problem in treating patients that develop pneumonia following an acute stroke, especially in pneumonia due to Pseudomonas aeruginosa. The findings from this project may help identify a host protein that lessens severity of Pseudomonas aeruginosa pneumonia in critically ill stroke patients and pave way for new therapy that circumvents antibiotic resistance in our stroke patients.

8. Budget

Expense Type Name :	Fringe
Yearly Fund :	1000.0, 1000.0
Expense Type Name :	Salary
Yearly Fund :	23844.0, 23844.0
Expense Type Name :	Project Support
Yearly Fund :	2000.0, 2000.0
Total :	53688.00

9. Funds Available

Funds Available Details

Do you have any active, pending, or approved awards?	false

10. Research Subjects / Assurances

Endorsement 1

Description :	Adult Human Stem Cells
Answer :	no
DocumentName :	

Endorsement 2

Description :	Biohazards (other than Recombinant DNA)
Answer :	no
DocumentName :	

Endorsement 3

Description :	Cloning
Answer :	no
DocumentName :	

Endorsement 4

Description :	Human Subjects
Answer :	no
DocumentName :	

Endorsement 5

Description :	Recombinant DNA
Answer :	no
DocumentName :	

Endorsement 6

Description :	Human Embryonic or Fetal Stem Cells
Answer :	no
DocumentName :	

Endorsement 7

Description :	Human Fetal Tissue
Answer :	no
DocumentName :	

Endorsement 8

Description :	Vertebrate Animal Subject
Answer :	yes
DocumentName :	AHA_Vertebrate Animals.pdf

Vertebrate Animal Subjects - AHA_Vertebrate Animals.pdf

VERTEBRATE ANIMAL SUBJECTS

1. **Provide a description of the proposed use of the animals in the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.**

Table outlining projected numbers of animals required for the funding period:

Strain	Aim 1	Aim 2
Thbs1$^{-/-}$	16	16
Thbs1$^{+/+}$	16	16
Mpl$^{-/-}$	16	N/A
Mpl$^{+/+}$	16	N/A
Thbs1$^{lox/lox}$	3	N/A
Pf4-Cre	9	N/A

Total mice needed = 108

We will use 6-12-week-old, gender-matched mice for experiments proposed. Male and female mice will be used for experiments.

Aim 1 involves administering purified TSP-1 to *Thbs1*$^{-/-}$, *Mpl*$^{-/-}$, and WT littermate controls following acute PA infection. We require 8 recipient mice/group x 4 groups of interest x 2 independent times performed = 64 mice required. Aim 1 also involves generating platelet-specific TSP-1 conditional knockout mice to assess contribution of platelet-derived TSP-1 to protection during PA-induced injury. We will require setting up 3 breeder cages with each cage containing 1 *Thbs1*$^{-/-}$ stud male and 3 harem *Pf4-Cre* females = 12 mice required.

Aim 2 will employ HBE cell culture to probe cellular effects of truncated IL-36γ generated by LasB. Aim 2 will also involve experiments confirming the role of IL-36γ signaling in PA-infected *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ mice. We will require 8 mice/group x 2 groups of interest x 2 independent times performed = 32 mice required.

2. **Justify the use of animals, the choice of species, and the numbers to be used. If animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and numbers.**

The most important reason for the animal experiments proposed is to determine the in vivo relevance of our pathway of interest in the setting of inflammation and injury to the lungs. The availability of mice with different genetic deficiencies provides a unique opportunity to test hypotheses in a biological system.

We will require group sizes of 8 for the in vivo experiments proposed below. The main outcomes are alveolar injury and bacterial burden at a given time point. The group size of 8 will achieve a power of 0.83 for a two-tailed test using numbers from our prior pneumonia studies where group 1 average = 8.214e6 cfu with std dev=1.184e7 cfu compared with group 2 average = 6.54e8 cfu with std dev = 6.275e8. This is using significance level (alpha) of 0.05. We realize that the estimated number of animals needed calculated by power analysis have limitations and are only projected values.

Vertebrate Animal Subjects - AHA_Vertebrate Animals.pdf

3. **Provide information on the veterinary care of the animals involved.**

   Each cage holds a maximum of 4 adult animals. Caged are cleaned weekly. All mice are handled according to the regulations of the American Association for the Accreditation of Laboratory Animal Care. University of Pittsburgh Medical Center is a member in good standing of this organization and strictly adheres to their regulations for the welfare and humane treatment of animals.

4. **Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices, where appropriate, to minimize discomfort, distress, pain, and injury.**

   During the studies, mice will be euthanized if they show signs of respiratory distress or have weight loss ≥ 20% body weight. Mice will be euthanized if they meet at least three of the following criteria: (1) Dehydration (evaluated by skin tenting test): (2) Lethargy and decreased movement or agitation and hyperactivity; (3) Show abnormal posture such as hunching and ruffled fur; (4) Have pale eyes; (5) Loose stools. Mice will also be euthanized if there is concern raised by the veterinarian regarding its welfare.

5. **Describe any method of euthanasia to be used and the reasons for its selection. State whether this method is consistent with the recommendation of the Panel on Euthanasia of the American Veterinary Medical Association.**

   At the specified times, mice will be euthanized with isoflurane inhalation in a closed system followed by cardiac puncture/exsanguination. These methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

11. Science and Evaluation Information

11.1. Applicant's Biographical Sketch

Applicant's Biographical Sketch - Biosketch_Tolani Olonisakin_AHA 2017.pdf

OMB No. 0925-0001 (Rev. 08/12 Approved Through 8/31/2015)

APPLICANT BIOGRAPHICAL SKETCH—Instructions

Use only for individual predoctoral and postdoctoral fellowships, dissertation research grants (R36),and Research Supplements to Promote Diversity in Health-Related Research (Admin Suppl). DO NOT EXCEED FIVE PAGES.

NAME OF APPLICANT: TOLANI OLONISAKIN

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE: MSTP Trainee

EDUCATION/TRAINING *(Most applicants will begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training and residency training if applicable. High school students should list their current institution and associated information. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	START DATE MM/YYYY	END DATE *(or expected end date)* MM/YYYY	FIELD OF STUDY
Fisk University	B.A.	08/2008	05/2012	Biology
University of Pittsburgh	M.D. Ph.D.	05/2013	Anticipated	Medicine

A. Personal Statement

My interest in basic biology propelled me to enter medical school as part of the Physician Scientist Training Program (PSTP), a structured 5-year scholarship program designed for medical students with interests in basic science research. As part of the protected research year provided by the PSTP, I joined Dr. Janet Lee's laboratory to probe host defense mechanisms in the lung. Under Dr. Lee's guidance, I presented my findings at several national meetings and published three papers (including a first-author paper in *The Journal of Infectious Diseases*). I discovered that I was intrigued by the intricacies of host-microbial interactions and that I enjoyed probing host defense mechanisms against pathogenic bacteria. Although I had grown significantly as a critical thinker and a creative writer through my inclusion in the PSTP, I recognized that I needed formal scientific training to deepen my skill set. Consequently, I applied and was accepted to the dual degree MD-PhD program to pursue studies in cellular and molecular mechanisms underlying cardiopulmonary pathologies.

This past year, I have come upon some exciting findings that I would love to further explore. We recently discovered in the lab that thrombocytopenic mice that lack the thrombopoietic receptor Mpl (*Mpl*$^{-/-}$) are prone to lung injury and impaired host defense against *Pseudomonas aeruginosa* infection, but show no visible sign of lung injury following *Klebsiella pneumoniae* infection compared to wildtype controls — highlighting a specific host-pathogen interaction. Though both extracellular gram-negative pathogens, *P. aeruginosa* secretes proteases and toxins that *K. pneumoniae* lacks. We posit that a host protein thrombospondin-1, a potent inhibitor of *P. aeruginosa*-secreted protease and neutrophil serine protease required to kill *K. pneumoniae*, may underlie the unanticipated finding. In particular, I plan on exploring the ability of thrombospondin-1 to curtail host-triggered inflammation in response to *P. aeruginosa* and enhance host defense against this pathogen. I am also quite fortunate to be in a very supportive lab and under the guidance of Dr. Lee. She encourages creativity, provides networking opportunities, and shares her experiences with me. Indeed, Dr. Lee has proven to be a superb mentor and a great role model, and I look forward to continuing my work with her.

Though my clinical interests have not been defined yet, I foresee myself applying for the American Board of Internal Medicine Research Pathway as a fourth year medical student and ultimately pursuing a subspecialty in Internal Medicine. The AHA Predoctoral Fellowship provides support for me as an MD-PhD student to not only focus on research for an extended period of time free from other commitments and to fully explore a research topic, but also opportunities to develop a strong basic science background in preparation for a research career. I am confident that the training and support that I will receive during the program will benefit me as I build my

career in Internal Medicine. Overall, my goals for the fellowship are to share my findings in the lab by getting published, build on the training that I have received thus far, and ultimately to make a small but significant contribution to scientific knowledge.

B. Positions and Honors

ACTIVITY/ OCCUPATION	START DATE (mm/yy)	END DATE (mm/yy)	FIELD	INSTITUTION/ COMPANY	SUPERVISOR/ EMPLOYER
Research Assistant	06/09	01/12	Physics, Materials science	Center for Physics and Chemistry of Materials, Fisk University	W. Eugene Collins, Ph.D.
Summer Intern	06/12	08/12	Pharmacology	University of California, San Diego	Joan Heller Brown, Ph.D.
Research Associate	09/12	05/13	Cardiovascular Disease	University of Alabama, Birmingham	Steven M. Pogwizd, M.D.
PSTP Trainee	06/13	10/13	Cardiovascular Disease	University of Pittsburgh	Hunter Champion, M.D. Ph.D.
PSTP Trainee	02/14	Present	Pulmonary Medicine	University of Pittsburgh	Janet S. Lee, M.D.
HHMI Medical Research Fellow	05/15	Present	Pulmonary Medicine	University of Pittsburgh	Janet S. Lee, M.D.
MSTP Trainee	05/17	—	Pulmonary Medicine	University of Pittsburgh	Janet S. Lee, M.D.

Honors

2008 Erastus Milo Cravath Presidential Scholar, Fisk University
2012 First place in Computer Science/Math/Physics/Engineering/Environmental Science presentations, 69th Joint Annual Meeting of NIS/BKX
2012 Phi Beta Kappa Society
2012 Salutatorian, Fisk University
2014 Best Poster Presentation Award in Graduate Student/Resident category, Department of Medicine, University of Pittsburgh
2015 Selected as Alternate, 2015-2016 American Association of University Women International Fellowship
2015 Abstract Scholarship, Assembly on Allergy, Immunology and Inflammation (AII) Travel Award, American Thoracic Society 2015 International Conference (declined due to acceptance of Minority Trainee Travel Award)
2015 Minority Trainee Development Scholarship, American Thoracic Society 2015 International Conference
2015 Year-Long Medical Research Fellowship, Howard Hughes Medical Institute
2016 Year-Long Medical Research Fellowship, Howard Hughes Medical Institute
2016 Abstract Scholarship, Assembly on Allergy, Immunology and Inflammation (AII) Travel Award, American Thoracic Society 2016 International Conference
2016 TYLENOL Future Care Scholarship
2017 Minority Trainee Development Scholarship, American Thoracic Society 2017 International Conference
2017 AAAS/Program for Excellence in Science

Professional Memberships and Leadership Positions

2013 Student Member, American Physiological Society

2013 – 2014 Historian, Student National Medical Association
2013 – 2014 Co-chair, Carolyn Carter Scholarship Banquet, Student National Medical Association
2015 Co-chair, PSTP retreat committee
2014 Student Member, American Medical Association
2014 Student Member, American Heart Association
2014 Student Member, American Thoracic Society
2016 Regional Co-chair, Medical Research Fellows Program, Howard Hughes Medical Institute

C. Contributions to Science

I. Mechanisms in host defense against gram-negative bacterial pneumonia.

1. Identified the scavenger receptor CD36 as a protective factor in pulmonary defense against *Klebisella pneumoniae*. (Mentor: Janet S. Lee, M.D.)

CD36 is a scavenger receptor expressed by a variety of cell types including platelets, endothelial cells, and macrophages. At least a dozen mutations in the coding sequence of the human CD36 gene have been described, with majority of CD36 deficiency phenotypes occurring in almost exclusively Asian and African populations. Concurrently, the gram-negative pathogen *Klebsiella pneumoniae* causes distinct community-acquired syndromes found in Asia and Africa, but rare in North America, Europe, or Australia. These severe invasive syndromes are characterized by necrotizing pneumonia, hepatic abscesses, bacteremia, endophthalmitis, and meningitis. Utilizing hypermucoviscous K1 isolate, K2 serotype research strain, and a carbapenemase-producing ST258 isolate of *K. pneumoniae*, we show that CD36 is required for optimal control of all 3 *K. pneumoniae* isolates in the lungs and extrapulmonary dissemination to distant organ sites. We demonstrate that CD36 facilitates cell surface recognition of *K. pneumoniae* LPS that is independent of polysaccharide capsular antigen, to enhance phagocytosis, and downstream cytokine production by alveolar macrophages. Our study provides new insights into host determinants of *K. pneumoniae* pathogenicity and suggests that CD36 deficiency may be a susceptibility factor for *K. pneumoniae* infection.

Publication

Olonisakin TF, Li H, Xiong Z, Kochman EJ, Yu M, Qu Y, Hulver M, Kolls JK, St. Croix C, Doi Y, Nguyen M, Shanks RMQ, Mallampalli RK, Kagan VE, Ray A, Silverstein RL, Ray P, Lee JS. CD36 provides host protection against Klebsiella pneumoniae intrapulmonary infection by enhancing LPS responsiveness and macrophage phagocytosis. *J Infectious Dis*. 2016 Dec 15;214(12):1865-1875. Epub 2016 Sep 28.

Poster presentation

Olonisakin TF, Xiong Z, Kochman EJ, Yu M, Qu Y, Hulver M, Mallampalli R, Kagan V, Ray A, Silverstein RL, Ray P, Lee JS. Defense against *Klebsiella pneumoniae*: Role of the scavenger receptor CD36. HHMI Science Meeting. HHMI Headquarters Conference Center. Chevy Chase, Maryland. November 2015.

2. Identified a novel role for thrombospondin-1 (TSP-1) in regulating neutrophil function during intrapulmonary *Klebsiella pneumoniae* infection. (Mentor: Janet S. Lee, M.D.)

Thrombospondin-1, a matricellular protein released during inflammation by platelets and myeloid-derived cells, has been shown to inhibit the catalytic activities of neutrophil elastase and cathepsin G *in vitro*. We showed that residues 794-801 within the type 3 repeats domain of TSP-1 can restrain neutrophil proteolytic function by effectively inhibiting neutrophil elastase and cathepsin G enzymatic activity, and enhance bacterial killing *in vivo*. We further demonstrated that early, effective host killing of pathogen in TSP-1 deficient mice improves lung bacterial clearance, limits bacterial dissemination, and enhances survival following intrapulmonary infection with *K. pneumoniae*. My contribution to the project was in helping identify the discrete region within the type 3 repeats domain of TSP-1 that influenced neutrophil function and in demonstrating that the major source of TSP-1 during inflammation is extracellular rather than neutrophil in origin.

Publication

Zhao Y, **Olonisakin TF**, Xiong Z, Hulver M, Sayeed S, Yu MT, Gregory AD, Kochman EJ, Chen BB, Mallampalli RK, Sun M, Silverstein RL, Stolz DB, Shapiro SD, Ray A, Ray P, Lee JS. Thrombospondin-

1 restrains neutrophil granule serine protease function and regulates the innate immune response during Klebsiella pneumoniae infection. *Mucosal Immunology* (2015) 8, 896–905. PMID: 25492474

Abstracts

Olonisakin TF, Zhao Y, Xiong Z, Hulver M, Lee JS. A Discrete Region Within the Type 3 Repeats Domain of Thrombospondin-1 Restrains Neutrophil Microbial Killing of Klebsiella Pneumoniae. *American Journal of Respiratory and Critical Care Medicine*. Abstract Issue. Vol 190 (7) May 2015. (Selected as oral presentation in Mini-Symposium: New Insights into Pulmonary Host Defense, American Thoracic Society International Conference, Denver, CO. May 17, 2015)

3. Developed a high-throughput assay to screen small molecule antagonists of thrombospondin-1's interaction with neutrophil elastase. (Mentor: Janet S. Lee, M.D.)

Thrombospondin-1 (TSP-1) is an endogenous neutrophil serine protease inhibitor that can restrain microbial killing of *Klebsiella pneumoniae* by limiting neutrophil elastase (NE) activity. We identified a discrete region within the type 3 repeats (T3R) domain of TSP-1 as the functional inhibitory domain of NE and conducted a small molecule library screen of about 500,000 compounds to identify candidates that block the interaction involving TSP-1 T3R domain and NE. Six high-ranking compounds were selected based on size, safety, and hydrophobicity. To screen the compounds, recombinant monomeric human TSP-1 (rhTSP-1) or purified trimeric human TSP-1 and purified human NE were incubated at 37oC in the presence of varying concentrations of the identified compounds. The reaction was started by the addition of N-(methoxysuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide (AAPV-pna) and the formation of 4-nitroaniline as a function of time was monitored continuously by measuring absorbance at 410nm. We showed that five of the six compounds effectively antagonized TSP-1 and enhanced NE hydrolysis of AAPV-pna, with the lead compound BC-1401 showing an IC_{50} of approximately 22μM. We also showed that BC-1401 itself had no effect on hydrolysis of AAPV-pna, indicating that the compound's ability to enhance NE activity was due to alterations in NE/TSP-1 interactions.

Poster presentations

Olonisakin TF, Qu Y, Hulver M, Xiong Z, Chen BB, Lee JS. Identifying small molecule compounds that enhance the host innate immune response to infection. Joint Meeting of the Association of American Physicians, the American Society for Clinical Investigation, and the American Physician Scientists Association. Chicago, IL. April 2016.

Olonisakin TF, Hulver M, Xiong Z, Lee JS. Development of a high-throughput assay to screen small molecule antagonists of thrombospondin-1's interaction with neutrophil elastase. 7th Annual Research Symposium, Physician Scientist Training Program, University of Pittsburgh. Pittsburgh, PA. September 2015.

II. Rigor and transparency in preclinical research.

Established pre-submission checklist incorporation as tool to enhance quality and transparency in reporting of preclinical research design and analysis. (Mentor: Janet S. Lee, M.D.)

Reproducibility in preclinical biomedical research has become an urgent concern, as greater than 75% of published preclinical studies have been deemed irreproducible. A joint workshop led by the NIH with leading science publishing groups conceived a set of principles emphasizing rigor and reproducibility, and a recommendation that journals utilize checklists during the editorial process to ensure transparent reporting of key methodological and analytical information. We tested whether incorporation of a checklist is effective in improving rigor and transparency in methodological reporting. Our findings show that reporting of important details such as sample size estimation and whether experiments are randomized or blinded is significantly enhanced with inclusion of a mandatory editorial checklist.

Publication

Han S, **Olonisakin TF**, Pribis JP, Zupetic J, Yoon JH, Holleran KM, Jeong K, Shaikh N, Rubio DM, Lee JS. A checklist is associated with increased quality of reporting preclinical biomedical research: A systematic review. *PLoS One*. 2017 Sep 13;12(9):e0183591. doi:10.1371/journal.pone.0183591. eCollection 2017.

Applicant's Biographical Sketch - Biosketch_Tolani Olonisakin_AHA 2017.pdf

III. G-protein coupled receptor (GPCR) signaling in astrocytes.

Investigated the role of sphingosine-1-phosphate (S1P) receptor subtypes in mediating inflammatory gene expression in astrocytes. (Mentor: Joan Heller Brown, Ph.D.)

Extracellular S1P functions in both a paracrine and autocrine fashion by binding to several high-affinity S1P receptors (S1PRs) that constitute a widely expressed, developmentally regulated family of G-protein coupled receptors. The activation of these S1PRs in astrocytes is crucial to the reactive astrogliosis and inflammation observed in multiple sclerosis. Interestingly however, receptors for S1P exhibit several different permutations in coupling to G proteins. We attempted to identify which of the receptors was responsible for the inflammatory signaling observed. Our results suggested that the S1P3 receptor may be the predominant receptor mediating the inflammatory signaling observed in astrocytes following S1P stimulation. I was responsible for performing all experiments related to the project, analyzing data generated, preparing a scientific report at the end of the project and presenting my work at the Department of Pharmacology Research Fellowship luncheon.

Abstracts

Olonisakin TF, Dusaban SS, Purcell NH, and Brown JH. Role of S1P receptor subtypes in mediating inflammatory signaling in astrocytes. Department of Pharmacology Research Fellowship Luncheon, University of California, San Diego. August 2012.

IV. Performance-limiting defects in microelectronic materials and devices.

Investigated passivation treatments of Silicon carbide (SiC)/Silicon dioxide (SiO2) interface defects. (Mentor: W. Eugene Collins, Ph.D)

Si-based microelectronics are inefficient for high-temperature and high-power applications such as those required in red-hot engines, solar inverters, and high-voltage power supplies. Silicon carbide (SiC), on the other hand, with its wider band gap and high thermal conductivity, has a large potential for high-temperature and high-power devices. Oxidation of SiC, however, releases carbon atoms, some of which are stuck at the interface as defects. Using X-ray photoelectron spectroscopy (XPS) and Infrared (IR) spectroscopy, we attempted to explain how certain nitridation techniques resulted in passivating interface defects by comparing two methods of nitridation. Our results suggested that samples annealed in NO may be superior to those annealed in N2/H2, as there was significantly higher C-C graphitization (intrusive carbon clusters) in the N2/H2 annealed samples when compared with the NO annealed samples. I worked with two postdoctoral fellows on the structural environment of nitrided silicon carbide-silicon dioxide (SiC-SiO2) interfaces. With their guidance, I performed all experiments during the project and analyzed the data generated.

Abstracts

Olonisakin TF, Barbosa R, Collins WE. Structural Investigation of Nitrided SiC-SiO2 Interfaces. 69th Joint Annual Meeting of National Institute of Science and Beta Kappa Chi Scientific Honor Society. Nashville, Tennessee. March 2012. (Awarded first place in Computer Science/Math/Physics /Engineering/Environmental Science poster presentations).

Olonisakin TF, Barbosa R, Collins WE. Structural Investigation of Nitrided SiC-SiO2 Interfaces. 13th Annual Fisk University Research Symposium. Nashville, Tennessee. April 2011.

D. Scholastic Performance: Please see academic record

11.2. Academic Record (Predoc)

Academic Record (Predoc) - ACADEMIC RECORD OF APPLICANT_OLONISAKIN.pdf

ACADEMIC RECORD OF PREDOCTORAL APPLICANT

Undergraduate Science Courses

Year	Course Title	Grade
2008	General Biology I	A
2008	General Biology I Lab	A
2008	Experiments in General Chemistry I	A
2008	General Chemistry I	A
2009	General Biology II	A
2009	General Biology II Lab	A
2009	Experiments in General Chemistry II	A
2009	General Chemistry II	A-
2009	Human Anatomy and Physiology I	A-
2009	Human Anatomy/Physiology I Lab	A
2009	Experimental Organic Chemistry I	A
2009	Organic Chemistry I	A
2010	Human Anatomy/Physiology II	A-
2010	Human Anatomy/Physiology II Lab	C
2010	Experimental Organic Chemistry II	A
2010	Organic Chemistry II	A
2010	Calculus I	A
2010	University Physics I	A
2010	Experiments in General Physics I	A
2010	Genetic Principles	A
2010	Genetic Principles Lab	A
2010	Natural Science	A
2010	Microbiology	A
2010	Microbiology Lab	A
2010	Molecular Cell Biology	A
2010	Molecular Cell Biology Lab	B-
2010	University Physics II	A
2010	Experiments in General Physics II	A
2011	Special Problems in Biological Research	A
2011	Undergraduate Research	A-
2011	Biochemistry I	A
2011	Biochemistry I Lab	A
2012	Biochemical Methods	A

Other Courses

Year	Course Title	Grade
2008	Elementary French I	A
2008	African-American Heritage I	A
2009	Elementary French II	A
2009	African-American Heritage II	A

Academic Record (Predoc) - ACADEMIC RECORD OF APPLICANT_OLONISAKIN.pdf

2009	Creative Arts	A
2009	Intensive Intermediate French	A
2010	General Psychology	A
2011	Humanities	A-
2011	Senior Seminar I	B+
2012	World and Its Peoples	A
2012	Bible as Literature	A
2012	Mythology	A
2012	Senior Seminar II	A

UNDERGRADUATE GRADING SYSTEM

A High distinction, 4.00 quality points
A- 3.70 quality points
AE Letter grade excluded from GPA calculation
B+ 3.30 quality points
B Very good work, 3.00 quality points
BE Letter grade excluded from GPA calculation
C+ 2.30 quality points
C Acceptable work, 2.00 quality points
C- 1.70 quality points
CE Letter grade excluded from GPA calculation
D Lowest passing, 1.00 quality points
DE Letter grade excluded from GPA calculation
E Failure
I Incomplete
WD Withdrawn
P/F Pass Fail
NG No grade

Medical School Courses

Year	Course Title	Grade
2013	Basic Science Fundamentals I	S
2013	Patient/Physician & Society I	S
2013	Basic Science Fundamentals 2	S
2013	Introduction to Patient Care 1	S
2013	Scientific Reasoning 1	S
2013	PSTP Research Basis of Medical Knowledge	S
2013	PSTP Work in Progress Seminar	S
2014	Basic Science Fundamentals 3	S
2014	Behavioral Medicine	S
2014	Neuroscience/Psychiatry	S
2014	Introduction to Patient Care 2	S
2014	PSTP Research Basis of Medical Knowledge 2	S
2014	Body Fluid Homesotasis	S
2014	GI/ENDO/HEM/SKIN/MUSK/REPROSCI	S

Academic Record (Predoc) - ACADEMIC RECORD OF APPLICANT_OLONISAKIN.pdf

2014	Pharmacology	S
2014	Introduction to Patient Care 3	S
2014	PPS3 Population Health	S
2014	PSTP Laboratory Research	S
2014	PSTP Professional Development 2	S
2014	PSTP Work In Progress Seminar	S
2015	Integrated Case Studies	S
2015	Introduction to Patient Care 4	S
2015	Mentored Project Interim Grade	S
2015	**USMLE STEP 1**	**242**
2015	PSTP Work In Progress Seminar	S
2016	Basics of Personalized Medicine	A-
2016	PSTP Work In Progress Seminar	S
2017	Molecular Mechanisms Tissue Growth & Differentiation	A+
2017	Preclerkship course	S
2017	Obstetrics & Gynecology Clerkship	Honors

SCHOOL OF MEDICINE GRADING SYSTEM
*Coursework in the first two years (2013 – 2015) of medical school at the University of Pittsburgh is evaluated as Satisfactory (S) or Unsatisfactory (US).

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11.4. Applicant's Publication 1

Applicant's Publication 1 - Accepted Editorial_AJRCMB.pdf

American Journal of Respiratory Cell and Molecular Biology/AJRCMB

Platelets as Agents of Host Defense?

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Subject Category:	10.06 Host Defenses to Microbial Pathogens < MICROBIOLOGY AND PULMONARY INFECTIONS, 10.07 Nosocomial Infections < MICROBIOLOGY AND PULMONARY INFECTIONS, 10.08 Opportunistic Infections < MICROBIOLOGY AND PULMONARY INFECTIONS, 10.12 Pneumonia: Bacterial Infections < MICROBIOLOGY AND PULMONARY INFECTIONS
Keywords:	

Platelets as Agents of Host Defense?

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Pseudomonas aeruginosa is an extracellular Gram-negative bacterial opportunistic pathogen that exploits the immunocompromised host. Though notorious for its ability to persist in the cystic fibrosis lung and establish chronic infection, *P. aeruginosa* is a common cause of acute lower respiratory tract infections in critically ill patients. Indeed, *P. aeruginosa* was identified as the most common gram-negative infection in a multi-center, international collaborative study of ICU infections from 75 countries (1), and *P. aeruginosa* infection is independently associated with increased ICU mortality (2). In this issue of AJRCMB, Amison et al. provide compelling evidence that platelets are required in host defense against *P. aeruginosa*. This may be surprising, as platelets are conventionally viewed as mediators of primary hemostasis. Yet, platelets are increasingly recognized as key players in innate defense against infection (3). Amison and colleagues demonstrate that acute intrapulmonary *P. aeruginosa* infection induces peripheral thrombocytopenia with accumulation of activated platelets in lung tissue. These platelets found in the airspaces show evidence of degranulation, with elevated PF-4 levels in the BAL fluid of infected mice. They further show that the accumulation of activated platelets in the lungs is protective rather than deleterious during *P. aeruginosa* infection, as platelet depletion in mice results in enhanced pathogenicity of *P. aeruginosa* with higher lung bacterial burden, increased weight loss, and increased mortality. Depletion of platelets also results in impaired leukocyte recruitment — most notably, neutrophils — in response to *P. aeruginosa*. Although the impaired neutrophil recruitment following infection may account for the worsened phenotype observed in platelet-depleted mice, the authors also suggest that platelets can limit bacterial growth in vitro. This finding invites the possibility that platelets may provide an additional function of direct host defense against bacterial pathogens.

Platelets are uniquely suited to survey the host for threats and rapidly respond to invaders via pattern recognition receptors (4). They are abundantly present in the bloodstream and are rich in granules, the contents of which can be mobilized to potentially facilitate a vast array of host defense functions (3).

While platelets can worsen inflammation and injury in experimental models of sterile lung injury (5), there is a growing body of evidence that suggests the contrary during infection (3). Activated platelets have been shown to directly bind and internalize infectious organisms within specific subcellular compartments analogous to phagosomes (6). Still others have shown that platelet TLR4 is activated in response to microbial trigger, resulting in platelet binding to adherent neutrophils and subsequent neutrophil activation with formation of neutrophil extracellular traps (NETs) (7). Moreover, activated platelets can tether recruited neutrophils via P-selectin leading to neutrophil integrin activation and induction of a neutrophilic inflammatory phenotype (8). Disruption of platelet-neutrophil interactions by depleting either platelets or neutrophils prevents release of NETs within liver sinusoids and enhances bacterial dissemination in an intraperitoneal *Escherichia coli* infection model (9). Depletion of platelets also results in enhanced bacterial growth and increased dissemination in an acute *Klebsiella pneumoniae* intrapulmonary infection model (10). However, platelets do not appear essential for pulmonary neutrophil recruitment or NET formation in the *Klebsiella pneumoniae* model, and differences in observed outcomes are not attributable to impaired neutrophil influx or activation. Thus, different pathogenic or injurious stimuli may induce distinct platelet-neutrophil responses and this prompts an investigation on the role of platelets in acute *P. aeruginosa* intrapulmonary infection. Here, Amison et al. contribute to this body of evidence by demonstrating that platelets are critical for host defense against *P. aeruginosa* in the lungs.

While the authors present experimental evidence supporting a critical role for platelets in host defense against *P. aeruginosa*, the mechanism by which platelets defend against this pathogen remains elusive. Impaired host defense and worsened survival in platelet-depleted mice following *P. aeruginosa* infection may be primarily a result of reduced neutrophil recruitment. Though targeted depletion of platelets may inadvertently deplete neutrophil population by reducing circulating platelet-neutrophil aggregates (11), the authors address this concern by demonstrating that peripheral blood neutrophil counts

are only minimally altered upon effective platelet depletion following anti-GPIb-α administration. They also show that peripheral blood neutrophil counts are unaltered in platelet-depleted mice, yet leukocyte recruitment into the airspaces are reduced during active infection. This bolsters the authors' claims that platelets, to some extent, actively facilitate neutrophil recruitment into the lungs. This finding is particularly relevant in *P. aeruginosa* pneumonia, where an exuberant neutrophilic influx occurs in a manner that is not completely understood (12). To definitively assess the contribution of platelet-mediated neutrophil recruitment to host defense against *P. aeruginosa*, future studies could assess whether disruption of platelet-neutrophil interactions (9) reproduce the phenotype in the model.

Amison and colleagues speculate that the impaired bacterial burden observed in platelet-depleted mice may be due to impaired NETosis. NET-mediated bacterial killing, however, remains controversial and the current paradigm dictates that NETs ensnare pathogens but do not kill (13, 14). Nevertheless, independent of NET formation, neutrophils are essential in host defense against *P. aeruginosa* (15). The authors also suggest that platelets may directly inhibit growth of *P. aeruginosa*. Indeed, activated platelets extrude contents contained within their granules that may exhibit microbicidal activity against bacterial and fungal pathogens (3). However, the relative contribution of platelet microbicidal activity to host defense remains unclear. It is also notable that mortality is increased in platelet-depleted mice — regardless of infectious inoculum administered — when compared to controls. This suggests that other mechanisms contribute to clinical toxicity and death in these mice, and platelet-mediated protection may be beyond simply defense against bacterial proliferation. The findings of Amison and colleagues thereby raise several important questions. What is the nature of the interaction between platelets and *P. aeruginosa*? Is there direct uptake of *P. aeruginosa* in stimulated platelets? Does *P. aeruginosa* trigger destruction of host tissue in a manner that necessitates protection provided by platelets? Are there specific components of platelets that mediate protection against *P. aeruginosa*-induced tissue degradation? While

Amison and colleagues shed light on the critical role that platelets play in host defense, future studies are needed to elucidate the mechanism by which platelets defend against *P. aeruginosa*.

Applicant's Publication 1 - Accepted Editorial_AJRCMB.pdf

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11.5. Applicant's Publication 2

Thrombospondin-1 restrains neutrophil granule serine protease function and regulates the innate immune response during *Klebsiella pneumoniae* infection

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Neutrophil elastase (NE) and cathepsin G (CG) contribute to intracellular microbial killing but, if left unchecked and released extracellularly, promote tissue damage. Conversely, mechanisms that constrain neutrophil serine protease activity protect against tissue damage but may have the untoward effect of disabling the microbial killing arsenal. The host elaborates thrombospondin-1 (TSP-1), a matricellular protein released during inflammation, but its role during neutrophil activation following microbial pathogen challenge remains uncertain. Mice deficient in TSP-1 (*thbs1*$^{-/-}$) showed enhanced lung bacterial clearance, reduced splenic dissemination, and increased survival compared with wild-type (WT) controls during intrapulmonary *Klebsiella pneumoniae* infection. More effective pathogen containment was associated with reduced burden of inflammation in *thbs1*$^{-/-}$ mouse lungs compared with WT controls. Lung NE activity was increased in *thbs1*$^{-/-}$ mice following *K. pneumoniae* challenge, and *thbs1*$^{-/-}$ neutrophils showed enhanced intracellular microbial killing that was abrogated with recombinant TSP-1 administration or WT serum. *Thbs1*$^{-/-}$ neutrophils exhibited enhanced NE and CG enzymatic activity, and a peptide corresponding to amino-acid residues 793–801 within the type-III repeat domain of TSP-1 bridled neutrophil proteolytic function and microbial killing *in vitro*. Thus, TSP-1 restrains proteolytic action during neutrophilic inflammation elicited by *K. pneumoniae*, providing a mechanism that may regulate the microbial killing arsenal.

INTRODUCTION

Thrombospondin-1 (TSP-1) is a matricellular protein that exists as a trimer of identical ~150–180 kDa subunits tethered together by disulfide bonds.[1,2] A major source of TSP-1 is platelet α-granules, with TSP-1 being released upon platelet activation.[3–5] TSP-1 also exists in plasma at low concentrations under basal conditions, and is made by numerous cells including myeloid-derived cells such as neutrophils.[6,7] Given its adhesive nature, TSP-1 can bind to the surface of platelets, extracellular matrix, and a number of other cells including fibroblasts, smooth muscle cells, endothelium, neutrophils, and macrophages, providing a multiplicity of potential cell–cell and cell–matrix interactions.[2] Thus, it is not surprising that TSP-1 has been implicated in a number of important biological functions such as embryogenesis, wound repair, tumor growth and metastasis, angiogenesis, hemostasis, and inflammation.[2,5,8–11]

Others have previously shown that platelet proteins such as TSP-1 are found in the airspace of patients with acute respiratory distress syndrome and TSP-1 concentrations correlate with composite injury scores that quantify the degree of lung injury.[12] The original description of mice deficient in TSP-1 (*thbs1*$^{-/-}$) showed spontaneous development of non-infectious pneumonia and the predisposition to develop inflammation due to impaired homeostasis.[13] We recently

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demonstrated that, although *thbs1*$^{-/-}$ mice do not spontaneously develop non-infectious pneumonia, these mice exhibit a defect in their ability to resolve from injurious stimuli to the lungs.[14] We have also shown that interleukin (IL)-10 is essential for recovery of lung inflammation during the late phase of bacterial infection.[14,15] In an experimental model of lung injury, we further show that the bridging function of TSP-1 is required for optimal triggering of macrophage IL-10 production following contact recognition of apoptotic neutrophils that is necessary for effective resolution of inflammation.[14,15] It remains unclear, however, what the role of TSP-1 is, if any, during bacterial pneumonia, an important cause of morbidity and mortality worldwide and a well-known risk factor for acute respiratory distress syndrome.[16]

Neutrophils are innate immune effector cells of microbial killing and are critical for pulmonary host defense against pathogen. Two major modes of intracellular killing are operant in neutrophils: (1) oxygen-dependent mechanism involving the recruitment and activation of the NADPH oxidase complex, the generation of oxygen free radicals and superoxide, resulting in myeloperoxidase-mediated halogenation to form hypochlorous acid; (2) the release of cytosolic granule contents within the phagosome, comprised of neutrophil serine proteases, the activation of these proteases within the phagosome,[17–19] and the contribution of antimicrobial proteins.[20] Neutrophil elastase (NE), a key granule serine protease, degrades outer membrane protein A on the surface of Gram-negative bacteria[21] and is an important contributor to the oxygen-independent arm of microbial killing. Although effective neutrophil microbial killing is required to thwart collateral tissue damage and organ injury induced by microbial–host interactions, the host also requires mechanisms to curtail its microbial killing arsenal to prevent subsequent collateral tissue damage and organ injury. Indeed, unbridled neutrophil protease activity is associated with lung injury or acute respiratory distress syndrome in humans.[22] Thus, a fine balance is required for effective neutrophil microbial-killing activity on one hand and the curtailment of an over-vigorous host inflammatory response on the other.

TSP-1 is a competitive inhibitor of serine proteases such as plasmin, preventing the cleavage of fibrinogen *in vitro* with stoichiometric predictions demonstrating 1 mol of TSP-1 interacting with 1 mol of plasmin.[23] This prompted further studies showing that TSP-1 binds and competitively inhibits the enzymatic activity of purified NE and cathepsin G (CG) *in vitro*.[3,4] These findings suggest a regulatory role for TSP-1 during inflammation, but how TSP-1 modulates neutrophil function during the innate immune response to bacterial pathogens is unclear.

RESULTS

Increased bacterial clearance from the lungs, reduced splenic dissemination, and enhanced survival following intratracheal instillation of *Klebsiella pneumoniae* in mice deficient in TSP-1

To investigate the role of TSP-1 in pulmonary host defense, *thbs1*$^{-/-}$ mice were inoculated with the bacterial pathogen *K. pneumoniae* at 4×10^3 colony-forming units (CFUs). At 48 and 72 h following intratracheal inoculation, *thbs1*$^{-/-}$ mice showed reduced bacterial burden in the lungs compared with wild-type (WT) mice, as measured by CFU/lung (**Figure 1a**). Consistent with the findings in the lungs, *thbs1*$^{-/-}$ mice showed reduced splenic dissemination compared with WT mice (**Figure 1b**). Thus, pulmonary host defense is enhanced during bacterial pneumonia in the absence of TSP-1 that is associated with reduced systemic dissemination. We next determined whether the enhanced bacterial clearance in *thbs1*$^{-/-}$ mice confers a survival advantage during bacterial pneumonia with *K. pneumoniae*. At an inoculum of 1.2×10^4 CFU, lethal dose (LD_{50}) was achieved at 72 h in WT mice (**Figure 1c**). Kaplan–Meier curve followed by the log-rank test showed enhanced survival in *thbs1*$^{-/-}$ mice compared with WT mice ($P = 0.02$, $n = 20$ per group). The median survival for *thbs1*$^{-/-}$ mice was 312 h (13 days), compared with 72 h (3 days) for WT mice. A kinetics study examining responses of WT and *thbs1*$^{-/-}$ mice 24, 48, 72, and 96 h following intratracheal inoculation (1.8×10^3 CFU) indicated that, by 72 and 96 h, *thbs1*$^{-/-}$ mice were clearing bacteria from the lungs faster than WT mice (**Supplementary Figure 1** online). Consistent with our prior findings,[24] *thbs1*$^{-/-}$ mice show deficiency in IL-10, which is required for optimal resolution of lung inflammation during the late

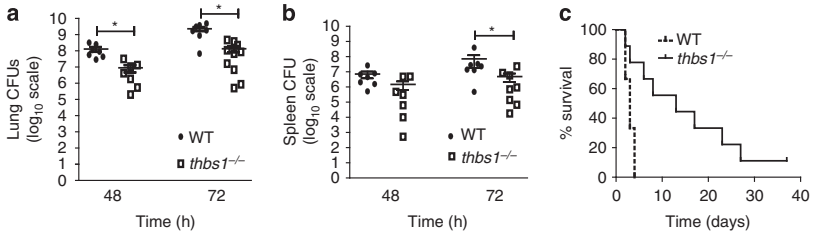

Figure 1 *thbs1*$^{-/-}$ mice show enhanced lung bacterial clearance, reduced splenic dissemination, and enhanced survival following intratracheal *K. pneumoniae* inoculation. Colony-forming units (CFU) obtained from (**a**) lung tissue homogenate and (**b**) splenic homogenate cultures of WT and *thbs1*$^{-/-}$ mice 48 and 72 h after intratracheal (i.t.) inoculation with *K. pneumoniae*, $n = 7$–10 mice per group. Kruskal–Wallis test with Dunn's multiple comparisons, $^*P < 0.05$. (**c**) A Kaplan–Meier survival curve of WT and *thbs1*$^{-/-}$ mice following i.t. infection with *K. pneumoniae* ($n = 20$ mice per genotype, $P = 0.02$). The median survival for WT mice was 72 h (3 days) and 312 h (13 days) for *thbs1*$^{-/-}$ mice. WT, wild type.

phase after infection.[15] Taken together, TSP-1 deficiency improves early lung bacterial clearance, reduces systemic dissemination, and confers increased survival during bacterial pneumonia induced by *K. pneumoniae*. However, *thbs1*$^{-/-}$ mice may later succumb to sub-optimal resolution from injury due to inability to trigger full IL-10 responses in the lungs.

thbs1$^{-/-}$ mice show enhanced lung NE activity and reduced parenchymal inflammation following intratracheal *K. pneumoniae*

TSP-1 can bind and induce the motility and chemotaxis of neutrophils *in vitro*,[25–28] and we have previously reported that *thbs1*$^{-/-}$ mice show slightly higher leukocyte counts in the airspaces under basal conditions.[24] Despite this, total leukocyte and neutrophil recruitment into the alveolar spaces were not different between *thbs1*$^{-/-}$ and WT mice following intratracheal *K. pneumoniae* (**Figure 2a, b**). Within the lung parenchymal compartment, *thbs1*$^{-/-}$ mice showed reduction in IL-6, IL-10, G-CSF, GM-CSF, KC, and MCP-1, but not TNF-α concentrations compared with WT controls (**Figure 2c**). Lung myeloperoxidase (MPO) activity, an indicator of total neutrophil content in tissue homogenates, showed no differences at 48 h (**Figure 2d**). By 72 h, however, *thbs1*$^{-/-}$ lungs showed reduced MPO activity in agreement with reduced total airspace protein concentrations (**Figure 2e**), a marker of lung injury. Moreover, histologic examination showed lower neutrophil burden in the lungs of *thbs1*$^{-/-}$ mice (**Figure 2f**; **Supplementary Figure 2**), and semi-quantitative morphometric analysis of lung tissue indicated a significantly lower inflammation score in *thbs1*$^{-/-}$ lungs compared with WT (**Figure 2g**). These findings suggest that a more effective pulmonary host defense and early containment of bacterial pathogen in *thbs1*$^{-/-}$ mice are associated with an overall reduction in the intensity of the lung inflammatory response. We next tested the possibility whether the absence of TSP-1 may contribute to enhanced neutrophil function and provide effective containment of bacterial pathogen.

Previous reports have shown the requirement for NE, a serine protease stored within azurophilic granules of neutrophils, for adequate bactericidal activity against Gram-negative bacteria such as *Escherichia coli* and *K. pneumoniae.*[21,29] Lung tissue homogenates of *thbs1*$^{-/-}$ mice showed enhanced NE compared with the lungs of WT mice at 48 and 72 h, following intratracheal challenge with *K. pneumoniae* (**Figure 2h**). Taken together, *thbs1*$^{-/-}$ mice show increased lung NE associated with reduced intensity in the inflammatory response, suggesting the possibility that TSP-1 may regulate NE activity to restrain microbial killing with consequent alterations in the host inflammatory response.

thbs1$^{-/-}$ neutrophils show enhanced microbial killing that is reversed by administration of recombinant TSP-1 or WT serum

To more closely examine intracellular microbial-killing capacity, *thbs1*$^{-/-}$ and WT neutrophils were obtained following intraperitoneal inoculation with *K. pneumoniae*. Neutrophils were washed and treated with gentamicin to remove extracellular, membrane-attached bacteria. At time 0, neutrophils from *thbs1*$^{-/-}$ and WT mice ingested similar numbers of bacteria as indicated by CFU following lysis of cells (**Figure 3a**). However, after 60 min incubation, *thbs1*$^{-/-}$ neutrophil lysates showed significantly less viable bacteria compared with WT neutrophil lysates (**Figure 3a**), indicating enhanced microbial killing in the absence of TSP-1. The addition of recombinant TSP-1 just prior to *K. pneumoniae* challenge *in vivo* eliminated the enhanced bacterial killing function of *thbs1*$^{-/-}$ neutrophils near to the level of WT neutrophils, suggesting a specific role of TSP-1 in restraining neutrophil microbial killing (**Figure 3b**). Moreover, whereas WT neutrophils showed a 2.3-fold reduction in viable bacteria over time with *ex vivo* incubation, *thbs1*$^{-/-}$ neutrophils showed a 17.8-fold reduction (mean-fold reduction in CFU/10^6 polymorphonuclear cells ± s.e.m.: 2.3 ± 0.2 vs. 17.8 ± 6.3, $P<0.05$). Compared with *thbs1*$^{-/-}$ neutrophils treated with vehicle alone, *thb1*$^{-/-}$ neutrophils pretreated with recombinant TSP-1 showed a 5.5-fold reduction over time with *ex vivo* incubation (mean-fold reduction in CFU/10^6 polymorphonuclear cells ± s.e.m.: 17.8 ± 6.3 vs. 5.5 ± 0.3, $P<0.05$). Thus, neutrophils show enhanced microbial killing in the absence of TSP-1, and addition of recombinant TSP-1 can mitigate this response.

We next examined microbial killing by WT and *thbs1*$^{-/-}$ neutrophils following *K. pneumoniae* infection *in vitro* to determine whether *in vitro* can recapitulate *in vivo* findings. At a multiplicity of infection of 50:1, *thbs1*$^{-/-}$ neutrophils showed enhanced microbial killing compared with WT neutrophils (**Figure 3c**). The enhanced microbial killing observed in *thbs1*$^{-/-}$ neutrophils could be reversed when bacteria incubated with WT serum rather than homologous serum was given to *thbs1*$^{-/-}$ neutrophils (**Figure 3d**). Moreover, when bacteria incubated in *thbs1*$^{-/-}$ serum was given to WT neutrophils, these neutrophils showed enhanced microbial killing compared with WT neutrophils that received bacteria incubated in homologous serum (**Figure 3e**). These experiments collectively indicate that neutrophil microbial killing is altered by TSP-1 and that the major source of TSP-1 is extracellular in origin.

thbs1$^{-/-}$ neutrophils show enhanced NE and CG activity, but normal respiratory burst and morphology

Previous reports have shown that TSP-1 can bind and competitively inhibit purified CG and NE *in vitro*.[3,4] However, it is unknown whether TSP-1 can constrain neutrophil proteolytic function by inhibiting granule serine protease activity. As NE is the predominant serine protease critical in non-oxidative effector killing of microbial pathogen and degrades outer membrane protein A (OmpA) on the surface of Gram-negative bacteria,[18,21,29,30] we examined the NE activity of tissue-recruited peritoneal-derived *thbs1*$^{-/-}$ and WT neutrophils. *Thbs1*$^{-/-}$ neutrophils showed enhanced NE activity compared with WT neutrophils, as measured by the rate of enzymatic hydrolysis of substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (**Figure 4a**). Whereas

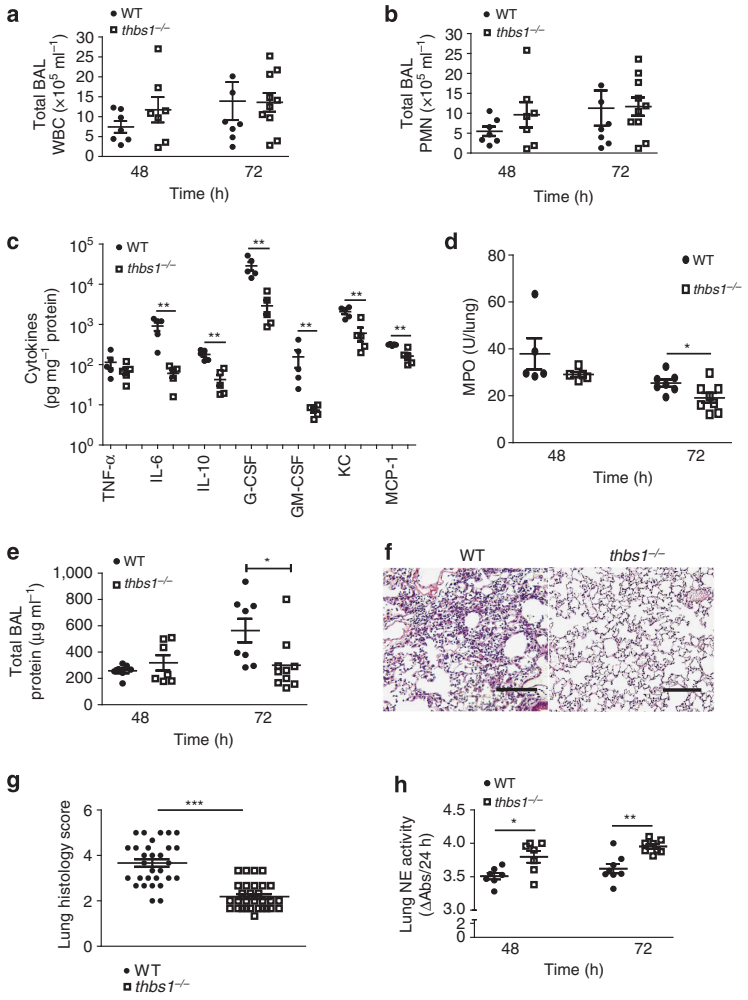

Figure 2 Reduced inflammatory response in the lung parenchyma of *thbs1*$^{-/-}$ mice is associated with enhanced neutrophil elastase (NE) activity following bacterial pneumonia with *K. pneumoniae*. (**a**) Total bronchoalveloar lavage (BAL) leukocyte counts and (**b**) Total BAL polymorphonuclear cell (PMN) counts obtained from wild-type (WT) and *thbs1*$^{-/-}$ mice 48 and 72 h following intratracheal (i.t.) inoculation with *K. pneumoniae*. (**c**) Lung cytokine measurements in pg ml^{-1} from 100 μg protein of tissue homogenates obtained from the left lung of mice 48 h following i.t. *K. pneumoniae*. Cytokines were measured individually by enzyme-linked immunosorbent assay, and data are presented in one graph for simplicity. (**d**) Myeloperoxidase (MPO) activity in left lung tissue homogenates measured as U/lung from WT and *thbs1*$^{-/-}$ mice 48 and 72 h after i.t. inoculation with *K. pneumoniae*. (**e**) Total BAL protein concentrations in μg ml^{-1} from WT and *thbs1*$^{-/-}$ mice 48 and 72 h after i.t. inoculation with *K. pneumoniae*. (**f**) Representative hematoxylin and eosin sections of R lung tissue obtained from WT and *thbs1*$^{-/-}$ mice at 72 h post *K. pneumoniae* instillation. Experimental mice underwent BAL of the lung prior to tissue fixation. Scale bar = 100 μm. (**g**) Lung histology shows a higher inflammation score in WT compared with *thbs1*$^{-/-}$ mouse lung tissue sections from 72 h post *K. pneumoniae* instillation, $n = 60$ random high-powered images from three sections per group scored by three blinded reviewers. The averaged score for each image is shown as an individual point. (**h**) NE activity measured in lung tissue homogenates at 48 and 72 h following i.t. *K. pneumoniae*. (**a**–**e** and **h**) $n = 7$–10 mice per group, Mann–Whitney *U*-rank sum test, ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$. G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; KC, keratinocyte-derived chemokine; MCP, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha; WBC, white blood cells.

N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide is an excellent substrate for NE but not CG,[31] protease 3 is a neutrophil serine protease contained within azurophilic granules that can also hydrolyze *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide.[32–34] However, neutrophils obtained from mice deficient in NE (*Ela2*$^{-/-}$) showed minimal to no measureable ability to hydrolyze *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, indicating that NE accounts for the serine protease activity involved in this reaction (**Figure 4a**).

A mixing experiment was conducted to test whether WT neutrophils possessed a factor that could inhibit the enhanced NE activity observed in *thbs1*$^{-/-}$ neutrophils. Increasing the ratio of WT neutrophil lysates (1:3, 2:3, 3:3) added to the

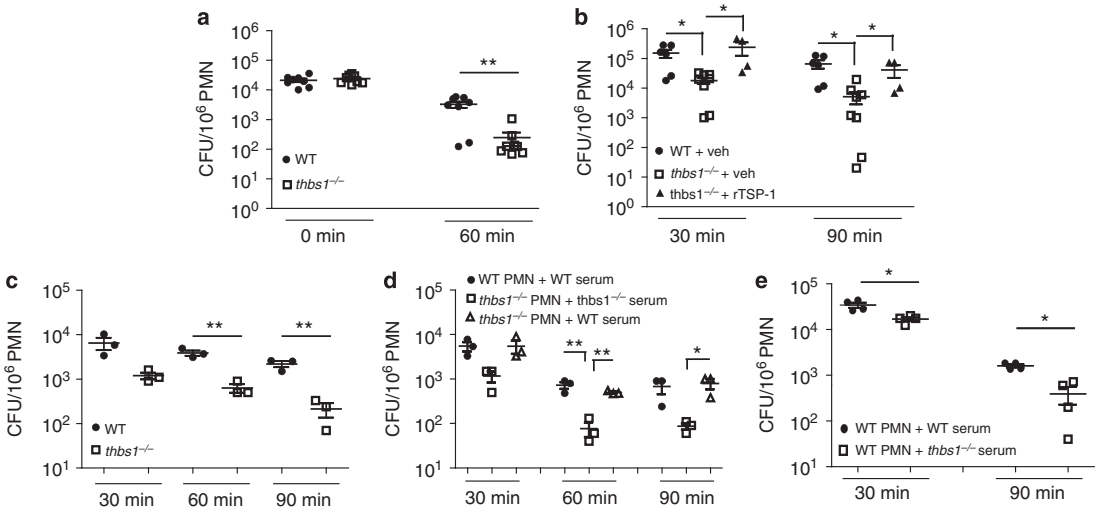

Figure 3 *thbs1*$^{-/-}$ neutrophils show enhanced intracellular microbial killing that is reversed by administration of recombinant thrombospondin-1 (TSP-1) or wild-type (WT) serum. (**a**) Intracellular killing by neutrophils from WT and *thbs1*$^{-/-}$ mice exposed to *K. pneumoniae* intraperitoneally. Neutrophils were immediately harvested and subsequently plated to quantify colony-forming units (CFUs) or incubated further *ex vivo* for an additional 60 min prior to plating. CFU/10^6 polymorphonuclear cell (PMN) was obtained for each sample. Data points indicate eight samples per group using harvested neutrophils from four *thbs1*$^{-/-}$ and four WT mice. (**b**) Mice were pretreated with recombinant TSP-1 (10 μg per mouse) or phosphate-buffered saline vehicle 30 min prior to intraperitoneal instillation of *K. pneumoniae*. Neutrophils were harvested and subsequently plated to quantify CFU at 30 min or incubated further *ex vivo* for an additional 60 min prior to plating. CFU/10^6 PMN was obtained for each sample. Data points indicate 4–8 samples per group using harvested neutrophils from six *thbs1*$^{-/-}$ and three WT mice. (**c**) *In vitro* microbial killing assay with WT and *thbs1*$^{-/-}$ neutrophils. Neutrophils were harvested from the peritoneum 6 h following 3% thioglycollate, as detailed in the Materials and Methods. *K. pneumoniae* was opsonized with 20% homologous serum on ice for 15 min. Neutrophils at 10^6 per well were infected with *K. pneumoniae* at a multiplicity of infection of 50 bacteria: 1 neutrophil *in vitro*. Neutrophils were washed with HBSS + gentamicin to remove extracellular or membrane-attached bacteria, lysed with 0.1% Triton-X, and CFU determined at the time points indicated. Data points indicate neutrophils harvested from individual mouse, $n = 3$ mice per group. (**d**) *In vitro* neutrophil microbial killing assay with cross-transfer of WT serum to *thbs1*$^{-/-}$ neutrophils. Conditions are the same as indicated in **c**, except *thbs1*$^{-/-}$ neutrophils received bacteria opsonized with either 20% homologous or WT serum. Data points indicate neutrophils harvested from individual mouse, $n = 3$ mice per group. (**e**) *In vitro* neutrophil microbial killing assay with WT neutrophils receiving either bacteria in WT or *thbs1*$^{-/-}$ serum. Data points indicate neutrophils harvested from individual mouse, $n = 4$ mice per group. Mann–Whitney *U*-rank sum test for two group comparisons, Kruskal–Wallis test followed by a Dunn's Multiple Comparisons test for three group comparisons, $^*P < 0.05$, $^{**}P < 0.01$.

substrate mixture containing a fixed amount of *thbs1*$^{-/-}$ neutrophil lysates dose-dependently reduced the NE activity to the level observed in WT neutrophils (**Figure 4b**). Thus, neutrophils derived from an *in vivo* environment that lacks TSP-1 show unrestrained NE activity, and reconstitution with lysates from neutrophils derived from mice with intact TSP-1 eliminated this effect. Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide is a chemical substrate for CG but not NE,[31] and *thbs1*$^{-/-}$ neutrophils also showed enhanced CG activity compared with WT neutrophils (**Figure 4c**). Taken together, the data indicate TSP-1 regulates both NE and CG activity in neutrophils.

To determine whether TSP-1 alters neutrophil respiratory burst, we measured the oxidation of dihydro-rhodamine to rhodamine in *thbs1*$^{-/-}$ and WT neutrophils, a clinically relevant test performed on patient neutrophils suspected of NADPH oxidase dysfunction. *thbs1*$^{-/-}$ neutrophils showed similar respiratory burst to WT neutrophils (**Figure 4d**), indicating that reactive oxygen species generation is not exaggerated in *thbs1*$^{-/-}$ neutrophils that may account for the altered pulmonary host defense. We also compared the ultrastructure of *thbs1*$^{-/-}$ neutrophils with WT neutrophils, and show similar morphology (**Figure 4e**). Furthermore, *thbs1*$^{-/-}$ neutrophils show similar *in vitro* phagocytosis of fluorescently labeled *E. coli* bioparticles compared with WT neutrophils (**Figure 4f**). Collectively, these findings suggest that TSP-1 impacts neutrophil proteolytic function by restraining NE and CG activity, but does not significantly alter the respiratory burst, phagocytosis, or morphology.

Peptides generated from the TSP-1 type-III repeat domain inhibit protease activity of neutrophils

The type-III repeat domain is a region within TSP-1 that harbors possible inhibitory reactive centers bearing striking similarity to consensus sequences derived from the Kazal and Streptomyces subtilisin inhibitor family members.[35,36] We generated peptides corresponding to residues 734–742 (DP-9 peptide) and 793–801 (DV-9 peptide), two stretches of eight amino acids within the type-III repeat domain, bearing reactive-site sequence similarity to select members of the Kazal family of subtilisin inhibitors. Moreover, a peptide corresponding to amino-acid residues 734–742 previously showed the ability to inhibit activity of purified CG *in vitro*, whereas a peptide corresponding to residues 793–801 showed

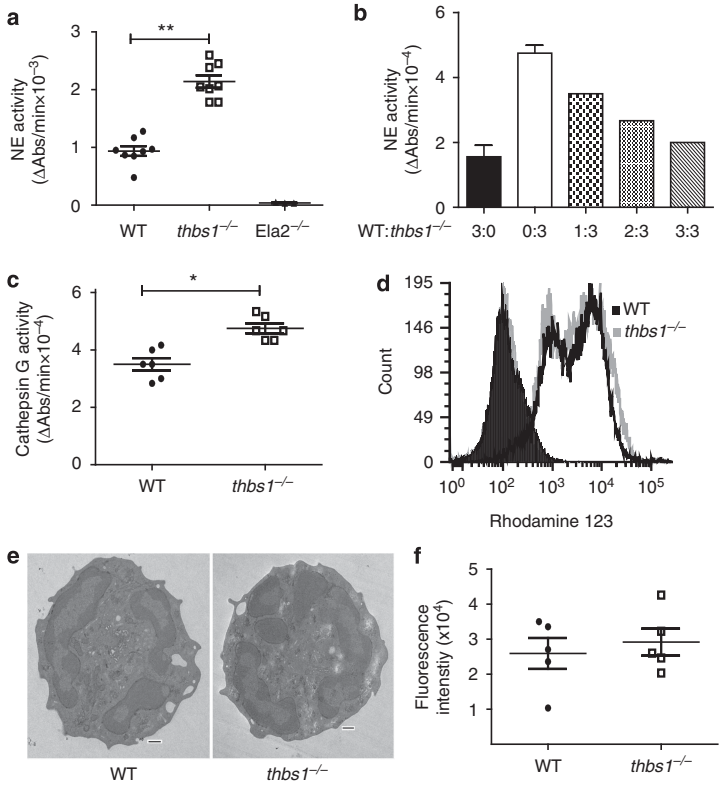

Figure 4 *thbs1*$^{-/-}$ neutrophils show increased neutrophil elastase (NE) and cathepsin G (CG) activity, but normal neutrophil oxidative burst, *in vitro* phagocytosis, and morphology. Neutrophils were harvested from the peritoneum 6 h following 3% thioglycollate injection. (**a**) NE activity measured as the rate of enzymatic hydrolysis of synthetic NE substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide reflected by the increase in absorbance (Abs) at 405 nm over time utilizing lysates obtained from wild-type (WT), *thbs1*$^{-/-}$, and Ela2$^{-/-}$ neutrophils. Data points indicate neutrophils obtained from four mice per group performed in duplicates. Student's *t*-test, $^{**}P<0.001$. (**b**) NE activity measured in WT, *thbs1*$^{-/-}$ lysates, and mixtures of WT:*thbs1*$^{-/-}$ neutrophil lysates at differing ratios normalized to protein concentrations. The mixture of neutrophil lysates was generated and pooled from WT and *thbs1*$^{-/-}$ mice at a ratio of 1:3, 2:3, 3:3, where three indicates 30 μl lysate containing 10 μg protein. Data obtained from WT (3:0, black bar graph) and *thbs1*$^{-/-}$ (0:3, white bar graph) neutrophil lysates. (**c**) CG activity measured as the rate of enzymatic hydrolysis of synthetic CG substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide reflected by the increase in absorbance (Abs) at 405 nm over time utilizing lysates obtained from WT and *thbs1*$^{-/-}$ neutrophils. Data points indicate neutrophils obtained from three mice per group performed in duplicates. Student's *t*-test, $^{*}P<0.01$. (**d**) Respiratory burst of WT and *thbs1*$^{-/-}$ peritoneal neutrophils as indicated by the shift in fluorescence following oxidation of dihydro-rhodamine (0.4 μg ml^{-1}) to rhodamine. Gray-filled histogram: *thbs1*$^{-/-}$ neutrophils at baseline; black-filled histogram: WT neutrophils at baseline; gray unfilled histogram: *thbs1*$^{-/-}$ neutrophils stimulated with phorbol myristate acetate (PMA) 2 μg ml^{-1}; black unfilled histogram: WT neutrophils stimulated with PMA 2 μg ml^{-1}. Data obtained from neutrophils pooled from five mice per group. (**e**) Transmission electron microscopy of WT and *thbs1*$^{-/-}$ neutrophils pooled from five mice per group. Images are representative of 15 neutrophils examined in each group. Scale bars = 500 nm. (**f**) *In vitro* phagocytosis of fluorescence-labeled *E. Coli* bioparticles by WT and *thbs1*$^{-/-}$ neutrophils, as indicated by fluorescence intensity. Data points indicate neutrophils obtained from five mice per group performed in quadruplicates.

inhibitory activity to both purified CG and NE.[35] We generated a third peptide corresponding to residues 854–862 (DA-9) without reactive-site sequence similarity or known inhibitory activity[35] that was used as control. Unlike *in vitro* conditions involving purified enzyme and inhibitor of known concentrations, activated neutrophils isolated from thioglycollate-stimulated peritoneum possess numerous proteases and enzymes that can reduce peptide efficacy. Thus, peptides were utilized in excess concentration to determine whether discrete regions of the type-III repeat domain exhibit inhibitory NE and CG activity by a mechanism of substrate competition. Lysates prepared from WT neutrophils were tested for NE activity, as measured by the rate of enzymatic hydrolysis of substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, in the presence or absence of peptides DV-9, DP-9, and control peptide DA-9.

NE activity was dose-dependently inhibited in the presence of DV-9. DV-9 reduced NE activity by 28% at 1.25 mM and 89% at 2.5 mM peptide, whereas DP-9 or DA-9 showed no effect (**Figure 5a**). CG activity, on the other hand, as measured by the rate of enzymatic hydrolysis of substrate *N*-Suc-Ala-Ala-Pro-Phe *p*-nitroanilide, was dose-dependently inhibited by DV-9 by 54% at 1.25 mM and 86% at 2.5 mM (**Figure 5b**). DP-9 at the higher concentration of 2.5 mM inhibited CG activity by 20%,

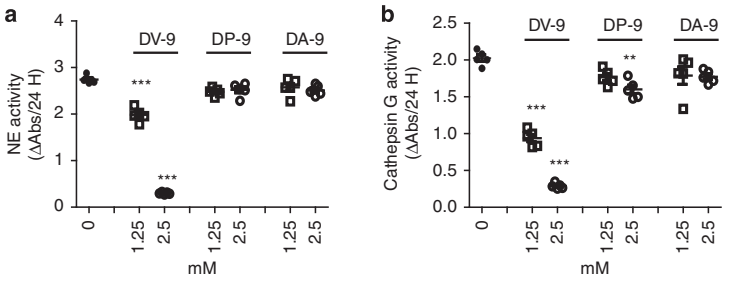

Figure 5 Peptides generated from the thrombospondin-1 type-III repeat domain inhibit neutrophil proteolytic function. (**a**) Neutrophil elastase (NE) activity measured in wild-type (WT) neutrophil lysates with 1.25 or 2.5 mM peptides DV-9 (DNCQYVYNV), DP-9 (DNCPFHYNP), and DA-9 (DNCPYVPNA). Data points indicate neutrophils obtained from five mice per group. (**b**) Cathepsin G activity measured in WT neutrophil lysates 1.25 or 2.5 mM peptides DV-9 (DNCQYVYNV), DP-9 (DNCPFHYNP), and DA-9 (DNCPYVPNA). Data points indicate neutrophils obtained from five mice per group. Analysis of variance with Bonferroni multiple comparisons test, $^{**}P<0.01$, $^{***}P<0.001$. Abs, absorbance.

whereas DA-9 showed no effect (**Figure 5b**). Thus, residues 793–801 within the break sequence of the calcium-binding loops, located in the type-III repeat domain of TSP-1, can restrain neutrophil proteolytic function by effectively inhibiting NE and CG enzymatic activity. Residues 734–742 of TSP-1 showed a weak inhibitory effect on CG enzymatic activity within neutrophils.

Peptides corresponding to residues 793–801 of the type-III repeat domain impair neutrophil microbial killing of *K. pneumoniae*

We next tested whether administration of peptide DV-9, showing inhibitory activity against NE and CG proteolytic function contained within neutrophil lysates, can alter neutrophil microbial killing. Neutrophils harvested from mice administered DV-9 peptide showed significantly higher viable bacteria at 30 and 90 min post bacterial inoculation than neutrophils from vehicle-treated mice (**Figure 6**). Neutrophils from vehicle-treated mice showed a 8.3-fold reduction in viable bacteria with *ex vivo* incubation over time, whereas neutrophils from peptide DV-9-treated mice showed a 3.5-fold reduction in viable bacteria over time (mean-fold reduction in CFU/10^6 polymorphonuclear cells ± s.e.: 8.3 ± 2.0 vs. 3.5 ± 0.6, $P<0.05$). Thus, the administration of a peptide corresponding to the type-III repeat region of TSP-1 can impair neutrophil microbial-killing machinery against *K. pneumoniae*.

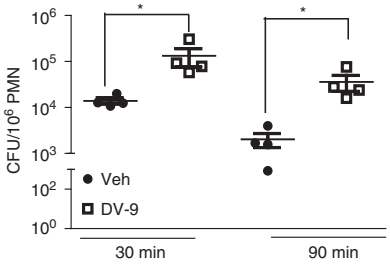

Figure 6 DV-9 peptide impairs neutrophil microbial killing of *K. pneumoniae.* Intracellular killing by peritoneal neutrophils from wild-type mice treated with peptide DV-9 or vehicle dimethyl sulfoxide just prior to instillation of *K. pneumoniae in vivo.* Thirty min represents the initial time point sampled, allowing for neutrophils to engulf bacteria *in vivo.* Neutrophils were harvested and subsequently plated to quantify colony-forming units (CFUs) at 30 min or incubated further *ex vivo* for an additional 60 min prior to plating. CFU/10^6 polymorphonuclear cell (PMN) was obtained for each sample. Data points indicate neutrophils harvested from individual mouse, $n=4$ mice per group. $^{*}P<0.05$. Mean-fold reduction in CFU/10^6 PMN ± s.e.m. over time: vehicle treatment, 8.3 + 2.0 compared with DV-9 treatment, 3.5 + 0.6, Mann–Whitney *U*-test, $^{*}P<0.05$.

DISCUSSION

Mice deficient in TSP-1 show enhanced bacterial clearance from the lungs induced by *K. pneumoniae*, reduced splenic dissemination, and increased survival compared with WT controls. Lungs of *thbs1*$^{-/-}$ mice show enhanced NE activity but reduced parenchymal inflammation and injury, suggesting that early containment of bacterial pathogen and microbial killing reduces overall intensity of the inflammatory response following intratracheal challenge with *K. pneumoniae*. In the complete absence of TSP-1, neutrophil serine protease NE and CG activity are intensified as is intracellular microbial killing in a model dependent upon an effective host response to a live bacterial pathogen. We suggest that TSP-1 provides an endogenous mechanism to curtail neutrophil proteolytic function during inflammation and identify a region within the break sequence of the calcium-binding loops, located in the type-III repeat domain of TSP-1, that may be involved in this interaction.

We have recently shown that mice deficient in TSP-1 are prone to experimental lipopolysaccharide-induced lung injury,[14] a well-known non-infectious inducer of inflammation and injury causing vascular leak. However, with a live bacterial pathogen, *thbs1*$^{-/-}$ mice show more effective containment of bacteria and reduced lung inflammation and injury. Susceptibility to sterile injury but enhanced ability to combat microbial challenge is a phenotype that is consistent with unbridled neutrophil protease activity. Others have shown improved host defense in *thbs1*$^{-/-}$ mice against live bacterial or fungal challenge,[37,38] but the mechanisms for these observations were not well understood. *thbs1*$^{-/-}$ mice showed improved survival in a cecal ligation puncture model of sepsis, involving polymicrobial infection in addition to an intraperitoneal

challenge with live *E. coli* bacteria.[37] In an experimental model of systemic candidasis, *thbs1*$^{-/-}$ mice also showed less dissemination of *Candida albicans* systemically and improved survival.[38] Although both studies suggested that TSP-1 impairs phagocytic capacity as a potential mechanism for survival differences following pathogen challenge, it remains unclear how TSP-1 would mediate the defect in phagocytosis. In contrast, we have previously reported WT and *thbs1*$^{-/-}$ alveolar macrophages show similar ability to phagocytize apoptotic neutrophils *in vivo*,[14] and herein, show no significant differences between WT and *thbs1*$^{-/-}$ neutrophils to phagocytize fluorescently labeled *E. coli* bioparticles *in vitro*. Similar to our findings, however, *thbs1*$^{-/-}$ mice showed reduced fungal burden and reduced host inflammatory response in the kidneys in the *C. albicans* model,[38] inviting speculation that effective containment of pathogen in *thbs1*$^{-/-}$ mice also reduced the burden of inflammation following systemic *C. albicans* challenge. Indeed, enhanced neutrophil intracellular microbial killing in *thbs1*$^{-/-}$ mice due to unopposed serine protease activity could potentially provide an explanation for the findings in the prior studies.

Non-oxygen-dependent neutrophil microbial killing mechanisms are a critical component of host defense. Mice deficient in NE (*Ela2*$^{-/-}$) show impaired host defense and survival in Gram-negative bacterial sepsis from intraperitoneal *K. pneumoniae* or *E. coli* infection,[29] systemic candidiasis from *C. albicans*,[18] and pneumonia from *Pseudomonas aeruginosa*.[39] Mice deficient in CG (*CtsG*$^{-/-}$) show impaired survival following *Staphylococcus aureus*[18] and *Streptococcus pneumoniae*[40] infection. Thus, it is not surprising that NE and CG double-knockout mice (*Ela2*$^{-/-}$ *CtsG*$^{-/-}$) show increased susceptibility to fungal infection from *Aspergillus fumigatus*, despite normal neutrophil recruitment and phagocytic activity,[30] and essentially worse survival than *CtsG*$^{-/-}$ mice from *S. pneumoniae* pneumonia with greater degree of lung injury from failure to effectively contain pathogen-derived virulence factors.[40]

Surprisingly, mice deficient in SERPINB1 (*serpinb1*$^{-/-}$), a serine protease inhibitor of NE, CG, and proteinase 3, fail to clear *P. aeruginosa* pneumonia and show worse survival and inflammation.[41] Although SERPINB1 is expressed in the cytoplasm of neutrophils, the data would indicate that SERPINB1 mainly functions to protect against the tissue injurious effects of extracellular neutrophil serine proteases and the degradation of host defense molecules such as surfactant protein D.[41,42] Interestingly, *Ela2*$^{-/-}$ *CtsG*$^{-/-}$ mice are resistant to endotoxic shock, and are protected from vascular leak and lung injury,[30] supporting the notion that unmitigated release of neutrophil serine proteases into the extracellular space can lead to fulminant tissue injury. Thus, the phenotype of *thbs1*$^{-/-}$ mice appears to be the reverse of *Ela2*$^{-/-}$ *CtsG*$^{-/-}$ mice. Given the TSP-1 effects on neutrophil microbial killing and intracellular protease activity, our data indicate that TSP-1 may be involved in the early regulation and compartmentalization of neutrophil granule serine proteases.

TSP-1 is a matricellular protein with numerous ligands and potential binding partners *in vitro*.[11] In kinetic studies examining TSP-1 inhibition of NE substrate hydrolysis, 1 mol of TSP-1 trimer binds to 2.7 ± 0.3 mol of NE with a site-binding constant of 57 ± 13 nM.[4] In assessing TSP-1 inhibition of CG substrate hydrolysis, 1 mol of TSP-1 trimer binds to 2.9 ± 0.4 mol of CG with a site-binding constant of 7.0 ± 3.5 nM.[3] These site-binding constants were obtained in the presence of calcium, as TSP-1 interactions with NE and CG are sensitive to calcium-dependent conformational changes within the type-III repeat domain of TSP-1.[3,4] In the absence of calcium, the reversible inhibition of NE and CG by TSP-1 is enhanced.[3,4] Given the complex nature of TSP-1 binding, predictions of physiological interactions remain challenging, and highlight the importance of clarifying the role of TSP-1 in the context of cell-based and *in vivo* studies. Various reports *in vitro* suggest TSP-1, either released in solution or adhered to a variety of ligands or binding partners, bind and induce motility of neutrophils, conceivably directing their migration in tissue.[25–28,43] We found no evidence that TSP-1 significantly contributes to neutrophil recruitment in our model. Although activated rabbit and human neutrophils produce TSP-1,[6] our findings indicate that the major contributor of TSP-1 is extracellular in origin, likely released in solution during inflammation and adhering to neutrophils to modulate their activity.

In conclusion, we have identified a novel role for TSP-1 in regulating neutrophil function during the innate immune response to *K. pneumoniae* infection. Our findings suggest that TSP-1 can bridle neutrophil proteolytic activity and microbial killing through the inhibitory action of its type-III repeat domain, providing one mechanism by which TSP-1 regulates the innate immune response. Although the host has evolved mechanisms to curtail the extracellular tissue-damaging effects of neutrophil serine proteases, TSP-1 appears to curb neutrophil proteolytic function with unintended consequences for host defense. Some chronic inflammatory lung diseases that demonstrate protease/anti-protease imbalance paradoxically show impaired ability to effectively kill colonizing bacteria, despite large numbers of neutrophils. Free NE activity in the bronchoalveloar lavage fluid of children, with cystic fibrosis early in life, is a strong independent risk factor for the development of bronchiectasis,[44] and inability to control infection precedes, initiates, and sustains inflammation in cystic fibrosis airways.[45] Neutrophils from cystic fibrosis patients show increase in TSP-1 gene expression by microarray compared with neutrophils from control healthy subjects.[46] Moreover, cystic fibrosis patients show increased circulating activated platelets with formation of heterotypic aggregates and surface P-selectin expression,[47,48] inviting speculation that excessive TSP-1 release in some inflammatory lung diseases may contribute to neutrophil dysfunction and impaired ability to effectively kill colonizing microbes.

METHODS

Animals. Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee at the

University of Pittsburgh. C57BL/6J (#000664) and *thbs1*$^{-/-}$ (B6.129S2-*Thbs1*^{tm1Hyn}/J, #006141) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Thbs1*$^{-/-}$ mice were backcrossed to C57Bl/6 mice nine times and a colony was subsequently established at the University of Pittsburgh. *Ela2*$^{-/-}$ (B6.129X1-*Ela2*^{tm1Sds}/J) mice were obtained from a colony at the University of Pittsburgh. All experimental procedures were performed in age- (8–12-week old) and gender-matched mice. The animals were housed and maintained in a pathogen-free environment.

Experimental bacterial pneumonia model. *K. pneumoniae* strain 43816, serotype 2 (American Type Culture Collection, Manassas, VA) was grown in tryptic soy broth overnight at 37 °C. One ml of this overnight culture was inoculated into fresh tryptic soy broth and grown for 2 h. A standard growth curve of bacterial culture measured by absorbance at 600 nm was generated to determine mid-log phase of growth. Inoculum concentration, measured in CFU, was determined by serial 10-fold dilutions of bacteria plated on tryptic soy agar plates (Sigma, St Louis, MO). Bacteria was harvested, washed, and resuspended in phosphate-buffered saline before use. WT and *thbs1*$^{-/-}$ mice were anesthetized with isoflurane, and 10^3 CFU of *K. pneumoniae* in a total volume of 100 µl was carefully administered intratracheally under direct visualization using a sterile 200-µl pipet with the filtered tip positioned just above the vocal cords. For mortality studies, 10^4 inoculum was utilized. The inoculums were confirmed by plating serial 10-fold dilutions on tryptic soy broth agar plates.

Bronchoalveloar lavage fluid collection. Animals were euthanized at predetermined time points with isoflurane-inhaled anesthetic overdose within a closed container system, followed by cardiac puncture and exsanguination. Methods for obtaining bronchoalveloar lavage total cell counts and differential and lung histology have been previously reported.[24]

Measurements of lung and spleen bacterial burden. The left lung and spleen were removed following euthanasia at specified time points. For enumerating bacterial CFUs in the lungs and spleens, tissue was homogenized in 1 ml of sterile deionized H_20. Homogenates (100 µl) were plated by 10-fold serial dilution on tryptic soy agar plates. Bacterial CFU was counted after an overnight incubation at 37 °C.

Measurement of lung cytokines and chemokines. Total protein in lung tissue homogenates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The volume of lung tissue homogenate corresponding to 100 µg total protein from each sample was used to perform enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay duoset antibodies for measuring TNF-α, IL-6, IL-10, G-CSF, GM-CSF, KC, MCP-1 were obtained from R&D Systems (Minneapolis, MN).

MPO assay. Lung tissue homogenates in 0.5% hexadecyl-trimethylammonium bromide (HTAB) buffer (5 g of hexadecyl-trimethylammonium bromide in 1 l of MPO buffer, where MPO buffer contains 6.8 g of KH_2PO_4 and 8.7 g of K_2HPO_4 in 1 l of water) were sonicated for 30 s and then centrifuged at 20,000 *g* for 4 min. Supernatant (7 µl) was transferred into a 96-well plate; 200 µl of *O*-dianisidine hydrochloride solution is added immediately prior to reading the optical density at 450 nm at 0 and 6 sec. The MPO activity was calculated using the following formula as previously described:[49] units of MPO activity in each well = (the change in absorbance (between 0 and 60 s)/time (min)) × 1.13×10^{-2}.

Neutrophil serine protease activity measurements. NE activity in lung tissue homogenates was measured utilizing a previously described method.[50] Briefly, 1 mg lung tissue homogenates were incubated with 0.1 mol l^{-1} Tris-HCl buffer (pH 8.0) containing 0.5 mol l^{-1} NaCl and 1 mmol l^{-1} *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma) at 37 °C for 24 h. The degradation of substrate in samples was measured by spectrophotometry at 405 nm. To measure serine protease activity within neutrophils, neutrophils were harvested from the peritoneum at 6 h following intraperitoneal injection with 2 ml of 3% thioglycollate. Cell counts were performed manually using a hemocytometer. Cytospins confirmed >90% neutrophils at 6 h harvest. Neutrophils, 1×10^6 per well, were seeded into a 24-well plate. Non-adherent cells were gently washed off after a 30-min incubation. Remaining adherent cells were lysed with lysis buffer containing leupeptin (Cell Signaling Technology, Danvers, MA). Ten µg total protein of lysates from each sample was incubated with 0.1 mol l^{-1} Tris-HCl buffer (pH 8.0) containing 0.5 mol l^{-1} NaCl and 1 mmol l^{-1} of NE substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma) or CG substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide at 37 °C for 1 h, utilizing a modified protocol from Niemann, *et al.*[51] Peptides DP-9 (Ac-DNCPFHYNP-NH2), DV-9 (Ac-DNCQYVYNV-NH2), and DA-9 (Ac-DNCPYVPNA-NH2), corresponding to amino-acid residues 734–742, 793–801, and 854–862 of human TSP-1, respectively, were generated and used in select experiments (Chi Scientific, Maynard, MA). Neutrophil lysates were incubated with 0, 1.25, or 2.5 mM peptides for 30 min before NE and CG substrates were added. The reaction was measured by spectrophotometry at 405 nm over time.

Statistics. Results are reported as the mean plus or minus s.e.m. A Student's two-tailed *t*-test was used to compare two groups and analysis of variance with Bonferroni multiple comparisons test was used for experiments involving >1 comparison. For data that was not normally distributed, the Mann–Whitney rank sum test was used to compare two groups and the Kruskal–Wallis test followed by a Dunn's Multiple Comparisons test was conducted for experiments involving >1 comparison. Log-rank test was performed to generate the Kaplan–Meier survival curve. A *P*-value <0.05 was considered significant using GraphPad Prism software version 5.0 (La Jolla, CA).

Methods for the transmission electron microscopy, phagocytosis assay, microbial killing assay, and lung histology inflammation scoring are detailed in the online **Supplementary Materials** section at http://www.nature.com/mi.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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11.6. Applicant's Publication 3

Applicant's Publication 3 - CD36_Publication 3.pdf

The Journal of Infectious Diseases

MAJOR ARTICLE

CD36 Provides Host Protection Against *Klebsiella pneumoniae* Intrapulmonary Infection by Enhancing Lipopolysaccharide Responsiveness and Macrophage Phagocytosis

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Klebsiella pneumoniae remains an important cause of intrapulmonary infection and invasive disease worldwide. *K. pneumoniae* can evade serum killing and phagocytosis primarily through the expression of a polysaccharide capsule, but its pathogenicity is also influenced by host factors. We examined whether CD36, a scavenger receptor that recognizes pathogen and modified self ligands, is a host determinant of *K. pneumoniae* pathogenicity. Despite differences in serum sensitivity and virulence of 3 distinct *K. pneumoniae* (hypermucoviscous K1, research K2, and carbapenemase-producing ST258) strains, the absence of CD36 significantly increased host susceptibility to acute intrapulmonary infection by *K. pneumoniae*, regardless of strain. We demonstrate that CD36 enhances LPS responsiveness to *K. pneumoniae* to increase downstream cytokine production and macrophage phagocytosis that is independent of polysaccharide capsular antigen. Our study provides new insights into host determinants of *K. pneumoniae* pathogenicity and raises the possibility that functional mutations in CD36 may predispose individuals to *K. pneumoniae* syndromes.

Keywords. CD36; *Klebsiella pneumoniae*; macrophage; host defense; multi-drug resistant *K. pneumoniae*; pneumonia; hypermucoviscous strains.

Bacterial pneumonia is a major cause of morbidity and mortality [1], and *Klebsiella pneumoniae* remains an important causative agent of gram-negative bacterial pneumonia [2]. The global emergence and nosocomial dissemination of multidrug-resistant, carbapenemase-producing strains of *K. pneumoniae* (KPC) have been associated with increased costs, length of hospitalization, and significant morbidity and mortality but are generally observed in critically ill patients [3, 4]. On the other hand, *K. pneumoniae* causes a distinct severe community-acquired invasive syndrome prevalent in parts of Asia and Africa but uncommon in North America, Europe, and Australia [5]. This invasive syndrome is characterized by the presence of necrotizing pneumonia, hepatic abscesses, bacteremia, endophthalmitis, and meningitis and is attributed to *K. pneumoniae* strains expressing the K1 or K2 capsular antigen [6]. K1/K2 serotype *K. pneumoniae* strains are especially virulent due to the presence of mucoviscosity-associated gene A (*magA*), the regulator of mucoid phenotype A gene (*rmpA*), and the capsular antigens K1 and K2, which promote resistance to phagocytosis by neutrophils and macrophages, promote evasion of killing by serum components, and enable disease in presumably healthy hosts [5, 6].

The early innate immune response to intrapulmonary *K. pneumoniae* infection involves phagocytosis and clearance of foreign pathogens with inflammation. Disruption of any part of this normal host response to infection can result in adverse outcomes, but specific host factors that influence susceptibility to *K. pneumoniae* infection are less known. Scavenger receptors play a key role in maintaining homeostasis through recognition and removal of foreign substances from the body. Initially identified as receptors for oxidized low-density lipoprotein (oxLDL) [7], scavenger receptors recognize a diverse range of ligands, including both modified endogenous molecules or danger-associated molecular patterns and exogenous pathogen-associated molecular patterns [8, 9]. Eight classes of scavenger receptors have been defined (classes A–H) and are distinguished from each other by their unique structural characteristics [10]. Class B, which includes scavenger receptor class B1, lysosomal integral membrane protein II, and CD36 (also known as glycoprotein IV), is structurally defined by possessing 2 transmembrane regions. CD36 is predominantly expressed by

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macrophages but is also expressed by other cell types, including platelets, monocytes, endothelial cells, adipocytes, myocytes, and epithelial cells [11]. At least 12 mutations in the coding sequence of the human CD36 gene have been described [12], with a majority of CD36 deficiency phenotypes occurring predominantly in Asian and African populations [12, 13].

Like most scavenger receptors, CD36 preferentially binds polyanionic ligands, including oxidized phosphatidylcholine of oxLDL, and contributes to foam cell formation and atherosclerosis [14]. CD36 has also been shown to contribute to the host innate immune response by facilitating recognition of certain pathogen-derived diacylglycerides that signal through the Toll-like receptor 2/6 (TLR2/6) heterodimer complex [15]. N-ethyl-N-nitrosourea–induced mutation resulting in a premature stop codon within the CD36 gene renders homozygous mutant mice (*obl/obl*) tumor necrosis factor (TNF)–hyporesponsive to diacylglyceride lipoproteins, lipoteichoic acid, and the R-enantiomer of macrophage activating lipopeptide 2 [15]. While o*bl/obl* mice show an inability to contain *Staphylococcus aureus* skin infection as compared to WT mice, the phenotype of *obl/obl* mutant mice is less severe than that of *Tlr2*$^{-/-}$ mice following lethal systemic infection with *S. aureus*, suggesting that CD36 plays an accessory rather than central role in TLR2-induced signaling [15].

Overall, the role of CD36 in facilitating innate immune responses against gram-positive bacteria such as *S. aureus* and *Streptococcus pneumoniae* is known, but whether CD36 is involved in the recognition of and host defense against gram-negative bacteria is less clear [15–18]. Although modified self components oxLDL and amyloid β bind CD36 and trigger TLR4/TLR6 complex to promote sterile inflammation [19], in vitro studies using a CD36 overexpression system in HeLa cells indicate that lipopolysaccharide (LPS) and the gram-negative bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium induce interleukin 8 expression and JNK phosphorylation independent of TLR2 and TLR4 [20]. Given the complex nature of the interaction between CD36 and various bacterial-derived ligands, relevant in vivo models are required to clarify the function of CD36, particularly with regard to the innate immune response against gram-negative bacteria.

Using 3 distinct serotypes of the gram-negative pathogen *K. pneumoniae*—a research strain that expresses the K2 capsular polysaccharide serotype and is virulent in mice (hereafter, the "K2 strain") [21], a clinical strain that expresses the hypermucoviscous K1 capsular serotype causing liver and lung abscesses in Asia and is highly virulent in mice and humans (hereafter, the "K1 strain") [22], and a KPC-producing multidrug-resistant clinical strain (hereafter, the "K41 strain")—we show that CD36 is required for optimal control of *K. pneumoniae* in the lung and extrapulmonary bacterial dissemination to distant organ sites. In addition, we show that CD36 mediates recognition of LPS, enhances phagocytosis of *K. pneumoniae* by alveolar macrophages, and enhances JNK activation for optimal cytokine production in the lung. Collectively, our findings demonstrate a host-protective role for CD36 and implicate CD36 as a host determinant of *K. pneumoniae* pathogenicity.

METHODS

Animals

A total of 8–12-week-old age- and sex-matched C57Bl/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Cd36*$^{-/-}$ (B6.129S1-Cd36tm1Mfe/J) mice that were backcrossed to the C57Bl/6J mice for 11 generations were obtained from the Jackson Laboratory. These *Cd36*$^{-/-}$ mice were subsequently crossed to *Cd36*$^{-/-}$ mice (also backcrossed to the C57Bl/6J mice for 11 generations) from the Blood Research Institute (Blood Center of Wisconsin, Milwaukee, WI) to establish a founder colony at the University of Pittsburgh. C57Bl/6J control mice were also bred and housed in specific-pathogen-free conditions with free access to food and water within the same animal care facility at the University of Pittsburgh. For in vivo pneumonia studies with predetermined time points of harvest, a group size of 8 per group was determined a priori to achieve a power of 0.83 for a 2-tailed test using an α of 0.05. Mice were randomly assigned, and a skilled technician who was blinded to the biological hypothesis performed the inoculations, monitoring, and harvest. Male and female mice aged 8–12 weeks were used for experiments. Survival study was conducted separately from pneumonia studies. The mice were monitored carefully and euthanized when they met predefined criteria for euthanasia. All procedures were performed with approval of the Institutional Animal Care and Use Committee at the University of Pittsburgh (protocol numbers 14013145 and 15086456).

K. pneumoniae Inoculation

K. pneumoniae strains were grown overnight in tryptic soy broth (TSB) for 18 hours at 37°C. A 1:100 dilution of bacteria in TSB was then incubated again at 37°C for 1.5 hours. Initial studies indicated that the *K. pneumoniae* K2 strain grown to early logarithmic phase but not at the late logarithmic phase induced a reproducible pathogenic phenotype in the lungs of wild-type (WT) mice, and subsequent studies used an $OD_{600\ nm}$ of 0.2 to prepare the bacterial inoculum. We have previously described a detailed method of intratracheal administration of bacteria by direct visualization [21, 23].

Bronchoalveolar Lavage

We have also previously described a detailed method of bronchoalveolar lavage [24].

Cytokine Enzyme-Linked Immunosorbent Assays (ELISAs)

ELISA Duo-sets for interleukin 6 (IL-6), interleukin 10 (IL-10), interferon γ (IFN-γ), monocyte chemoattractant protein 1 (MCP-1), interleukin 12p70 (IL-12p70), interleukin 17A (IL-17A), interleukin 1β (IL-1β), and TNF-α were purchased from R&D Systems (Minneapolis, Minnesota).

Serum Killing Assay

Serum from healthy volunteers was obtained from peripheral whole blood following collection of a 20-mL blood sample by venipuncture. All subjects underwent venipuncture after providing informed written consent, and ethnicities and sex were identified by self-reporting. Following collection, serum was processed from whole blood and used in the in vitro study in a deidentified manner. The Institutional Review Board of the University of Pittsburgh approved the study (protocol number IRB0410173). Overnight cultures of *K. pneumoniae* strains were diluted 100-fold in TSB and incubated at 37°C until reaching an OD_{600} of 0.2. A total of 10^6 bacterial cells in early logarithmic phase, 5% sterile TSB, and 85% nonimmune human serum were incubated at 37°C. Bacterial growth in nonimmune human serum was determined by measurement at OD_{600} and, in select experiments, confirmed by determining colony-forming units (CFU) via bacterial serial plating.

Statistical Analysis

A Student *t* test was performed for comparisons between 2 groups. For data that were not normally distributed, a 2-tailed Mann–Whitney *U* test was used, and differences were considered significant for *P* values of $< .05$. Statistical analysis was performed using Graph Pad Prism 6 software (La Jolla, California).

Additional Assays

Detailed descriptions of bacterial strains, Western blot analysis, ex vivo stimulation of macrophages, cytotoxicity detection, and phagocytosis are provided in the Supplementary Materials.

RESULTS

Cd36$^{-/-}$ Mice Show Higher Lung Bacterial Burden and Greater Extrapulmonary Dissemination Following Intrapulmonary Infection With the K2 Strain

CD36 is a scavenger receptor that binds various polyanionic ligands and recognizes lipid signatures expressed by pathogen and modified self-ligand. We sought to determine the contribution of this receptor to pulmonary host defense against the K2 strain, which is a well-known ATCC research strain that is pathogenic in mice [21]. At 24 and 48 hours following bacterial inoculation of 2×10^3 CFU, *Cd36*$^{-/-}$ mice showed a higher bacterial burden in the lungs, compared with WT mice (Figure 1*A*). By 48 hours, *Cd36*$^{-/-}$ mice showed greater bacterial dissemination to the spleen, liver, and blood (Figure 1*A*). An inability to optimally control the bacterial load in *Cd36*$^{-/-}$ mice was reflected by a higher level of lung myeloperoxidase, a marker of neutrophilic inflammation following infection (Figure 1*B*). Impaired host defense led us to examine the effects of CD36 on mortality during intrapulmonary *K. pneumoniae* infection. At an inoculum of 7×10^3 CFU, *Cd36*$^{-/-}$ mice showed greater susceptibility to *K. pneumoniae* (Figure 1*C*). By 72 hours, 79% of *Cd36*$^{-/-}$ mice were dead following intratracheal instillation, in contrast to 44% of WT mice, with a few WT mice surviving the virulent *K. pneumoniae* infection for up to 20 days when the study was terminated ($P = .01$ by the log-rank test; Figure 1*C*).

Cd36$^{-/-}$ Mice Have a Higher Lung Bacterial Burden and Greater Extrapulmonary Dissemination Following Intrapulmonary Infection With the K1 Strain

The K1 strain causes liver abscesses and disseminated disease in humans [25–27]. Indeed, *K. pneumoniae* virulence is associated with capsular serotypes K1 and K2 in mice and humans [28, 29], but host factors that contribute to aggressive disease remain largely unknown. Thus, we tested the responses of WT and *Cd36*$^{-/-}$ mice to the K1 strain. At an inoculum of 1×10^3 CFU, *Cd36*$^{-/-}$ mice showed a higher bacterial burden in the lungs, early liver dissemination, increased neutrophilic inflammation, and greater weight loss as compared to WT mice (Figure 2*A*–*C*). Taken together, these findings indicate that CD36 is required for optimal control of virulent *K. pneumoniae* in the lung to prevent extrapulmonary dissemination and mitigate systemic toxicity.

KPC-Producing *K. pneumoniae* ST258 Is Susceptible to Serum Killing but Shows Enhanced Pathogenicity in *Cd36*$^{-/-}$ Mice Following Acute Intrapulmonary Infection

K. pneumoniae virulence determinants include the ability to evade complement-mediated killing and phagocytosis that is dictated primarily by the polysaccharide capsular structure [30] and lipopolysaccharide (LPS) [31]. Both factors contribute to bacteremia and lethality in murine models of experimental pneumonia [30, 32]. In contrast, KPC-producing strains generally lack virulence determinants such as K1 and K2 capsular serotypes and do not induce lethality in mouse septicemia models [33, 34]. We examined the ability of nonimmune serum obtained from healthy volunteers to inhibit the growth of various *K. pneumoniae* strains and determined their relative virulence in vitro. Although all strains showed similar growth curves in culture broth, K1 and K2 strains exhibited continued growth in human serum, as measured by OD_{600}, whereas human serum from healthy subjects inhibited growth of all KPC strains tested (Figure 3*A*). The subject demographic characteristics are shown in Figure 3*B*. Representative data examining CFU indicated KPC-producing strains were susceptible to human serum killing, whereas K1 and K2 strains escaped human serum killing (Figure 3*C*). Inactivation of heat-labile serum factors abrogated the susceptibility of KPC-producing strains and enabled growth in human serum (Figure 3*C*). As these findings highlight the role of soluble factors in providing defense to control growth of KPC-producing strains in blood, we sought to determine whether CD36 contributes to host defense against KPC-producing strains in the lung, where the pathogen is initially encountered.

When compared with WT mice, *Cd36*$^{-/-}$ mice showed impaired bacterial burden and increased extrapulmonary dissemination to the spleen and liver following intrapulmonary infection with KPC (Figure 4*A*). Moreover, bronchoalveolar lavage (BAL) concentrations of the cytokines IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, MCP-1, and TNF-α were significantly

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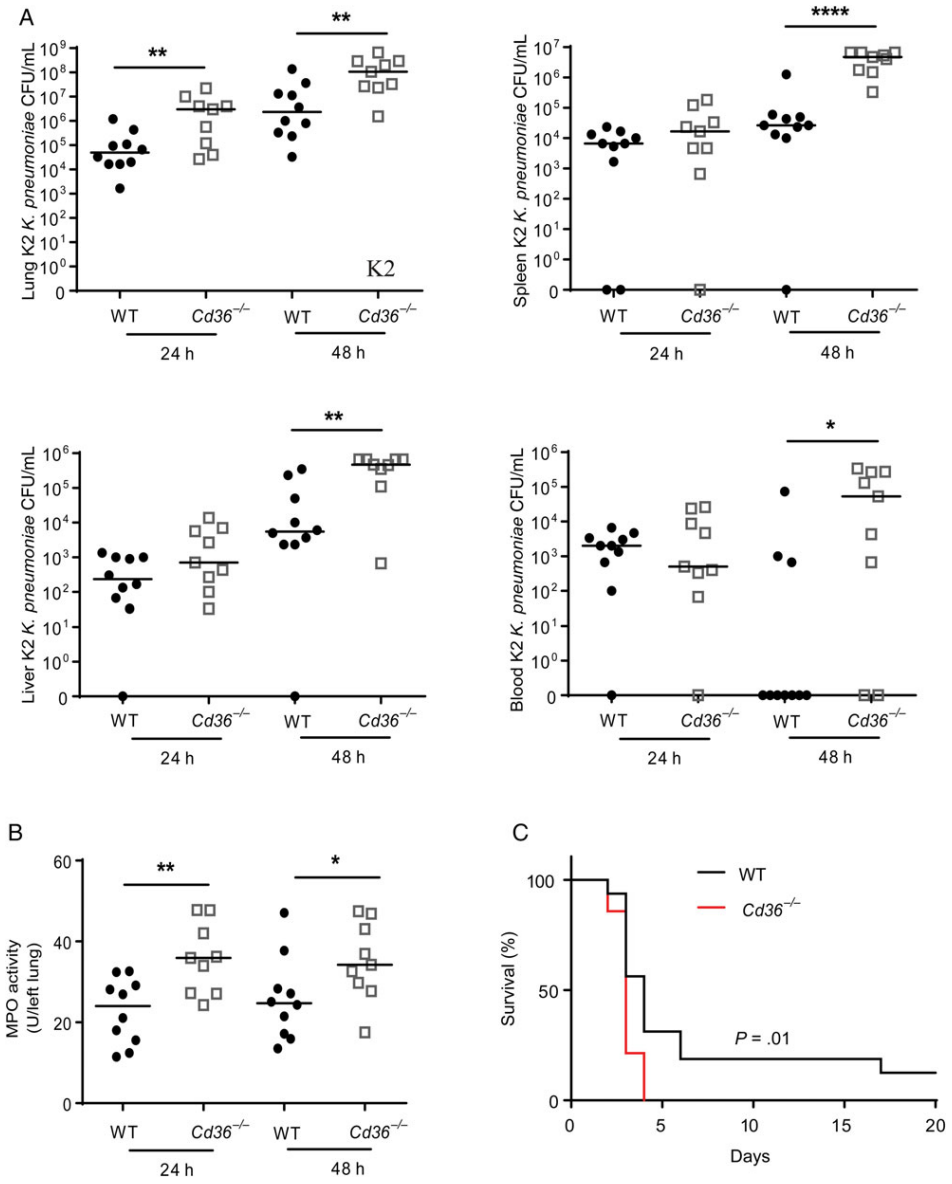


Figure 1. *Cd36*$^{-/-}$ mice show impaired lung bacterial burden, enhanced extrapulmonary dissemination, increased neutrophilic inflammation, and impaired survival following intrapulmonary infection with *Klebsiella pneumoniae*, capsular serotype K2. *A*, Colony-forming units (CFU) obtained from lung tissue homogenates, spleen homogenates, liver homogenates, and blood cultures of wild-type (WT) and *Cd36*$^{-/-}$ mice 24 and 48 hours following intratracheal inoculation with *K. pneumoniae* (inoculum, 2×10^3 CFU). *B*, Lung tissue myeloperoxidase (MPO) content. *A* and *B*, Dots and squares represent individual mice. Lines indicate median values. **P* < .05, ***P* < .01, and *****P* < .0001, by the 2-tailed Mann–Whitney *U* test. *C*, Survival curve of WT (n = 16) and *Cd36*$^{-/-}$ (n = 14) mice following intratracheal instillation of *K. pneumoniae* (7×10^3 CFU inoculum). *P* = .01, by the Mantel-Cox log-rank test.

reduced in *Cd36*$^{-/-}$ mice as compared to WT mice during infection (Figure 4*B*). For a similar inoculum given, it was notable that the bacterial burden in the lungs of WT mice following infection by KPC-producing strains was substantially less than that observed with virulent *K. pneumoniae* strains (Figures 1*A*, 2*A*, and 4*A*). Despite the muted virulence of KPC-producing strains relative to the virulence of K1 and K2 strains, the absence of CD36 enhanced host susceptibility to acute infection by KPC-producing *K. pneumoniae* and impaired lung cytokine production.

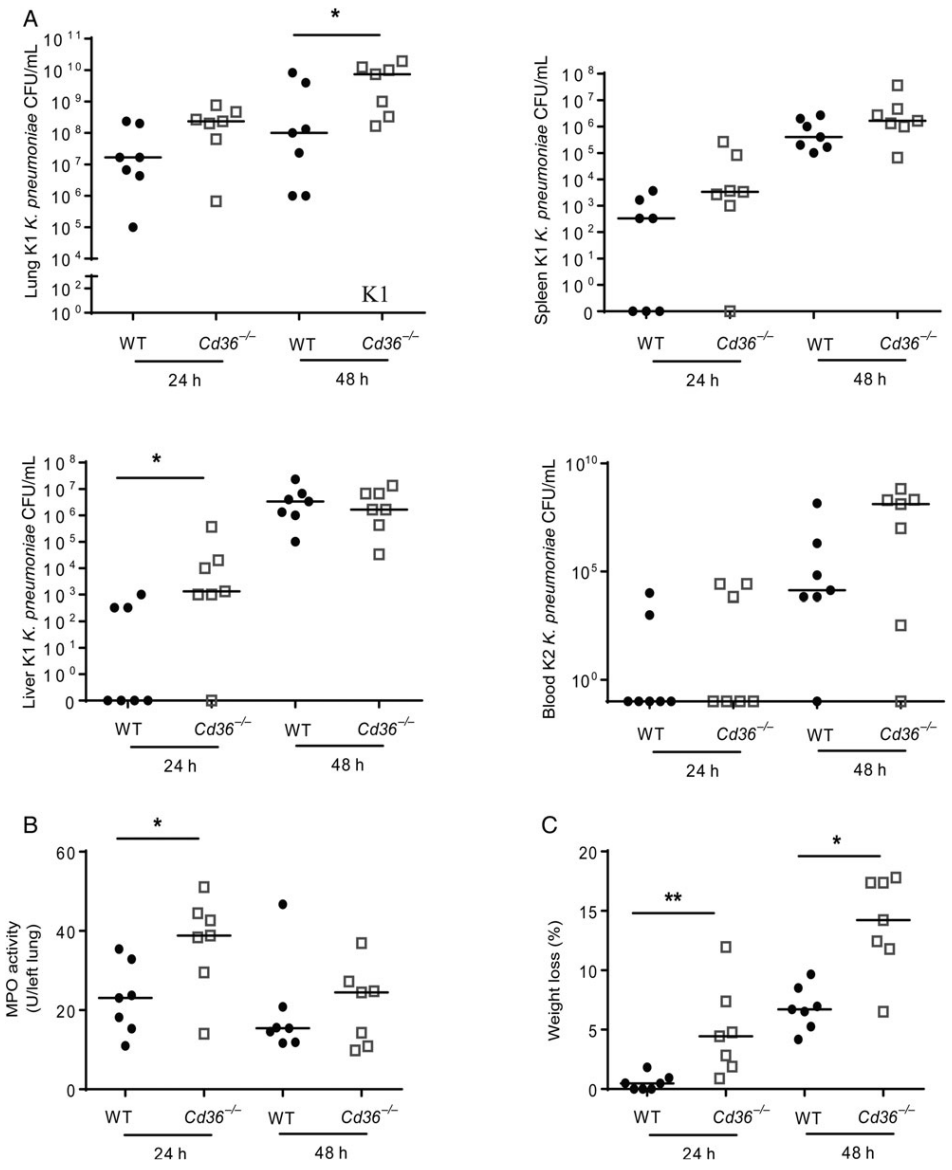
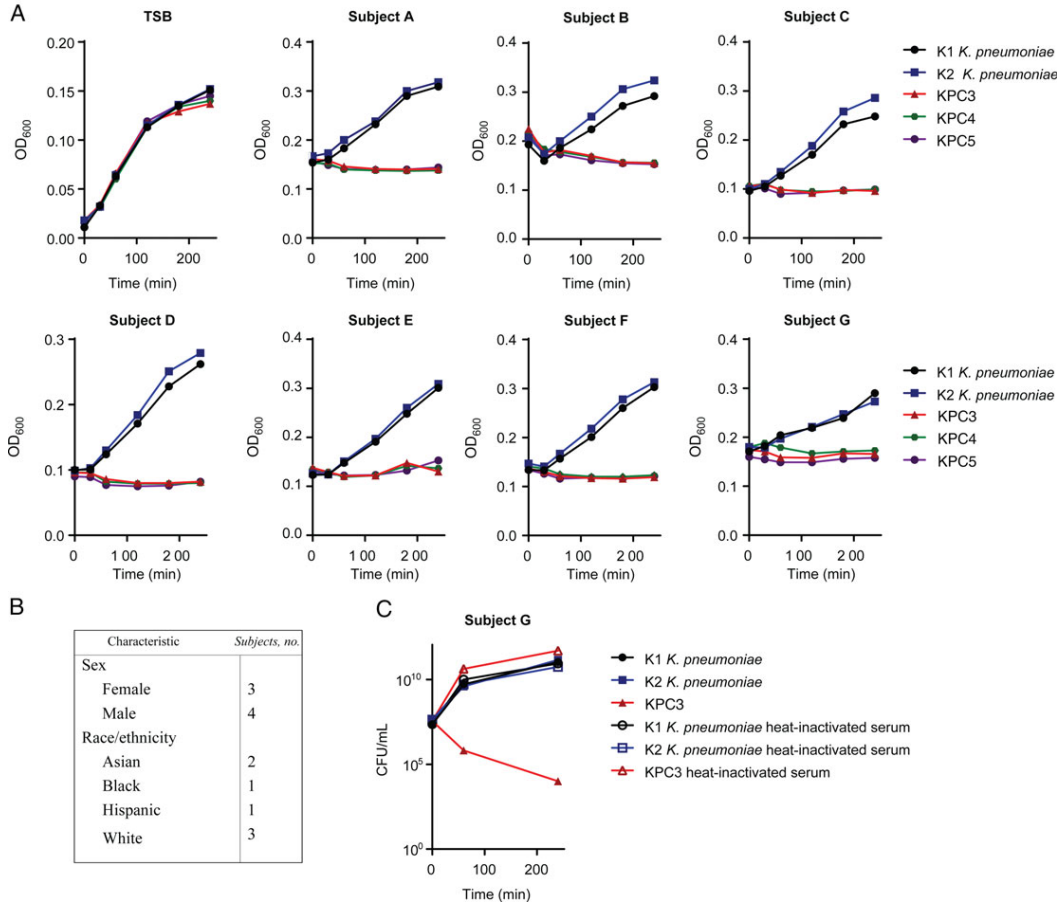


Figure 2. *Cd36*$^{-/-}$ mice show impaired lung bacterial burden, early liver dissemination, increased neutrophilic inflammation, and increased weight loss following intrapulmonary infection with hypervirulent *Klebsiella pneumoniae*, capsular serotype K1. A, Colony-forming units (CFU) obtained from lung tissue homogenates, spleen homogenates, liver homogenates, and blood cultures of wild-type (WT) and *Cd36*$^{-/-}$ mice 24 and 48 hours following intratracheal inoculation with *K. pneumoniae* (inoculum, 1.3×10^3 CFU). *B*, Lung tissue myeloperoxidase (MPO) content. *C*, Mice were monitored 24 and 48 hours following intratracheal inoculation with *K. pneumoniae*, and weights were recorded as the percentage of weight lost from baseline. Dots and squares represent individual mice. Lines indicates median values. **P* < .05 and ***P* < .01, by the 2-tailed Mann–Whitney *U* test.

Cd36$^{-/-}$ Macrophages Show Blunted Cytokine Production in Response to *K. pneumoniae* LPS That Is Independent of Capsular Serotype

Given the impaired cytokine response observed in the airspaces of *Cd36*$^{-/-}$ mice following infection, we next examined whether CD36 expressed by macrophages is involved in the recognition of *K. pneumoniae*. As the capsular polysaccharide and LPS are secreted [35], we exposed WT and *Cd36*$^{-/-}$ macrophages to cell-free supernatants obtained from K2 and K41 strains following a culture for 18 hours in TSB. We measured cytokine production as a readout to test CD36 function as a pattern-recognition



Characteristic	*Subjects, no.*
Sex	
Female	3
Male	4
Race/ethnicity	
Asian	2
Black	1
Hispanic	1
White	3

Figure 3. Carbapenemase-producing *Klebsiella pneumoniae* (KPC) clinical strains exhibit muted virulence and are susceptible to human nonimmune serum killing. *A*, Bacterial growth was determined by measurement at OD_{600} following incubation with tryptic soy broth alone or 85% normal human serum (% v/v) obtained from healthy subjects. Data are indicative of 2 independent experiments. *B*, Subject demographic characteristics. *C*, Representative colony-forming units (CFU) obtained after bacteria were exposed to either 85% normal human serum or 85% human serum heat-inactivated for 30 minutes at 56°C. Data are indicative of 2 independent experiments.

receptor. Incubation in TSB alone failed to induce cytokine production by WT and *Cd36*$^{-/-}$ macrophages (Figure 5*A*). In contrast, cell-free supernatants from K2 and K41 strains induced IL-10 and IL-6 production in WT macrophages, but this response was notably attenuated in *Cd36*$^{-/-}$ macrophages (Figure 5*A*). Similarly, K1, K2, and K41 supernatants induced TNF-α production in WT macrophages, and the response was attenuated in *Cd36*$^{-/-}$ macrophages (Figure 5*A*). Residual cytokine release in the absence of CD36 is due to TLR4 (Supplementary Figure 1) [36]. Reduction of capsular polysaccharide synthesis by sodium salicylate [37] did not alter *K. pneumoniae*–induced TNF-α production in WT and *Cd36*$^{-/-}$ macrophages, indicating an alternative bacterial component recognized by macrophages (Figure 5*A*). Bacterial cell lysates obtained from K1, K2, and K41 recapitulated IL-10 and IL-6 responses in WT macrophages observed with cell-free supernatants, and cytokine production was blunted in *Cd36*$^{-/-}$ macrophages (Figure 5*B*). At neutral pH, polylysine exhibits a high affinity for LPS ($K_D = 1.1 \times 10^{-11}$ M) [38]. Removal of LPS by treatment of K2 and K41 cell lysates by spinning in a polylysine column resulted in negligible endotoxin levels (<8 endotoxin units/mL by the *Limulus* amebocyte lysate test; data not shown) and abrogated macrophage cytokine production (Figure 5*B*). Moreover, incubation with purified LPS from K2 and K41 strains recapitulated macrophage cytokine response observed with *K. pneumoniae* supernatant in WT but not in *Cd36*$^{-/-}$ macrophages (Figure 5*C*). The attenuated cytokine response in *Cd36*$^{-/-}$ macrophages was not due to appreciable differences in cytotoxicity, as measured by lactate dehydrogenase release (Figure 5*D*). Collectively, these findings indicate CD36

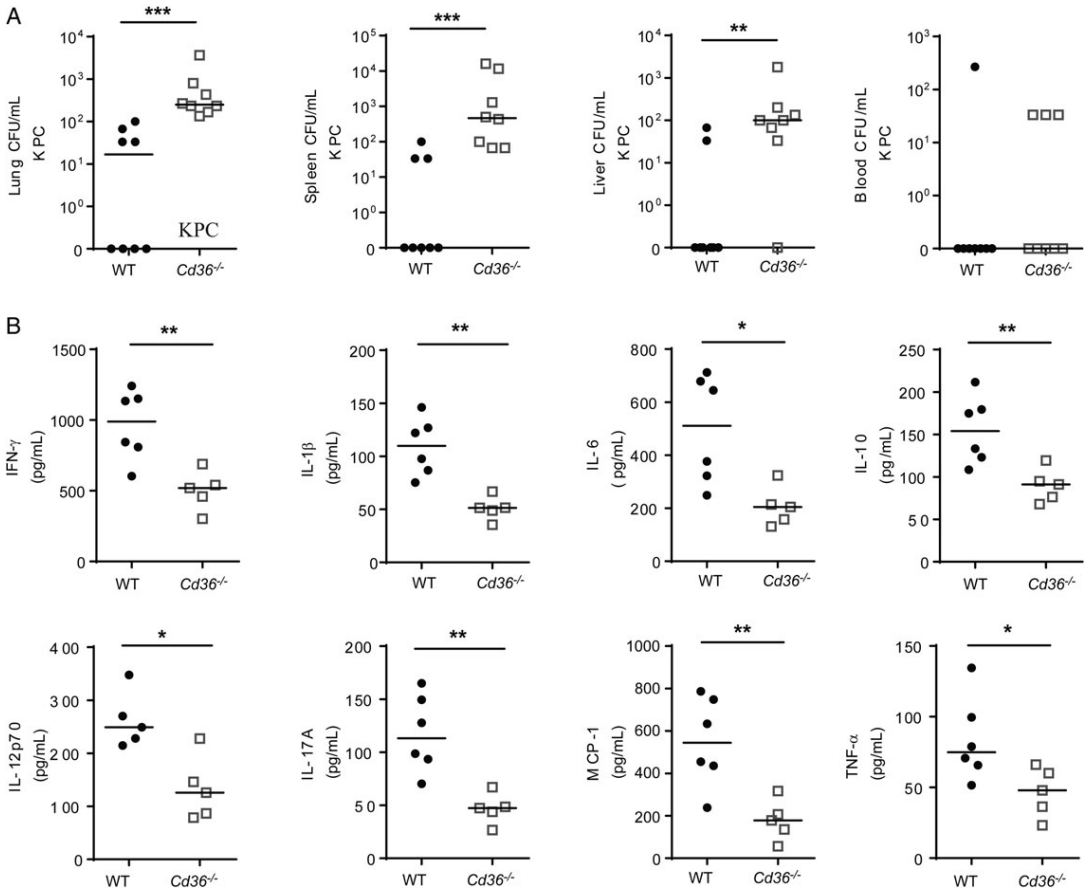


Figure 4. *Cd36*$^{-/-}$ mice show enhanced lung bacterial burden, enhanced extrapulmonary dissemination, and impaired cytokine production in the airspaces following infection with carbapenemase-producing *Klebsiella pneumoniae* (KPC). *A*, Colony-forming units (CFU) measured in lung tissue homogenates, spleen homogenates, liver homogenates, and blood cultures of wild-type (WT) and *Cd36*$^{-/-}$ mice 24 hours following intratracheal inoculation with *K. pneumoniae* (inoculum, 2.6×10^3 CFU). *B*, Concentrations of interferon γ (IFN-γ), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12p70 (IL-12p70), interleukin 17A (IL-17A), monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor α (TNF-α) were measured in cell-free bronchoalveolar lavage fluid from WT and *Cd36*$^{-/-}$ mice 24 hours after KPC infection. Dots and squares represent individual mice. Lines indicate median values. **P* < .05 and ***P* < .01, by the 2-tailed Mann–Whitney *U* test.

mediates LPS responsiveness and macrophage cytokine production that is independent of the capsular polysaccharide antigen.

Cd36$^{-/-}$ Macrophages Show Impaired Phagocytosis and JNK Activation

A main virulence mechanism of extracellular *K. pneumoniae* is related to the ability to evade phagocytosis [39]. To further examine CD36-mediated host protection, we evaluated CD36 involvement in *K. pneumoniae* phagocytosis and the downstream signaling pathway. We isolated alveolar macrophages from WT and *Cd36*$^{-/-}$ mice and exposed them to a tdTomato plasmid–expressing *K. pneumoniae* clinical strain to visualize internalization within F4/80-positive macrophages by quantitative confocal microscopy. To ensure an unbiased approach, the 4 corners of each cover slip were imaged and analyzed by a binary region of interest function within Nikon Instrument digital software. Compared with WT macrophages, *Cd36*$^{-/-}$ macrophages showed significant reduction in the uptake of *K. pneumoniae* (Figure 6*A*), indicating that CD36 is involved in the recognition and phagocytosis of *K. pneumoniae* [20]. We next determined whether impaired recognition results in impaired signaling. Macrophages isolated from *Cd36*$^{-/-}$ mice previously showed reduced activation of JNK but not p38 in response to oxLDL [40], and CD36-overexpressing HEK293 cells showed higher levels of JNK phosphorylation following treatment with LPS or *E. coli* in vitro [20]. JNK signaling is tightly linked to cytokine production [41, 42], and inhibition of JNK activity in macrophages impairs cytokine production following

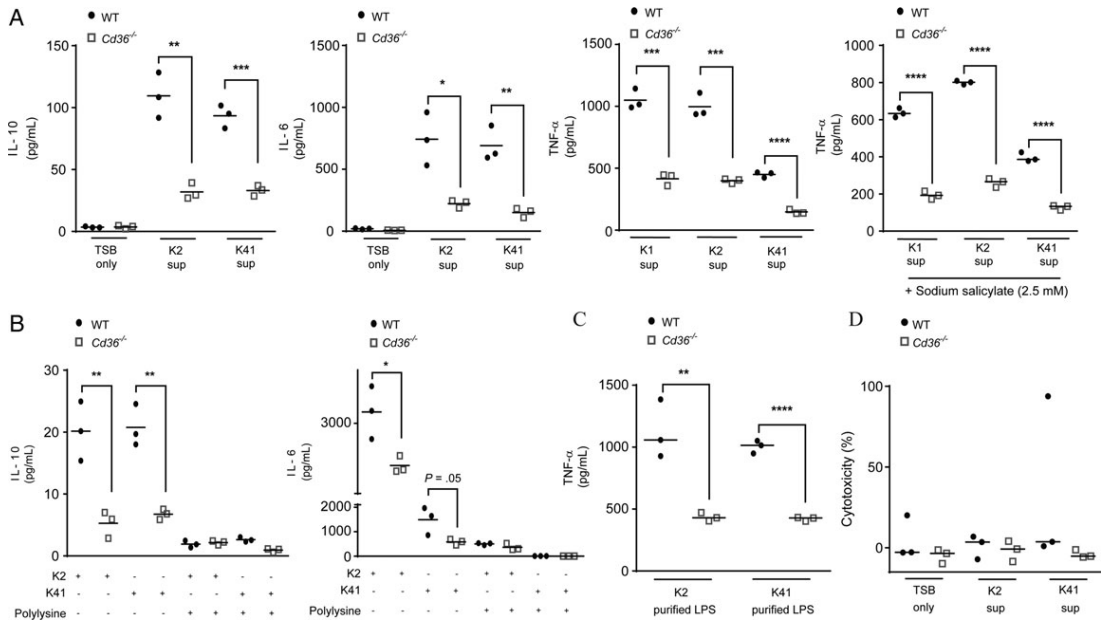


Figure 5. *Cd36*$^{-/-}$ macrophages show blunted cytokine production in response to *Klebsiella pneumoniae* lipopolysaccharide (LPS) that is independent of capsular serotype. *A*, Peritoneal macrophages pooled from wild-type (WT) mice (n = 3) and *Cd36*$^{-/-}$ mice (n = 4) were incubated with cell-free supernatants from overnight cultures of serotype K2 *K. pneumoniae* (K2 sup), K41 serotype carbapenemase-producing *K. pneumoniae* (K41 sup), or K1 serotype *K. pneumoniae* (K1 sup) for 6 hours. Macrophages were also incubated with tryptic soy broth (TSB) in the absence of bacterial growth for 6 hours. Interleukin 10 (IL-10), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) release were measured in the supernatant of cell culture medium by enzyme-linked immunosorbent assay (ELISA). Data are indicative of 2 independent experiments. *B*, Macrophages pooled from WT mice (n = 3) and *Cd36*$^{-/-}$ mice (n = 4) were incubated with diluted bacterial lysate or diluted bacterial lysate treated through a polylysine column for 6 hours. A total of 10^9 cells of K2 and K41 strains were lysed, diluted 1000-fold, and spun through a column containing cellulose beads coated with polylysine. IL-10 and IL-6 release were measured in the supernatant of cell culture medium by ELISA. Data are indicative of 2 independent experiments. *C*, Macrophages pooled from WT mice (n = 3) and *Cd36*$^{-/-}$ mice (n = 3) were incubated with purified LPS from 10^8 cells of K2 and K41 strains for 6 hours. TNF-α production was measured by ELISA. *D*, Cytotoxicity was determined by measuring lactate dehydrogenase release in the supernatant of cell culture medium. Macrophages associated with panels *A*–*D* were incubated in culture medium with 10% fetal bovine serum and 1% penicillin-streptomycin. Dots and squares represent technical replicates from pooled macrophages in each mouse group. Line indicates mean values. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, by the 2-tailed Student *t* test.

K. pneumoniae stimulation (Supplementary Figure 2). It remains unclear, however, whether CD36-dependent JNK activation occurs in primary alveolar macrophages ex vivo and in the lung in vivo following stimulation with a gram-negative pathogen. We isolated and pooled alveolar macrophages from BAL fluid of WT and *Cd36*$^{-/-}$ mice and exposed them to *K. pneumoniae* (multiplicity of infection, 10) in vitro. Under basal conditions, both WT and *Cd36*$^{-/-}$ alveolar macrophages showed minimal JNK phosphorylation (Figure 6*B*). Following exposure to *K. pneumoniae* ex vivo, *Cd36*$^{-/-}$ macrophages showed impaired JNK activation as compared to WT macrophages (Figure 6*B*). In contrast, there were no significant differences observed in phosphorylated or total p38 between WT and *Cd36*$^{-/-}$ alveolar macrophages at various multiplicities of infection (Figure 6*C*). We next examined JNK activation in the lung tissues of WT and *Cd36*$^{-/-}$ mice following infection with *K. pneumoniae* in vivo. *Cd36*$^{-/-}$ lungs showed reduced levels of p-JNK when compared to WT lungs (Figure 6*D*). There were no differences in levels of total JNK levels between the 2 groups (Figure 6*D*), suggesting that although JNK is detectable, CD36 is required for optimal activation of JNK in response to *K. pneumoniae*.

DISCUSSION

We investigated the role of the scavenger receptor CD36 in the innate immune response to *Klebsiella pneumoniae* using 3 different strains (K2, K1, and K41) in an acute bacterial pneumonia model. Mice defective in CD36 showed impaired ability to clear the K2 strain from the lungs and displayed increased dissemination into extrapulmonary sites such as the blood, liver, and spleen. Inability to adequately contain the infection within the lung was associated with increased mortality. A similar finding was observed following intrapulmonary infection with the K1 strain, where *Cd36*$^{-/-}$ mice showed higher bacterial burden in the lungs, increased liver dissemination and systemic toxicity compared to WT mice. Importantly, the K41 strain showed

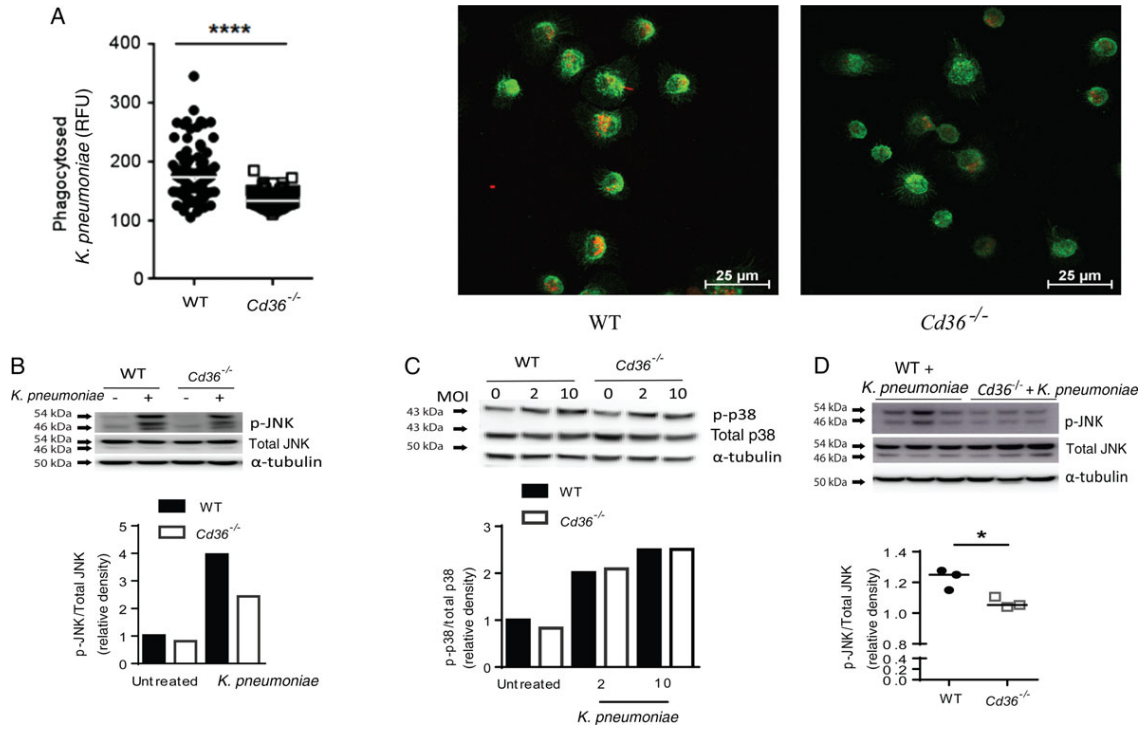


Figure 6. *Cd36*$^{-/-}$ macrophages show impaired uptake of *Klebsiella pneumoniae*, and *Cd36*$^{-/-}$ mice show impaired JNK activation following *K. pneumoniae* infection. *A*, Alveolar macrophages pooled from wild-type (WT) mice (n = 6) and *Cd36*$^{-/-}$ mice (n = 6) were stimulated with tdTomato plasmid–expressing *K. pneumoniae* clinical strain 883 (multiplicity of infection [MOI], 50) for 1 hour. Cells were washed and subsequently fixed. *Left*, The middle slice of each *z*-stack confocal image was analyzed to determined the mean *K. pneumoniae* intensity per cell. Dots and squares represent individual cells (WT, 87 cells; *Cd36*$^{-/-}$, 85 cells). Lines indicate median values. ****$P < .0001$, by the 2-tailed Mann–Whitney *U* test. *Right*, Representative slices of *z*-stack confocal images are shown from WT and *Cd36*$^{-/-}$ macrophages following stimulation with tdTomato plasmid–expressing *K. pneumoniae*. Green, F4/80-positive macrophages; red, tdTomato plasmid–expressing *K. pneumoniae*. *B*, p-JNK, total JNK, and α-tubulin expression in alveolar macrophages pooled from naive WT (n = 16) or *Cd36*$^{-/-}$ mouse lungs (n = 12) stimulated ex vivo in the presence or absence of *K. pneumoniae* (MOI, 10) for 1 hour. Data in the bar graph indicate the relative density of p-JNK (normalized to total JNK) in pooled alveolar macrophages exposed to *K. pneumoniae* (MOI, 10) for 1 hour. *C*, p-p38, total p38, and α-tubulin expression in alveolar macrophages pooled from naive WT (n = 16) or *Cd36*$^{-/-}$ (n = 12) mouse lungs stimulated ex vivo with *K. pneumoniae* (MOI, 0, 2, and 10) for 1 hour. The graph indicates the relative density of p-p38 (normalized to total p38) in pooled alveolar macrophages exposed to *K. pneumoniae* (MOI, 10) for 1 hour. *D*, p-JNK, total JNK, and α-tubulin expression in WT and *Cd36*$^{-/-}$ lungs following intratracheal instillation of *K. pneumoniae*. Lung tissue homogenates were obtained from perfused WT and *Cd36*$^{-/-}$ lungs harvested 0.5 hours following intratracheal instillation of *K. pneumoniae* (1×10^8 CFU). Each lane indicates lung tissue homogenate from an individual mouse. Dots and squares represent lungs of individual mice. Lines indicate median values. *$P < .05$, by the 2-tailed Student *t* test. Abbreviation: RFU, relative fluorescence units.

enhanced pathogenicity in mice defective in CD36 that is associated with impaired lung cytokine production. Our findings further indicate that CD36 functions to enhance LPS responsiveness and increase phagocytosis and macrophage cytokine production, which play crucial roles in eliminating the extracellular bacteria *K. pneumoniae* from the lung. These findings are independent of the capsular serotype and strongly support the contention that CD36 is a host determinant of *K. pneumoniae* pathogenicity.

CD36 is involved in many critical aspects of innate immunity, including the recognition and clearance of pathogen-derived phospholipids, modified host-derived lipoproteins, and apoptotic cells to maintain cellular homeostasis [10]. Depending on the ligand stimulus, the cell type, and the binding partners that CD36 engages, CD36 has the ability to influence the course of inflammation [10, 11]. For example, CD36 mediates endocytosis of oxLDL and amyloid-β into lysosomes [43] and regulates TLR4/TLR6 complex formation to potentiate nuclear factor κB activity in response to oxLDL and amyloid-β in HEK293 cells [19]. However, CD36 can also facilitate recognition and clearance of apoptotic cells, with resolution of inflammation through production of the antiinflammatory cytokine IL-10 [44, 45]. Following stimulation with oxLDL, the carboxyl terminal domain of CD36 containing the CXCX5K motif interacts with a signaling complex containing Lyn kinase and MEKK2 with downstream JNK 1/2 activation in macrophages [40].

Moreover, CD36 has been shown to enhance recognition of LPS and gram-negative bacteria in HeLa and HEK293 cells and to mediate cytokine signaling through JNK 1/2 activation that is independent of TLR4 [20, 46]. The evidence thus far indicates that JNK1/2 phosphorylation is an important signaling pathway that triggers CD36-dependent proinflammatory cytokine responses in macrophages [40]. In this study, we show that CD36 contributes to JNK but not p38 activation in alveolar macrophages ex vivo and in lung in vivo following *K. pneumoniae* challenge.

A pattern-recognition receptor for components of microbial pathogens such as *Plasmodium falciparum* erythrocyte membrane protein 1 and certain microbial-derived diacylglycerides [11, 15, 18], CD36 promotes diverse responses depending on the type of pathogen the host encounters. Although CD36 activates the TLR2 signaling pathway through recognition of *S. aureus* and lipoteichoic acid to enhance inflammatory cytokine responses [15], others have shown that CD36 suppresses inflammation induced by *S. pneumoniae* by binding to phosphocholine residues of lipoteichoic acid [16]. These studies suggest a pathogen-specific interaction with host CD36 during infection that dictates the subsequent inflammatory response and, in some cases, host outcome following infection. These findings also highlight the need for in vivo studies using relevant models to properly ascertain the biology of CD36. Herein, our findings indicate that, in response to a live gram-negative pathogen, *K. pneumoniae*, CD36 contributes to an effective host defense strategy following intrapulmonary infection. Our data also indicate that CD36 enhances LPS signaling by macrophages, phagocytosis, and subsequent cytokine production. Approximately 3%–11% of Asians, 2.4% of African Americans, and 7%–8% of sub-Saharan Africans lack platelet CD36 (type II deficiency) [12, 47–49], a deficiency originally identified as the Nak^{a-} phenotype that is associated with refractoriness to HLA-matched platelet transfusions [50]. Of those individuals with the Nak^{a-} phenotype, 10% presumably lack CD36 in all cells (type I deficiency) [12, 47]. It remains to be seen whether type I or type II deficiency in CD36 contributes to host-pathogen interactions that explain geographic differences in *K. pneumoniae*–associated severe community-acquired pneumonia and invasive syndromes of endophthalmitis, meningitis, or liver abscesses occurring almost exclusively in portions of Asia and Africa [5].

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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11.7. Research Plan 5 Pages

Research Plan 5 Pages - AHA Research Proposal_FINAL .pdf

1. Specific Aims: Stroke-associated pneumonia (SAP) remains one of the leading causes of death following acute stroke[1,2] and *Pseudomonas aeruginosa* (PA) is a major causative agent of SAP[3]. PA secretes toxic exoproducts such as Pseudomonas elastase B (LasB) that enhance its virulence[4] and disrupt pulmonary epithelia[5,6], yet host factors that disarm PA-encoded virulence factors and protect against injury are not fully understood. Our preliminary findings indicate that platelets secrete soluble factor(s) that significantly inhibit LasB in a dose-dependent manner. These findings proved relevant in vivo, as we observed that natively thrombocytopenic mice exhibit gross alveolar hemorrhage upon instillation of PA cell-free exoproduct, and that disruption of LasB activity in PA exoproduct mitigates PA-induced injury in platelet-deficient mice. Although platelets are involved in the acute thrombotic event leading to stroke[7], they have recently been implicated as agents of host defense and ascribed multiple roles in innate immunity[8,9]. Identifying discrete factors or components of platelets that are protective may provide novel therapeutic strategies that can be selectively utilized during nosocomial bacterial infection following stroke. Our findings implicate thrombospondin-1 (TSP-1), a major constituent of platelet alpha granules found in the airspaces following severe lung inflammatory injury[10], as the platelet factor that mediates potent inhibition of LasB and protects against PA-induced lung injury. We have previously shown that TSP-1 promotes resolution of experimental lung injury[11] and that TSP-1 regulates the innate immune response during bacterial infection[12]. **Thus, we hypothesize that beyond repairing vascular injury induced by PA, platelets provide protection through the release of TSP-1 to disarm pathogen-encoded protease and curtail lung inflammation.** This hypothesis will be tested by the following specific aims:

Aim 1: To assess the contribution of platelet TSP-1 to host protection during PA-induced lung injury. We show that TSP-1 potently inhibits LasB activity and that mice deficient in TSP-1 (*Thbs1*$^{-/-}$) show worsened neutrophilic inflammation and impaired host defense to virulent PA infection. Accordingly, we will administer TSP-1 purified from platelets to *Thbs1*$^{-/-}$ mice, natively thrombocytopenic *Mpl*$^{-/-}$, and WT littermates to determine whether TSP-1 is sufficient to rescue mice from PA-induced lung injury. To definitively assess contribution of platelet-derived TSP-1 to host protection, we have generated *Thbs1*$^{fl/fl}$ mice using CRISPR/Cas9 and I will cross these mice with PF4-cre mice to generate novel platelet-specific TSP-1 conditional knockout mice.

Aim 2: To elucidate the mechanism by which LasB propagates PA-induced inflammation and determine whether TSP-1 curtails LasB-mediated PA inflammation. Our preliminary findings suggest that LasB propagates PA-induced neutrophilic inflammation and that LasB cleaves IL-36γ, a proinflammatory cytokine in the IL-1 family known to amplify inflammation upon proteolytic processing[13]. It remains unclear, however, whether N-terminal processing of IL-36γ by LasB amplifies inflammation. As human bronchial epithelial (HBE) cells have been shown to express the IL-36 receptor[14], we will utilize these cells to determine whether LasB-generated cleaved IL-36γ amplifies inflammatory cytokine production. We will then examine the ability of purified platelet TSP-1 to restrict LasB-cleavage of IL-36γ and dampen HBE cytokine response.

2. Background and significance: Bacterial pneumonia is a major complication following acute stroke that is associated with increased mortality[1,2] and hospital costs of patients with stroke-associated pneumonia (SAP) are estimated at $459 million annually[15]. Microbiological data indicate that PA is a recurrent cause of SAP[3]. However, unlike other causative agents of SAP, mortality in nosocomial pneumonia due to PA is distinctively high and approaches 40%[16]. Though an opportunistic pathogen that typically exploits critically ill hosts, PA employs an arsenal of virulence factors to exert injury on the susceptible host[4]. Increasing Pseudomonal

resistance to antibiotics and limited therapeutic options have now made the quest for alternative strategies to combat PA infection an urgent concern[17,18].

TSP-1 is a multifunctional, matricellular glycoprotein implicated in diverse biological functions including wound repair, tumor growth and metastasis, and inflammation[19–21]. It is stored primarily in platelet alpha-granules where it is released upon platelet activation increasing TSP-1 plasma concentrations by 100-fold[22,23]. It is also found in the airspaces during lung injury suggesting that it may influence the local inflammatory response[10]. We have shown that TSP-1 tempers neutrophil proteolytic function and regulates the innate immune response during *Klebsiella pneumoniae* infection[12]. We now present evidence that TSP-1 disarms the pathogen-encoded protease LasB and propose that TSP-1's inhibition of LasB is a proximal host protective mechanism during PA-induced lung injury.

Though traditionally viewed as cellular orchestrators of primary hemostasis, platelets are increasingly recognized as key players in innate defense against infection[8,24]. They are rich in granules, contents of which can be rapidly mobilized for extracellular degranulation to facilitate an integrated array of host defense functions[8]. TSP-1 is one of the most highly expressed proteins in platelet alpha-granules (approximately 101,000 copies per cell)[25,26] and has been shown to modulate platelet activity[26,27]. The proposed project aims to establish the mechanisms by which platelets, in part through the release of TSP-1, provide protection against PA-mediated injury beyond hemostasis. Our work highlights TSP-1, a principal constituent of platelets, as an endogenous host factor that may lessen severity of PA-induced lung injury in critically ill infected stroke patients and opens the possibility of novel therapeutics design in the future.

3. Preliminary studies: Our preliminary studies show (1) Cell-free exoproduct from PA is sufficient to induce gross alveolar hemorrhage in thrombocytopenic mice, and injury is attenuated in the absence of LasB, (2) LasB is the predominant protease secreted by PA, and is inhibited by platelet releasate and specifically by purified TSP-1, (3) Mice deficient in TSP-1 (*Thbs1*$^{-/-}$) show impaired host defense to acute intrapulmonary PA infection, (4) LasB preferentially cleaves full-length IL-36γ, (5) Disruption of LasB mitigates PA-induced inflammation, (6) *Thbs1*$^{-/-}$ mice show worsened inflammation following PA infection. These studies are included as part of Section 4. I conducted experiments shown in Figures 1, 2, 5 & 6.

4. Research Design and Methods.

4.1. Approach to Aim 1: To assess the contribution of platelet TSP-1 to host protection during PA-induced injury. Rationale: Amison et al. recently demonstrated that activated platelets accumulate in the lungs during PA infection and depletion of platelets impairs murine host defense to PA infection[24]. Our preliminary data affirm their observations and further indicate that natively thrombocytopenic *Mpl*$^{-/-}$ mice — which lack the thrombopoietin receptor Mpl, show <10% circulating platelet counts but demonstrate no other defects in peripheral blood cell counts, and do not spontaneously bleed[28] — exhibit enhanced susceptibility to injury induced by PA even in the absence of bacterial cells (Figure 1A), suggesting that injury is induced by a secreted bacterial product.

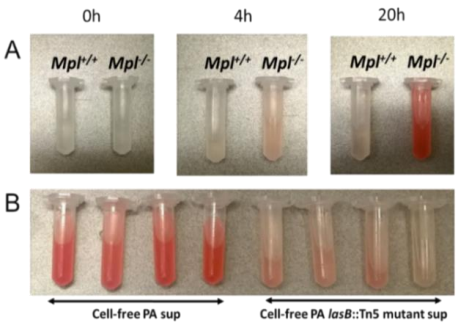
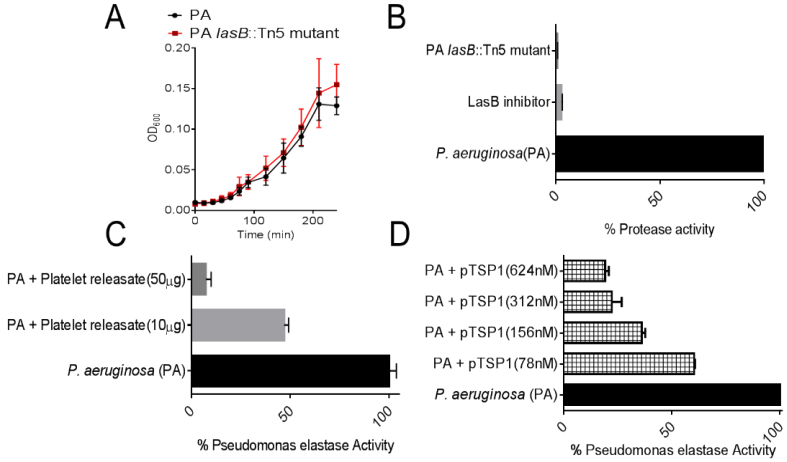


Figure 1: Thrombocytopenic mice are prone to acute PA-driven lung injury, even in the absence of bacterial cells. PA was grown overnight, bacterial cells were pelleted, and residual bacterial cells in supernatant were filtered with a 0.22μm filter to obtain cell-free supernatant (sup). (A) Representative

bronchoalveolar lavage (BAL) fluid obtained from *Mpl*$^{+/+}$ and *Mpl*$^{-/-}$ littermates (n=4 mice per group at 0, 4, and 20h following cell-free PA sup intratracheal instillation. (B) BAL fluid from *Mpl*$^{-/-}$ mice 20h following cell-free PA sup or PA *lasB*::Tn5 (a non-redundant transposon insertion mutant with deficient LasB activity) sup instillation.

Selective depletion of Pseudomonas Elastase B (LasB) activity, utilizing both transposon mutagenesis and pharmacologic inhibition, suggest that LasB is the predominant protease secreted by PA (Figure 2B) and disruption of LasB activity attenuates PA-induced injury in thrombocytopenic mice (Figure 1B). Furthermore, soluble factor(s) obtained from activated platelets effectively inhibit Pseudomonas elastase activity and nanomolar quantities of purified TSP-1 from platelets recapitulate Pseudomonas elastase inhibition observed with platelet releasate (Figure 2C, D).

Figure 2: TSP-1 inhibits Pseudomonas elastase activity. (A) PA and PA *lasB*::Tn5 mutant show similar growth patterns. Growth curve was obtained by measuring optical density at 600nm for each bacterial strain cultured in Luria broth at 37^{o}C, 250RPM over 4h. (B) Cell-free supernatant was tested for protease activity using fluorogenic casein substrate in the presence or absence of specific LasB inhibitor (N-mercaptoacetyl-Phe-Tyr-amide)[29]. PA *lasB*::Tn5 mutant strain is deficient in protease activity. (C-D) Dose-dependent inhibition of Pseudomonas elastase activity by thrombin-induced platelet releasate and purified TSP-1 from platelet releasate using fluorogenic elastin substrate.



<u>Design:</u> To assess the relevance of TSP-1 in protection against PA-induced injury, we will purify TSP-1 from platelets and administer purified TSP-1 to *Thbs1*$^{-/-}$ and *Mpl*$^{-/-}$ mice following acute intratracheal PA instillation. WT littermates will be used as controls. BAL and lung tissue will be harvested 20h post-infection and BAL hemoglobin content, protein concentration, and lung histology will be assessed for evidence of alveolar injury. CFU in BAL, lung, and splenic tissue will also be enumerated at 20h. We show that platelet releasates are enriched for TSP-1 and that *Thbs1*$^{-/-}$ mice show impaired host defense to PA infection (Figure 3). I have been working with Dr. Qu (PhD Research Associate in the lab) who is proficient in TSP-1 purification using a Heparin-Sepharose column according to the methods of Lawler et al[30].

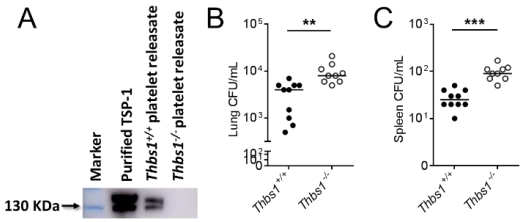


Figure 3: Mice deficient in TSP-1 (*Thbs1*$^{-/-}$) show impaired host defense to PA infection. (A) Western blot of TSP-1 expression in in-house purified TSP-1, *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ platelet releasate. (B-C) Colony-forming units (CFU) in lung and splenic tissue homogenate obtained 20h from *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ mice inoculated with 10^6 CFU PA. Data points indicate individual mice. Line indicates median. **p<0.01, ***p<0.001 by Mann-Whitney U test.

To definitively assess contribution of platelet-derived TSP-1 to protection during PA-induced injury, we have begun generating platelet-specific TSP-1 conditional knockout mice under the guidance of CRISPR-Cas9 expert, Dr. Sebastian Gingras from the Innovative Technologies

Development Core at the University of Pittsburgh[31]. This approach is feasible and within the time constraints of the fellowship, as we have already successfully generated four mice carrying inserted LoxP sites at designated introns 3 and 7 (Figure 4). These *Thbs1*$^{lox/lox}$ mice are already being bred to confirm germline transmission and progeny will be crossed with commercially available platelet-factor 4 (PF4) cre-recombinase mice to generate platelet-specific TSP-1 conditional knockout mice.

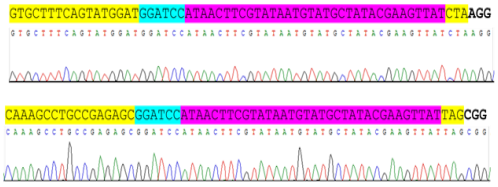


Figure 4: Sanger sequence chromatogram confirming precise insertion of LoxP sites in introns 3 (above) and 7 (below). Sequence for LoxP site (34bp, pink) and a SalI restriction enzyme (6bp, cyan) introduced at the position of the double strand break (DSB) induced by Cas9, which is 3 bp upstream of the protospacer adjacent motif (PAM) sequence (NGG, bold), disrupting the sgRNA target sequence (yellow).

Interpretation of results, potential pitfalls, and alternative approaches: We anticipate that *Thbs1*$^{-/-}$ and *Mpl*$^{-/-}$ mice administered TSP-1 will show resistance to PA-induced injury, as evidenced by diminished BAL hemorrhage, protein leakage into the alveolar space, lung and splenic bacterial burden. I have been training with Ms. Hulver (laboratory technician) and Dr. Xiong (Research Assistant Professor) in animal handling over the last year and anticipate completion in the first 6 months. While we predict that platelet-specific TSP-1 conditional knockout mice will show enhanced susceptibility to PA-induced injury, it is possible that other sources of TSP-1 in the lung — particularly the endothelium[32]— mediate host protection in response to PA. If so, we will utilize endothelial-specific TSP-1 conditional knockout mice. The Cdh5(PAC)-CreERT2 line was originally created by Ralf Adams (Max Planck Institute, Germany), and has been shown to reliably induce endothelial-specific deletion of floxed allele when given tamoxifen[33]. Cdh5(PAC)-CreERT2 mice are in-house and ready to be bred with *Thbs1*$^{lox/lox}$ mice. I am particularly interested in learning the fundamentals of transgenic mice creation and anticipate experimental completion within 2 years.

4.2. Approach to Aim 2: To elucidate the mechanism by which LasB propagates PA-induced inflammation and determine whether TSP-1 curtails LasB-mediated PA inflammation. Rationale: Airway inflammation is a hallmark of PA infection, yet the mechanism by which PA amplifies the host immune response to infection is not well-defined. The early host transcriptional response to PA comprises expression of IL-1 family of cytokines, most notably IL-1F9 (now formally, IL-36γ), in HBE cells[34]. Like other IL-36 family members, both murine and human IL-36γ possess biological activity in their full-length state[13,35]. Their potencies, however, are increased ~500 to 1000-fold upon N-terminal truncation[13,36]. While we observed some degradation of IL-36α and IL-36β following PA exoproduct incubation (Figure 5B), our findings indicate that complete N-terminal truncation (determined by Edman degradation, data not shown) occurs primarily upon co-incubation of PA exoproduct with IL-36γ and cleavage is attenuated in the absence of LasB activity (Figure 5A).

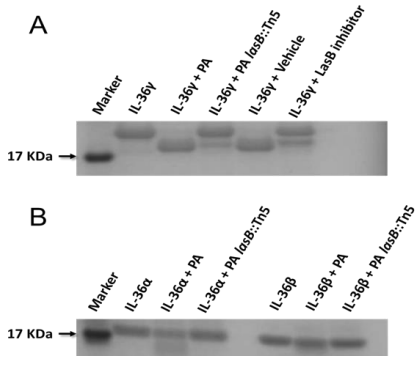


Figure 5: LasB preferentially cleaves IL-36γ. Cell-free supernatant obtained from PA or PA *lasB*::Tn5 mutant was incubated with 3µg of recombinant human (A) IL-36γ, (B) IL-36α, IL-36β for 2h at 37°C. 100µM of LasB inhibitor was utilized where indicated. Samples were analyzed by Coomassie-stained gels following SDS-PAGE.

Furthermore, we show that PA-induced pulmonary neutrophilic inflammation is significantly attenuated in the absence of LasB activity (Figure 6), inviting the possibility that LasB amplifies inflammation through N-terminal cleavage of IL-36γ.

Design: HBE cells express the IL-36 receptor and secrete IL-6 and neutrophil chemoattractant IL-8 in response to IL-36γ treatment[14]. To determine whether truncated IL-36γ derived from LasB cleavage amplifies epithelial cell cytokine production, we will utilize primary HBE cells obtained from Dr. J. Pilewski, Director of the Human Airway Cell and Tissue Core at the University of Pittsburgh (see letter of support). We obtain these cells on a weekly basis and have established our ability to maintain culture on human placental collagen-coated Costar transwell permeable supports. IL-36γ-induced cytokines IL-6, G-CSF, IL-8 will be measured in cell culture medium by ELISA following purified LasB + IL-36γ incubation. Recombinant full-length and truncated human IL-36γ will serve as controls. Dosage- and time-dependent assays will be undertaken to optimize experimental procedure.

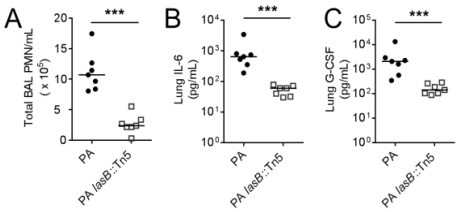


Figure 6: PA-induced neutrophilic inflammation is attenuated in the absence of LasB activity. (A) Total BAL neutrophil (PMN) count, (B) Lung IL-6, and (C) Lung G-CSF obtained 20h from WT mice inoculated with 1 x 10^6 CFU PA or 2.67 x 10^6 CFU PA *lasB*::Tn5 mutant strain. Data points indicate individual mice. Line indicates median. ***p<0.001 by Mann-Whitney U test.

As *Thbs1*$^{-/-}$ mice show amplified airspace neutrophil recruitment and increased IL-6, G-CSF production in response to PA infection (Figure 7), we will test whether introduction of purified TSP-1 from human platelets tempers LasB-mediated IL-36γ amplified cytokine response.

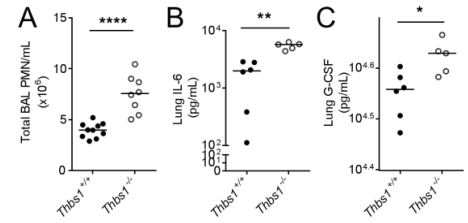


Figure 7: *Thbs1*$^{-/-}$ mice show worsened neutrophilic inflammation following PA infection. (A) Total BAL PMN count, (B) Lung IL-6, and (C) Lung G-CSF obtained 20h from *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ mice inoculated with 10^6 CFU PA. Data points indicate individual mice. Line indicates median. *p<0.05, **p<0.01, ****p<0.0001 by Mann-Whitney U test.

Interpretation of results, potential pitfalls, and alternative approaches: While we hypothesize that LasB-cleaved IL-36γ will amplify cytokine production in epithelial cells, it is possible that LasB alone is sufficient to induce epithelial cell cytokine production and that LasB amplifies inflammatory response independent of IL-36γ. To confirm the role of IL-36γ in our experimental model, we will negate cellular effects of IL-36γ utilizing IL-36γ neutralizing antibody[37] (Dr. Standiford, see letter of support) in our murine PA-infection model and IL-36γ-induced cytokines will be assessed.

4.3 Statistical analysis: For both specific aims, comparisons of more than two groups will be made using a one-way analysis of variance (ANOVA) with subsequent comparisons made with Tukey test. If assumption of normality fails, Mann-Whitney U two-tailed test will be used to compare two groups and Kruskal-Wallis analysis of ranks to compare more than two groups. For all tests, differences will be considered significant when p<0.05.

5. Ethical aspects of proposed research: There are approved institutional review board (IRB) protocols (PRO11070367, IRB970946) that allow for use of human biologic material proposed in Aim 2. Rationale for use of vertebrate animals in achieving proposed aims is described in detail in the Vertebrate Animals section.

12. Supporting Documentation

12.1. Consultant 1 (Documents)

Name :	JosephPilewski
Position Title :	Associate Professor of Medicine, Cell Biology & Pediatrics
Institution :	University of Pittsburgh Medical Center
Document Name 1 :	Olonisakin Consultant letter Pilewski for AHA 2017 (1).pdf
Status 1 :	Uploaded

Consultant - Olonisakin Consultant letter Pilewski for AHA 2017 (1).pdf

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October 24, 2017

AHA Predoctoral Fellowship Committee

Dear Committee Members,

This letter is to formalize my enthusuiasm to serve as a consultant for Tolani Olonisakin's application for the AHA Predoctoral Fellowship Program. I am an Associate Professor in the Division of Pulmonary, Allergy, and Critical Care Medicine and have been a long-time colleague and collaborator of Tolani's mentor, Dr. Janet Lee. I am the co-Director of the Cystic Fibrosis Program at the University of Pittsburgh Medical Center and Children's Hospital of Pittsburgh of UPMC, and have directed an Airway Cell Core for ~20 years for our Cystic Fibrosis Research Center. I have extensive experience in primary airway epithelial cell and organotypic culture techniques. My laboratory has processed thousands of normal and diseased human lungs, and isolates primary airway cells for investigators in our Center and collaborating institutions, under an IRB approved protocol that permits de-identified cells to be provided without cost to the investigator. The Cell Core has been continuously funded by the NIH and Cystic Fibrosis Foundation since the mid-1990s. We have assisted numerous investigators over the years by providing polarized human bronchial airway epithelial cells at air-liquid interface. As consultant, I will provide the infrastructure for creating and providing primary human bronchial epithelial cultures relevant to this important application focused on Pseudomonas pneumonia and lung injury, and provide intellectual assistance with airway epithelial biology.

Sincerely,

Joseph M. Pilewski, MD
Associate Professor of Medicine, Cell Biology & Pediatrics
University of Pittsburgh
Associate Chief for Clinical Affairs, Division of Pulmonary, Allergy & Critical Care Medicine
University of Pittsburgh Medical Center
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12.2. Consultant 2 (Documents)

Name :	TheodoreStandiford
Position Title :	Henry Sewall Professor of Medicine and Chief
Institution :	University of Michigan Medical Center
Document Name 1 :	Letter AHA 2017.pdf
Status 1 :	Uploaded

Consultant - Letter AHA 2017.pdf

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October 26, 2017

AHA Predoctoral Fellowship Review Committee

Dear Committee Members,

I am delighted to provide my expertise in Tolani Olonisakin's project entitled, "Role of thrombospondin-1 in platelet-mediated protection during *Pseudomonas aeruginosa*-induced injury". Tolani was introduced to me by Dr. Janet Lee at this year's Joint Meeting of the American Society for Clinical Investigation and Association of American Physicians. Tolani's work entails probing the mechanism by which *P. aeruginosa* amplifies host inflammation through the pathogen-encoded protease, Pseudomonas elastase B (LasB), and IL-36γ. I have already begun collaborating with Dr. Lee and Tolani on this project and have provided them with recently generated rabbit anti-mouse polyclonal IL-36γ neutralizing antibody.

My research focuses on the mechanisms underlying generation of protective innate immune responses in the lung. Specifically, I am interested in delineating the role of novel IL-1 family members such as IL-36 in lung innate immunity. I have published several articles on the contribution of IL-36 to lung immunity, most recently in *Mucosal Immunology*, where we demonstrate that IL-36γ drives type-1 responses and promotes classical macrophage activation during bacterial pneumonia. As Tolani's project attempts to uncover a novel mechanism by which a pathogen co-opts host IL-36γ to propagate inflammation, I am delighted to advise her and contribute my scientific expertise to this exciting work.

Best regards,

Theodore J. Standiford, M.D.
Henry Sewall Professor of Medicine and Chief
Division of Pulmonary and Critical Care Medicine
University of Michigan Medical Center
Ann Arbor, MI 48109

12.3. Consultants 1 (Documents)

Name :	JosephPilewski
Position Title :	Associate Professor of Medicine, Cell Biology & Pediatrics
Institution :	University of Pittsburgh Medical Center
Document Name 1 :	Olonisakin Consultant letter Pilewski for AHA 2017 (1).pdf
Status 1 :	Uploaded

Consultants - Olonisakin Consultant letter Pilewski for AHA 2017 (1).pdf

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October 24, 2017

AHA Predoctoral Fellowship Committee

Dear Committee Members,

This letter is to formalize my enthusuiasm to serve as a consultant for Tolani Olonisakin's application for the AHA Predoctoral Fellowship Program. I am an Associate Professor in the Division of Pulmonary, Allergy, and Critical Care Medicine and have been a long-time colleague and collaborator of Tolani's mentor, Dr. Janet Lee. I am the co-Director of the Cystic Fibrosis Program at the University of Pittsburgh Medical Center and Children's Hospital of Pittsburgh of UPMC, and have directed an Airway Cell Core for ~20 years for our Cystic Fibrosis Research Center. I have extensive experience in primary airway epithelial cell and organotypic culture techniques. My laboratory has processed thousands of normal and diseased human lungs, and isolates primary airway cells for investigators in our Center and collaborating institutions, under an IRB approved protocol that permits de-identified cells to be provided without cost to the investigator. The Cell Core has been continuously funded by the NIH and Cystic Fibrosis Foundation since the mid-1990s. We have assisted numerous investigators over the years by providing polarized human bronchial airway epithelial cells at air-liquid interface. As consultant, I will provide the infrastructure for creating and providing primary human bronchial epithelial cultures relevant to this important application focused on Pseudomonas pneumonia and lung injury, and provide intellectual assistance with airway epithelial biology.

Sincerely,

Joseph M. Pilewski, MD
Associate Professor of Medicine, Cell Biology & Pediatrics
University of Pittsburgh
Associate Chief for Clinical Affairs, Division of Pulmonary, Allergy & Critical Care Medicine
University of Pittsburgh Medical Center
412-647-8477
pilewskijm@upmc.edu

12.4. Consultants 2 (Documents)

Name :	TheodoreStandiford
Position Title :	Henry Sewall Professor of Medicine and Chief
Institution :	University of Michigan Medical Center
Document Name 1 :	Letter AHA 2017.pdf
Status 1 :	Uploaded

Consultants - Letter AHA 2017.pdf

MICHIGAN MEDICINE
UNIVERSITY OF MICHIGAN

Division of Pulmonary and Critical Care Medicine
Department of Internal Medicine
6301 MSRB III
1150 W. Medical Center Drive, SPC 5642
Ann Arbor, Michigan 48109-5642
734-936-5010 Office
734-764-4556 Fax

October 26, 2017

AHA Predoctoral Fellowship Review Committee

Dear Committee Members,

I am delighted to provide my expertise in Tolani Olonisakin's project entitled, "Role of thrombospondin-1 in platelet-mediated protection during *Pseudomonas aeruginosa*-induced injury". Tolani was introduced to me by Dr. Janet Lee at this year's Joint Meeting of the American Society for Clinical Investigation and Association of American Physicians. Tolani's work entails probing the mechanism by which *P. aeruginosa* amplifies host inflammation through the pathogen-encoded protease, Pseudomonas elastase B (LasB), and IL-36γ. I have already begun collaborating with Dr. Lee and Tolani on this project and have provided them with recently generated rabbit anti-mouse polyclonal IL-36γ neutralizing antibody.

My research focuses on the mechanisms underlying generation of protective innate immune responses in the lung. Specifically, I am interested in delineating the role of novel IL-1 family members such as IL-36 in lung innate immunity. I have published several articles on the contribution of IL-36 to lung immunity, most recently in *Mucosal Immunology*, where we demonstrate that IL-36γ drives type-1 responses and promotes classical macrophage activation during bacterial pneumonia. As Tolani's project attempts to uncover a novel mechanism by which a pathogen co-opts host IL-36γ to propagate inflammation, I am delighted to advise her and contribute my scientific expertise to this exciting work.

Best regards,

Theodore J. Standiford, M.D.
Henry Sewall Professor of Medicine and Chief
Division of Pulmonary and Critical Care Medicine
University of Michigan Medical Center
Ann Arbor, MI 48109

12.5. Referent 1 (Documents)

Name :	RichardSteinman
Position Title :	Associate Professor of Medicine and Pharmacology
Institution :	University of Pittsburgh
Document Name 1 :	TOLANI AHA_pdf.pdf
Status 1 :	Uploaded

12.6. Referent 2 (Documents)

Name :	SallyWenzel
Position Title :	Professor of Medicine
Institution :	University of Pittsburgh
Document Name 1 :	
Status 1 :	Not Uploaded

12.7. Referent 3 (Documents)

Name :	NuriaPastor-Soler
Position Title :	Associate Professor of Medicine
Institution :	University of Southern California
Document Name 1 :	
Status 1 :	Not Uploaded

12.8. Sponsor 1 (Documents)

Name :	JanetLee
Position Title :	Professor of Medicine
Institution :	University of Pittsburgh
Document Name 1 :	LeeJS_Biosketch_102317.pdf
Status 1 :	Uploaded

Sponsor - LeeJS_Biosketch_102317.pdf

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: LEE, Janet S.

eRA COMMONS USER NAME (credential, e.g., agency login): LEEXJS3

POSITION TITLE: Professor of Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
The Johns Hopkins University, Baltimore, MD	BA	1987-1991	Natural Sciences
Georgetown University, Washington, DC	MD	1991-1995	Medicine
University of Alabama, Birmingham, AL	Residency	1995-1998	Internal Medicine
University of Washington, Seattle, WA	Fellowship	1998-2001	Pulmonary/ Critical Care Medicine
VA Pulmonary Research Laboratories, University of Washington, Seattle, WA	Postdoctoral Fellowship	1999-2004	Pulmonary/ Critical Care Medicine

A. Personal Statement

I am a physician-scientist with a strong track record of examining host determinants of lung injury, pulmonary host defense mechanisms, and chemokine/cytokine biology. My research focuses upon effector functions of mononuclear phagocytes and neutrophils, their roles in the initiation and resolution process of injury in tissue sites such as the lungs, and their potential relevance to human disease. I have also had a long-standing interest in the intersection between vascular medicine and lung biology, particularly with regards to factors derived from hematopoietic cells such as platelets and red blood cells that can influence the course of inflammation. A primary objective of my laboratory is to define the molecular and cellular basis of host defenses in the lungs, with recent focus upon products of extracellular gram negative pathogens such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and their ability to degrade or subvert host defenses utilizing molecular genetic approaches, in vitro biochemical assays, in vivo murine models, and human bio-samples. In the capacity of T1 Translational Track Director, I advise and mentor junior faculty members across the School of Medicine pursuing a Master of Science Degree in Clinical Research interested in laboratory training through the T1 Track pathway and combining this with formal coursework in bio-statistics. I also train PhD and medical students as a faculty member within the Cellular and Molecular Pathology Graduate Training Program. I am a practicing pulmonologist and intensivist specializing in the care of patients with acute respiratory failure such as ARDS and severe pneumonia in the ICU. My prior and current research accomplishments and background in training and mentoring makes me well suited to serve as primary mentor to Tolani Olonisakin in navigating the field of innate immunity and inflammation from the perspective of a physician scientist and lung biologist.

a. Zhao Y, Xiong Z, Lechner EJ, Klenotic PA, Hamburg BJ, Hulver M, Khare A, Oriss T, Mangalmurti N, Chan Y, Zhang Y, Ross MA, Stolz DB, Rosengart MR, Pilewski J, Ray P, Ray A, Silverstein RL, **Lee JS***. Thrombospondin-1 triggers macrophage IL-10 production and promotes resolution of experimental lung injury. *Mucosal Immunol*. 2014 Mar;7(2):440-8. PMID: 24045574; PMCID: PMC3945733.

b. Zhao Y, Olonisakin TF, Xiong Z, Hulver M, Sayeed S, Yu MT, Gregory AD, Kochman EJ, Chen BB, Mallampalli RK, Sun M, Silverstein RL, Stolz DB, Shapiro SD, Ray A, Ray P, **Lee JS***. Thrombospondin-1 restrains neutrophil granule serine protease function and regulates the innate immune response during *Klebsiella pneumoniae* infection. *Mucosal Immunol*. 2015 Jul;8(4):896-905. PMID: 25492474. PMCID:PMC4465063

c. Pinilla-Vera M, Xiong Z, Zhao Y, Zhao J, Donahoe MP, Barge S, Horne WT, Kolls JK, McVerry BJ, Birukova A, Tighe RM, Foster WM, Hollingsworth J, Ray A, Mallampalli R, Ray P, **Lee JS***. Full Spectrum of LPS Activation in Human Alveolar Macrophages by Whole Transcriptomic Profiling. *PLOS ONE* 2016 Jul 19;11(7):e0159329. doi: 10.1371/journal.pone.0159329. eCollection 2016. PMID: 27434537; PMCID:PMC4951018

d. Olonisakin T, Li HH, Xiong Z, Kochman E.J.K., Yu MT, Qu Y, Hulver M, Kolls JK, St. Croix C, Doi Y, Nguyen MH, Shanks RMQ, Mallampalli RK, Kagan VE, Ray A, Silverstein RL, **Ray P**, **Lee JS***. CD36 provides host protection against *Klebsiella pneumoniae* intrapulmonary infection by enhancing LPS responsiveness and macrophage phagocytosis. *J Infectious Dis* 2016 Sep 28. PMID: 27683817; PMCID: PMC5142085

B. Positions and Honors.

Positions and Employment:

1995 Intern, Internal Medicine, University of Alabama at Birmingham, Birmingham, AL
1996 Resident, Internal Medicine, University of Alabama at Birmingham, Birmingham, AL
1998 Fellow, Pulmonary and Critical Care Medicine, University of Washington, Seattle, WA
2004 Instructor, Pulmonary and Critical Care Medicine, University of Washington, Seattle, WA
2004 Assistant Professor of Medicine, University of Pittsburgh, Pittsburgh, PA
2010 Member, Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA
2011 Associate Professor of Medicine, University of Pittsburgh School of Medicine (tenure, 2014)
2011 Associate Professor, Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health (secondary appointment)
2014 Member, Career Advisor/Steering Committee MSTP Training, University of Pittsburgh
2014 Faculty Member, Cellular and Molecular Pathology Graduate Training Program, U Pittsburgh
2014 T1 Translational Track Director, Masters of Science in Clinical Research Program, ICRE
2016 Director, Pulmonary Translational Research Core, University of Pittsburgh
2016 Professor of Medicine with tenure, University of Pittsburgh

Other Experience and Professional Memberships

2011-16 Member, American Thoracic Society Research Advisory Committee
2012 Co-Chair, Special Emphasis Panel, NHLBI initiatives ZRG1 VH-F 50 (S): Selected Topics in Transfusion Medicine, June 12-13, 2012 and Chair, Special Emphasis Panel, NHLBI initiatives ZRG1 VH-A 50 (S): Selected Topics in Transfusion Medicine, June 13, 2012
2013-14 Ad Hoc Reviewer, Mentored Transition to Independence: K99/R00 NHLBI study section, November 21-22, 2013; March 5-6, 2014
2013 Member, American Thoracic Society Membership Committee
2014 Ad hoc External Grant Reviewer, Scientific Advisory Council of the Dutch Landsteiner Foundation for Blood Transfusion Research, June 2014
2014-15 Ad hoc Reviewer, Innate Immunity and Inflammation NIH Standing Study Section
2015 Vice Chair, American Thoracic Society Membership Committee, American Thoracic Society
2015 Permanent Member, Innate Immunity and Inflammation NIH Standing Study Section, July 2015-June 2019
2016 Member, American Thoracic Society Finance Committee, American Thoracic Society
2017 Chair, American Thoracic Society Membership Committee, American Thoracic Society

Honors:

1994 Alpha Omega Alpha, Georgetown University School of Medicine
1995 Janet M. Glasgow Memorial Achievement Citation for Scholastic Achievement, American Medical Women's Association
1995 *cum laude*, Georgetown University School of Medicine
1998 Outstanding Senior Resident, William E. Dismukes Award of Excellence, University of Alabama at Birmingham
2004 Recipient, NIH Loan Repayment Program
2013 Elected Member, American Society for Clinical Investigation (ASCI)
2015 Robert M. Rogers, MD Outstanding Mentor Award, Division of PACCM, University of Pittsburgh

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C. Contribution to Science (* indicates first or senior author)

1. Host-microbial interactions that exerted pressure on the human genome: Effector function of atypical chemokine receptor Duffy antigen in lung inflammation: My initial work focused on the minor RBC blood group antigen determinant Fy, or Duffy antigen, which is the receptor for the malarial parasite *Plasmodium vivax* and an atypical 7-transmembrane spanning chemokine receptor that binds to CC and ELR$^+$ CXC chemokines with high affinity. I previously cloned the human Duffy antigen cDNA from a pancreas cDNA library (GenBank Accession no. AY167991), stably expressed Duffy antigen in a HUVEC cell line to examine K_d and IC_{50} of various ^{125}I-chemokine ligands in addition to visualizing its location along the plasma membrane and co-localization to LAMP-1 associated endosomes following ligand endocytosis. My work indicated that red cell Duffy antigen functions as a dynamic chemokine reservoir in vivo, scavenging and releasing chemokines based upon the dissociation constant (Kd) of the ligand to the receptor, and its relationship to the concentrations of competing ligands within the surrounding environment that influences pulmonary neutrophil recruitment. I also showed that human Duffy antigen functional polymorphisms contribute to inter-individual variability in systemic and tissue chemokine responses during neutrophilic inflammation, utilizing a unique blend of *in vitro* studies, *in vivo* models, and translational studies using whole blood obtained from volunteers with Duffy "loss of function" phenotypes. This concept of red cell Duffy antigen as regulator of systemic inflammatory chemokine levels was validated by others in a large scale human genome wide association analysis for CCL2 in 3 independent cohorts.

a. **Lee JS***, Frevert CW, Thorning DR, Segerer S, Alpers CE, Cartron JP, Colin Y, Wong VA, Martin TR, Goodman RB. Enhanced expression of Duffy antigen in the lungs during suppurative pneumonia. J Histochem Cytochem. 2003 Feb;51(2):159-66. PMID: 12533524.

b. **Lee JS***, Frevert CW, Wurfel MM, Peiper SC, Wong VA, Ballman KK, Ruzinski JT, Rhim JS, Martin TR, Goodman RB. Duffy antigen facilitates movement of chemokine across the endothelium in vitro and promotes neutrophil transmigration in vitro and in vivo. J Immunol. 2003 May 15;170(10):5244-51. PMID: 12734373; PMCID: PMC4357319.

c. **Lee JS***, Wurfel MM, Matute-Bello G, Frevert CW, Rosengart MR, Ranganathan M, Wong VW, Holden T, Sutlief S, Richmond A, Peiper S, Martin TR. The Duffy antigen modifies systemic and local tissue chemokine responses following lipopolysaccharide stimulation. J Immunol. 2006 Dec 1;177(11):8086-94. PMID: 17114483; PMCID: PMC2665269.

d. Zhao Y, Mangalmurti NS, Xiong Z, Prakash B, Guo F, Stolz DB, **Lee JS***. Duffy antigen receptor for chemokines mediates chemokine endocytosis through a macropinocytosis-like process in endothelial cells. PLoS One. 2011;6(12):e29624. PMID: 22216333; PMCID: PMC3246497.

2. Host Modifiers of Lung Inflammation and Injury: Red cell transfusion in acute lung inflammation and injury. My laboratory has further shown that red cell storage impairs the functional chemokine scavenging property of Duffy antigen (Fy) and enhances lung neutrophilic inflammation in a model of red cell transfusion-associated injury. Consistent with our basic findings, others have shown that the GATA-1 box$^{T\text{-}46C}$ polymorphism leading to the selective ablation of Duffy antigen on RBCs in individuals of African ancestry (and confers host resistance to *P. vivax* malarial infection) is associated with increased mortality among African Americans with acute lung injury. Thus, a genetic mutation that provides host-protection against malarial infection may predispose at risk individuals to the development of lung injury in the modern world.

a. Mangalmurti NS, Xiong Z, Hulver M, Ranganathan M, Liu XH, Oriss T, Fitzpatrick M, Rubin M, Triulzi D, Choi A, **Lee JS***. Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation. Blood. 2009 Jan 29;113(5):1158-66. PMID: 19064726; PMCID: PMC2635081.

b. Mangalmurti NS, Chatterjee S, Cheng G, Andersen E, Mohammed A, Siegel DL, Schmidt AM, Albelda SM, **Lee JS***. Advanced glycation end products on stored red blood cells increase endothelial reactive oxygen species generation through interaction with receptor for advanced glycation end products. Transfusion. 2010 Nov;50(11):2353-61. PMID: 20492604; PMCID: PMC3010325.

c. **Lee JS***, Gladwin MT. Bad Blood: The Risks of Red Cell Storage. Bedside to Bench Invited Commentary. *Nature Medicine* 2010 Apr;16(4):381-2. PMID:20376046; PMCID: PMC5320870

d. **Lee JS**, Kim-Shapiro D. Stored Blood: How Old is Too Old? *J Clin Invest*. 2016 Dec 12. pii: 91309. doi: 10.1172/JCI91309. [Epub ahead of print]. PMID: 27941251; PMCID: PMC5199682

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3. Host immune responses contributing to cigarette smoke-induced lung tissue injury: Chronic obstructive pulmonary disease is defined pathologically by chronic inflammation of the airways and lung parenchymal destruction. My laboratory examines the innate immune basis for persistent inflammation with particular focus on cytokine and chemokine factors that trigger recruitment of mononuclear phagocyte subsets with tissue-destructive responses. My laboratory's work on the chemokine CX3CL1 (otherwise known as human fractalkine or mouse neurotactin) and its cognate receptor CX3CR1 in mononuclear phagocyte signaling during cigarette smoke–induced inflammation is a bedside-to-bench approach to examine relevant pathways in COPD, as CX3CL1 gene upregulation was originally identified in the lung tissue of humans with the disease and in airway epithelial cells from cigarette smokers.

a. **Lee JS***, Rosengart MR, Kondragunta V, Zhang Y, McMurray J, Branch RA, Choi AM, Sciurba FC. Inverse association of plasma IL-13 and inflammatory chemokines with lung function impairment in stable COPD: a cross-sectional cohort study. Respir Res. 2007 Sep 14;8:64. PMID: 17868461; PMCID: PMC2064925.
b. McComb JG, Ranganathan M, Liu XH, Pilewski JM, Ray P, Watkins SC, Choi AM, **Lee JS***. CX3CL1 up-regulation is associated with recruitment of CX3CR1+ mononuclear phagocytes and T lymphocytes in the lungs during cigarette smoke-induced emphysema. Am J Pathol. 2008 Oct;173(4):949-61. PMID: 18772344; PMCID: PMC2543064.
c. Xiong Z, Leme AS, Ray P, Shapiro SD, **Lee JS***. CX3CR1+ lung mononuclear phagocytes spatially confined to the interstitium produce TNF-α and IL-6 and promote cigarette smoke-induced emphysema. J Immunol. 2011 Mar 1;186(5):3206-14. PMID: 21278339; PMCID: PMC3912553.
d. **Lee JS***. Heterogeneity of lung mononuclear phagocytes in chronic obstructive pulmonary disease. J Innate Immun. 2012;4(5-6):489-97. PMID: 22572241; PMCID: PMC3804221.

4. Host Defense Against Pathogen-induced Lung Injury: I examine the innate arm of immunity in the lungs and how cells recognize and respond to exogenous pathogen associated molecular patterns, and the triggering of the resolution process following injury. Utilizing relevant murine models of injury to the lungs, we have discovered novel mechanisms by which cells protect, promote repair, and aid in the resolution of inflammation. We have shown that the host response to protect and promote repair following injury may serve to curtail inflammation on the one hand but can lead to impaired pulmonary host defense and immunosuppression on the other. This finding is particularly relevant to the critically ill population where even previously immunocompetent patients may develop an immunosuppressive phenotype during prolonged sepsis.

a. Balasubramanian KK, Maeda A, **Lee JS**, Mohammadyani D, Tyurin VA, Tyurina YY, Ray P, Klein-Seetharaman J, Mallampalli RK, Bayir H, Fadeel B, Kagan VE. Dichotomous roles for externalized cardiolipin in extracellular signaling: Promotion of phagocytosis and attenuation of innate immunity. *Science Signal.* 2015 Sep 22;8(395):ra95. PMID:26396268; PMC4760701
b. Chakraborty K, Raundhal M, Chen B, Morse C, Tyurina YY, Khare A, Oriss TB, Huff R, **Lee JS**, St. Croix CM, Watkins S, Mallampalli RK, Kagan V, Ray A, Ray P. The Mito-DAMP Cardiolipin Blocks IL-10 Production Causing Persistent Inflammation During Bacterial Pneumonia. *Nature Communications* 2017 Jan 11;8:13944. doi: 10.1038/ncomms13944. PMID:2807481; PMCID: PMC5241690

c. Flitter BA, Hvorecny KL, Ono E, Eddens T, Yang J, Kwak DH, Bahl CD, Hampton T, Morisseau C, Hammock BD, Liu X, **Lee JS**, Kolls JK, Levy BD, Madden DR, Bomberger JM. *Pseudomonas aeruginosa* sabotages the generation of host pro-resolving lipid mediators. *Proc Natl Acad Sci U S A.* 2016 Dec 15. pii: 201610242. [Epub ahead of print]. PMID: 27980032; PMCID:PMC5224368
d. Chen K, Eddens T, Trevejo-Nunez G, Way EE, Elsegeiny W, Ricks DM, Garg AV, Erb CJ, Bo M, Wang T, Chen W, **Lee JS**, Gaffen SL, Kolls JK. Epithelial IL-17R signaling is required for mucosal chemokine gradients and pulmonary host defense against *K. pneumoniae*. *Cell Host & Microbe*. 2016 Nov 9;20(5):596-605. doi: 10.1016/j.chom.2016.10.003. PMID: 27923703; PMCID:PMC5149406

Complete list of Published Work:

http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/47846252/?sort=date&direction=descending

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D. Research Support
Active Research Support (federal)

1 R01 HL136143-01 Lee, JS (PI) 07/01/17 - 06/30/21
NIH/NHLBI
Mechanisms of Host Protection Against Pathogen-Secreted Proteases in Acute Lung Injury
The broad, long term objective is to identify host-protective mechanisms that counter pathogen-initiated lung inflammation and injury utilizing Pseudomonas aeruginosa pneumonia as a model system.
Role: PI

5R01HL086884-10A1 Lee, JS (PI) 07/01/13-03/31/18
NIH/NHLBI
Red cell transfusion: Modifier of Systemic and Lung Inflammatory Responses
Utilizing red cell transfusion as a relevant model to study injury in the lungs, this project aims to better understand regulatory mechanisms employed by phagocytes to curtail innate immune activation following exposure to injurious stimuli and focuses upon the role of thrombospondin-1 as a bridging molecule.
Role: PI

1R21AI119042-02 Lee, JS (PI) 07/01/2015- 05/31/2018 (NCE)
NIH/NIAID
Enhancing Neutrophil Responses to Counter MDR Gram Negative Bacterial Pneumonia
The major hypothesis of this application is that small molecule compounds that potentially disrupt TSP-1/neutrophil serine protease interaction can alter microbial killing of MDR *Klebsiella pneumoniae.* This is in response to an R21/R33 Phased Innovation Award: Development of Novel Therapeutics for Select Pathogens.
Role: PI

2R01HL098032-06 Gladwin, MT (PI) and Kim-Shapiro, D (PI) 08/01/13-05/31/18
Storage Lesion in Banked Blood Due to Disruption of Nitric Oxide Homeostasis
The focus of this proposal centers around the hypothesis that the red cell storage lesion is largely due to dysregulation of nitric oxide homeostasis in the blood. The proposal will examine the exact mechanisms of loss of NO bioavailability, as well as down-stream effects of this loss, particularly platelet activation utilizing mouse models, a canine model, and human studies.
Role: Co-I

1P01HL114453-01A1 Mallampalli, R (PI) 01/03/2014 – 12/31/2018
Cardiolipin as a Novel Mediator of Acute Lung Injury
Project 3: Dysregulation of innate immune response in bacterial pneumonia by cardiolipin
The focus of Project 3 is to examine the mechanism by which the novel DAMP cardiolipin compromises host immunity in the lungs by suppressing production of the anti-inflammatory cytokine IL-10.
Role: Co-I

Vascular Medicine Institute Scholar Lee, JS (PI) 07/01/13-6/30/20
Institute for Transfusion Medicine
Hemostasis and Vascular Biology Research Institute
The project aims to determine the molecular mechanisms underlying clinically important biomedical problems of hemostasis, thrombosis, transfusion medicine and vascular biology.
Role: PI

Name :	JanetLee

Position Title :	Professor of Medicine
Institution :	University of Pittsburgh
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SPONSOR'S LIST OF PAST AND CURRENT TRAINEES

Past / Current Trainee	Trainee Name (in Alphabetical Order)	Type of Trainee	Dates	Prior Academic Degree(s)	Prior Academic Degree Institution(s), Year(s)	Title of Research Project	Current Position of Past Trainees / Source of Support of Current Trainees
Past	Meghan Fitzpatrick	Post-doctoral	2009	M.D.	Temple University School of Medicine, 2006	2,3 DPG determination of stored murine red cells	Instructor of Medicine, Pulmonary, Allergy, & Critical Care Medicine, University of Pittsburgh
Past	Elizabeth J. Lechner Kochman	Post-doctoral	2011- 2014	M.D.	University of Maryland School of Medicine, 2008	Role of CD36 in Bacterial Pneumonia	Member, The Lung Center at Mercy Medical Center/University of Maryland
Past	Nilam Patel Mangalmurti	Post-doctoral	2005-2008	M.D.	Temple University School of Medicine, 2002	Erythrocyte Transfusion in Acute Lung Injury During Sepsis	Assistant Professor of Medicine, Division of Pulmonary and Critical Care Medicine, University of Pennsylvania
Past	Jennifer Gonzalez McComb	Post-doctoral	2005-2008	M.D.	Albany Medicine College, 2002	$CX3CR1^{+}$ Mononuclear Phagocytes and T Lymphocytes in Cigarette Smoke Induced Emphysema	Section Chief and Clinical Associate Professor of Medicine, UPMC Shadyside Campus Hospital, Pittsburgh, PA

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Past / Current Trainee	Trainee Name (in Alphabetical Order)	Type of Trainee	Dates	Prior Academic Degree(s)	Prior Academic Degree Institution(s), Year(s)	Title of Research Project	Current Position of Past Trainees / Source of Support of Current Trainees
Past	Heather E. Metz	Pre-doctoral	2005-2006	B.A., Biological Sciences Ph.D., Molecular Cellular Pathology	University of Pittsburgh, 2006 University of Pittsburgh, 2016	Cyr61 and CX3CL1 in in vitro and in vivo models of cigarette smoke inflammation	Post-doctoral Fellow, Fred Hutchinson Cancer Research Center, Seattle, WA
Past (as co-mentor with Mark Gladwin, M.D.)	David Osei-Hwedieh	Pre-doctoral	2013-current	B.A., Genetics and Philosophy Ph.D., Pharmacology and Chemical Biology	Rutgers University, 2009 University of Pittsburgh, 2016	The Effect of Donor Genetic Background on Red Blood Cell Post-transfusion Survival-Sickle Cell Trait	Medical Student ('20), University of Pittsburgh School of Medicine
Past	Miguel Pinilla-Vera	Post-Doctoral	2013-2016	M.D.	Pontifical Javeriana University School of Medicine, 2008	Whole Transcriptomic Profiling of Human BAL cells of Healthy Volunteers	Clinical Fellow, Division of Cardiology, The Johns Hopkins University
Past	Zeyu Xiong	Post-Doctoral	2007-2013	M.D.	Shaanxi College of Medicine, China, 1988	Mononuclear phagocyte function in lung inflammation	Research Assistant Professor, Division of Pulmonary, Allergy, Critical Care Medicine, University of Pittsburgh

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Past / Current Trainee	Trainee Name (in Alphabetical Order)	Type of Trainee	Dates	Prior Academic Degree(s)	Prior Academic Degree Institution(s), Year(s)	Title of Research Project	Current Position of Past Trainees / Source of Support of Current Trainees
Current	Min Ting Yu	Pre-doctoral	2013-2017	B.A., Biochemistry	University of Pittsburgh, 2016	CD36-dependent phagocytosis in macrophages	Medical Student ('20), University of Pittsburgh School of Medicine
Past	Yani Zhao	Post-doctoral	2010-2014	M.D. Ph.D.	Peking University Health Science Center, 1997 Peking University First Hospital, Dept of Medicine, 2002	Mechanism of chemokine endocytosis in endothelial cells Resolution of Inflammation and Injury in the Lungs	Resident, Department of Pathology, New York University School of Medicine, New York, NY
Current	Tolani Olonisakin	Pre-doctoral	2014-current	B.A., Biology	Fisk University, 2012	Thrombospondin-1 mediated protection during *P. aeruginosa* induced injury	MSTP, University of Pittsburgh/Cellular and Molecular Pathology Graduate Program 2017-current/ Institutional Seed account
Current	William Bain	Post-doctoral	2016-	B.A., Earth and Planetary Sciences M.D.	Harvard University, Cambridge, MA, 1998 Columbia University School of Medicine, 2008	Epithelial-platelet interactions in P. aeruginosa lung infection	Pulmonary Fellow, Division of Pulmonary, Allergy, Critical Care Medicine/T32 Translational Training Program in Pulmonary Biology and Medicine

Name :	JanetLee
Position Title :	Professor of Medicine
Institution :	University of Pittsburgh

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Sponsor's Training Plan:

Research Progress in Sponsor's Laboratory: The research in progress in the Sponsor's laboratory focuses on lung innate immunity and inflammation. My laboratory studies effector functions of mononuclear phagocytes and neutrophils, their roles in the initiation and resolution process of injury at tissue sites such as the lungs, and their potential relevance to human disease. I have also had a long-standing research interest at the intersection of vascular biology and inflammation, particularly with regards to factors derived from hematopoietic cells such as platelets and red blood cells that can influence vascular integrity and tissue inflammation at barrier sites. It is within this intersection that Tolani will pursue her training and one that is relevant to the mission of the AHA.

Training Plan: The Training Plan is comprised of 3 key components: 1) Tolani's Research Project; 2) The Training Environment; 3) Didactic Training and Continuing Scientific Development. Tolani joined my lab as a medical student within the University of Pittsburgh School of Medicine Physician Scientist Training Program (PSTP), a structured 5-year program, designed to position graduates for careers in academic medicine. The PSTP program provides the opportunity for dedicated laboratory research investigation for one year, between their 2nd and 3rd year of medical school. However, Tolani was remarkably industrious and was awarded an HHMI Medical Research Fellows award to protect her time for 2 years (the maximum allotted time, May 2015-May 2017). In that time, Tolani published three papers (*Mucosal Immunol* 2015 where she is 2nd author, *J Infect Dis* 2016 where she is 1st author, and *PLoS ONE* where she is 2nd author). Tolani first-authored an editorial in *Am J Respir Cell Mol Biol* that is currently In Press. She has another manuscript that has been invited as a fast-track submission to *JCI Insight*, and two other manuscripts that are currently in preparation. Tolani has developed a passion for science but also recognized that, for her to successfully pursue a physician-scientist track long-term, she will require additional time and training to acquire the knowledge, skills, and experience to begin a career in biomedical science. Tolani formally applied to our MSTP program and matriculated into the MD PhD Cellular and Molecular Pathology program in May 2017. My goal is to provide a closely guided mentored experience and a rich network of opportunities for her laboratory training to foster her development into a physician scientist.

The Research Project: Tolani's research project will address two aims that focus on novel aspects of thrombospondin-1 (TSP-1) biology and are designed to develop critical skills and techniques. TSP-1 is a matricellular protein released by a variety of cells during inflammation, but the major source of TSP-1 within the circulation is platelets and constituting the most abundant protein per mass within α-granules. Tolani's research project focuses on how the host protects against aggressive lung injury triggered by infection through the release of soluble factors from platelets. When this protective mechanism goes awry, such as in the case when platelet counts fall below a threshold level during overwhelming critical illness, the host is unable to adequately repair the air sacs responsible for gas exchange. The basis for Tolani's studies originally stems from recent work in our lab showing that mice deficient in TSP-1 (*Thbs1*$^{-/-}$) are prone to *P. aeruginosa* (PA) acute intrapulmonary injury and enhanced neutrophilic inflammation. Given that platelets are a major reservoir for TSP-1, Tolani began pursuing studies in natively thrombocytopenic *Mpl*$^{-/-}$ mice and found that these mice show profound alveolar injury and early mortality following *P. aeruginosa* infection. Tolani's training will center upon defining molecular mechanisms underlying protection mediated by platelet TSP-1 in PA injury.

Aim 1 will build upon preliminary findings that TSP-1 dose-dependently inhibits activity of the pathogen-derived metalloprotease LasB, a well-known virulence factor of *P. aeruginosa,* that accounts for its elastolytic property. Tolani will examine whether administration of purified TSP-

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1 from platelets sufficiently rescues *Thbs1*$^{-/-}$ and *Mpl*$^{-/-}$ mice from *P. aeruginosa* acute intrapulmonary injury. Although Tolani has been involved in aspects of mouse studies, her project will provide real-life opportunity to learn mouse genetic tools in greater detail with the *Thbs1*$^{-/-}$, *Mpl*$^{-/-}$, and platelet-specific Thbs1 conditional knockout mice, design in vivo studies, become technically proficient with in vivo models, troubleshoot, and understand capabilities as well as limitations of techniques utilized. She will be given increasing personal responsibility of the mouse project and the conduct of the research.

Aim 2 will examine whether LasB propagates *P. aeruginosa*-induced neutrophilic inflammation through proteolytic processing of IL-36γ and determine whether the host protein TSP-1 effectively counters this response. We have enlisted the expertise of one of my colleagues at the University of Michigan, Dr. Theodore Standiford, who has kindly provided us with in-house generated IL-36γ neutralizing antibody. We have also enlisted the expertise of Dr. Joseph Pilewski, who has provided us with the infrastructure for obtaining clinically relevant primary human bronchial epithelial cells to serve as culture models. Tolani will focus upon experimental design, interpretation of data, troubleshoot and gain competence in both *in vitro* and *in vivo* techniques. The goal is to teach Tolani to effectively critique her own work, interpret data by weighing the strength of the evidence, acknowledging limitations, examining existing literature, testing alternative approaches, and creating solutions to enhance her scientific work.

Training Environment: My laboratory will provide the direct training environment. Dr. Zeyu Xiong (Research Assistant Professor), Dr. Yan Yan Qu (Research Associate), Dr. Huihua Li (Research Associate) and Mei Hulver (laboratory technician) will provide the additional layers of day-to-day technical training and instruction. My clinical responsibilities are 6 weeks of in-patient service and no out-patient clinic duties, so I have considerable time to devote to research and trainees. Tolani will be paired with Dr. Yan Yan Qu who has expertise in molecular and cellular immunology. Dr. Qu's project relates to *Thbs1*$^{-/-}$ mice and the potential role of TSP-1 in titrating TH_{17} and γδ T cell responses during pathogenic inflammation in the lungs. Dr. Qu will provide the direct day-to-day technical supervision on cell-based and *in vivo* studies proposed in Aims 1 and 2. Ms. Hulver, the technician who oversees our laboratory's mouse colony management and performs the *in vivo* models, will teach Tolani how to perform the pneumonia studies. Tolani will also learn basic principles of breeding, PCR genotyping, colony management of mice. Dr. Zeyu Xiong oversees the entire day-to-day operations of our laboratory. His expertise is molecular biology techniques and flow cytometry. He will assist Tolani with protein and RNA isolation from cells and tissue, primer design, qPCR, cell transfection techniques, siRNA studies, plasmid construction, and basic flow cytometry that will expand upon the *in vivo* and cell-based training proposed in Aim 1 and Aim 2. Dr. Huihua Li has considerable expertise in mouse genetics, bioinformatics, and mouse models of lung injury. Our goal is to help Tolani become directly familiar with the techniques common to molecular biology and immunology so that she has the necessary tools to build upon this foundation as a physician scientist. Our laboratory meetings are weekly on Mondays at 9-11 am. Tolani will be expected to present briefly each week, discuss the raw data, provide her analysis and potential next steps.

Additional Experts: Tolani's PhD thesis committee consists of 1) Dr. Wendy Mars, co-Director of the Cellular Molecular Pathology program, whose expertise is in the realm of cellular interactions influenced by soluble factors urokinase, uPA, tPA which will be relevant to Tolani's work with *P. aeruginosa*; 2) Dr. Jennifer Bomberger, whose group recently demonstrated that a secreted *P. aeruginosa* protein, Cif, prevents resolution of airway inflammation by impairing 15-epi lipoxin A_4 production; 3) Dr. Sally Wenzel, Chair of Translational Airway Biology and Director of the University of Pittsburgh Asthma Institute, who has served as Tolani's career advisor since

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January 2015; 4) Dr. Grant Bullock, whose expertise is in hematopathology, specifically in mechanisms underlying regulation of red blood cell and platelet function. In addition to the interactions with committee members and consultations with Drs. Standiford and Pilewski, the training environment will also include experts with whom I closely collaborate and who will help advance Tolani's scientific progress. Drs. Prabir and Anuradha Ray are eminent scientists whose research interests lie in the immunoregulatory mechanisms of lung inflammation. Dr. Prabir Ray's scientific contributions include the development of lung-specific inducible transgenic mice based upon the tet-inducible system, impairment of Treg function by RSV and the development of allergic asthma, and most recently a role for myeloid-derived suppressor-like cells in promoting resolution of non-resolving bacterial pneumonia. Dr. Anuradha Ray is the co-discoverer of GATA-3 as a master regulator of Th2 cells, which promote allergic diseases including asthma. She has also identified a key role for Tregs expressing membrane-bound TGF-β and cross-talk with the Notch pathway in regulation of immune tolerance in the airways. I am co-investigator on Dr. Prabir Ray's Project 3 of a P01 in Acute Lung Injury, as is Dr. Anuradha Ray. My office is next door to Dr. Anuradha Ray's and Dr. Prabir Ray's office. We interact virtually every day on an informal basis and the Rays often provide feedback on Tolani's presentations and progress.

Enrichment of Student's Scientific and Career Development: For career development and networking opportunities, Tolani will be expected to attend the Basic Translational Research Conference each Tuesday, an outstanding forum in which invited lecturers from within and outside the institution present state-of-the art research relevant to Pulmonary, Cardiovascular, Hematology and Immunology fields. As part of the MD-PhD program, Tolani will be expected to partake in a discussion-based ethics course each summer, guided by faculty preceptors, where students review literature and discuss ethically challenging situations with their peers. Tolani will also partake in the longitudinal clinical clerkship offered through the MSTP, a half-day per week elective, that allows her to explore her clinical interests and teaches her how to synthesize her clinical and research skills. Furthermore, Tolani will submit abstracts to two conferences each year, one of which is the American Thoracic Society International Conference. Tolani is also expected to submit abstracts locally to the annual Department of Medicine Research Day Forum and the MSTP Research Day Forum. I am active in the ATS and will provide Tolani opportunity to network with leaders in the field.

Assessment of Applicant and Role of Applicant in the Development of the Research Proposal: Tolani is an individual of outstanding intellect who possesses enormous work ethic, scientific curiosity, motivation and maturity. Tolani and I discussed the ideas that became her 2 aims, and the experimental approaches she would need to carefully consider. However, Tolani wrote the grant application entirely, and sent me completed drafts that I edited for clarity of presentation. I believe she has high potential to become an independent investigator in the future. Given her keen scientific ability and exemplary personal attributes, Tolani is the kind of candidate we want to train as a future physician scientist and deserving of this prestigious award.

Financial Resources: I have adequate financial resources, including computer resources, to support Tolani's project during the entire fellowship term. Currently, I have R01 HL136143 (till 6/30/21), 5R01HL086884-10 (till 3/31/18- enough funds for NCE), 1R21AI119042-02 (ending 5/31/18), and another R01 HL142084 that was competitively scored (9%, awaiting NHLBI review). Tolani's salary support currently comes from my institutional seed account (no time limit).

Name :	JanetLee

Position Title :	Professor of Medicine
Institution :	University of Pittsburgh
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Status 3 :	Uploaded
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Sponsor's Research Project Environment

Laboratory:

Dr. Lee's lab laboratory consists of ~1500 ft^2 area of space (2 lab rooms comprising 1047 ft^2: W631, W640 in addition to ~450 ft^2: W632, W633) in Montefiore University Hospital, 6th floor. There are 6 PC desktop computers, 9 work benchtop spaces and 4 office desks in this area. W633 includes a dedicated tissue culture room containing two laminar flow hoods and W632 is a short-term animal experimental room. Tolani occupies an office desk with desktop computer connected to the network that is in W631 and dedicated benchtop space. The laboratory is BSL-2 laboratory outfitted within a short walking distance from the main Division office where Dr. Lee's office is located. Members of the laboratory have full access to the facilities and equipments located within the West Wing. This includes a chemical hood, a shared walk-in cold room, -80^{o}C freezer room that also contains an ice making machine and centrifuges, and shared liquid nitrogen freezing unit. All personnel, including Tolani, have been trained in BSL-2 safety practices and handling on entry to the laboratory and on an annual basis.

Computer:

Dr. Lee has a HP Compaq D530 CMT Desktop that is connected to the Biomedical Computing Facility of the University of Pittsburgh Medical Center and MacBook 5.1 laptop. Dr. Lee has access to several laser printers that is connected by the Division network, 2 copiers, and secretarial support located within the Division offices.

Office:

Dr. Lee's office is located in the Division offices of Pulmonary, Allergy and Critical Care Medicine at 628 NW Montefiore University Hospital of the University of Pittsburgh Medical Center. The Division office is a short walking distance away and on the same floor as the West Wing where Dr. Lee's laboratory is located.

Clinical:

Not applicable

Animals:

Mice will be housed at the Biomedical South Tower (BST) Animal Services Room S-1033 under specific pathogen-free conditions. Experiments can be conducted either in the dedicated animal procedure room located in S-1035 or, for short-term experiments, the approved animal space of the West Wing. The S-BST tower is located within 2 minutes walking distance and is directly connected by an in-door bridge to the Montefiore University Hospital where the laboratory is located. All animal facilities at the University of Pittsburgh are under the direction of full time veterinarians and have been fully accredited by the American Association for Accreditation of Laboratory Animal Care since 1971.

Equipment at the University of Pittsburgh

Dr. Lee's laboratory contains a BD FACSCalibur and Beckman Model TJ-6 refrigerated centrifuge are available in the short-term experimental animal room located in W632. Tissue Culture hoods/SterilGARD III Advance are available in a dedicated tissue culture room located in W633, next door from the main laboratory. Centrifuges SORVALL Super T21, Eppendorf Centrifuge 5810 R, Beckman Microfuge R centrifuges are in the common use room W630, across the door from the laboratory. Forma Scientific -86^{o}C upright Freezer is also located in the common use room W630, across the door from the laboratory. Protein Array System BioPlex (Luminex machine) is owned and used by members of the Division and located in the

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NW wing of the 9th floor, just 3 floors directly above the main laboratory. Beckman L-60 Ultracentrifuge is located just outside of NW640, just the adjacent wing from the laboratory. SpectraMax i3x Multi-Mode Microplate Detection platform with luminescence, fluorescence and UV-Vis absorbance capabilities is available for in vitro enzymatic assays, and spectrophotometric work in measuring bacterial OD readings, ELISA plates is in one of Dr. Lee's laboratory rooms (W640).

A Unified Flow core is now available and operated by the Department of Immunology, and just 3 minutes walking distance from Dr. Lee's laboratory. The core is in BST-East Building, 10th floor. The core has 3 LSR Fortessas (1 bio-contained), 2 LSR IIs, 3 FACSAria cell sorters (2 bio-contained) and 1 ImageStream Mark II. The Core is available 24/7 to trained users.

Other:
Center for Biologic Imaging facilities
www.cbi.pitt.edu
The CBI is housed in the medical research facility of the University of Pittsburgh Medical School in approximately 6,500 sq ft. of space, within 2 minutes from Dr. Lee's office. This space has been designed as a dedicated, state of the art imaging center, and has fully equipped microscopy suites, computer labs, and wet and dry bench space for light and electron microscopic preparations. The center currently employs 4 faculty and 20 staff including multiple post-doctoral fellows, students and technicians. The CBI has an installed base of over 19 confocal microscopes of different types, as well as multiple systems dedicated to super-resolution imaging, and several electron microscopes. Essentially the Center is designed to be a "one stop shop" for all optical imaging needs. CBI also houses multiple new devices including a high speed multiphoton and live cell confocal microscopes (funded through the NCRR) and microscopes for large area scanning of slides in either brightfield or using up to 5 different fluorophores.

BIOHAZARDS
Dr. Lee's laboratory and imaging facility are approved for BSL2 materials, including human, non-human primate, rodent cells, *P. aeruginosa* and LPS, and short-term animal experiments. Experiments will be performed in accordance with CDC/NIH guidelines, *Biosafety in Microbiological and Biomedical Laboratories* (5th ed.) requirements for biological safety. Dr. Lee's laboratory is also approved for ^{125}I, ^{51}Cr, ^{99}Tm work in a dedicated radioactivity room containing a gamma counter (E633 Montefiore, just 60 feet from Dr. Lee's laboratory that is kept locked). All personal Personnel must demonstrate competency and proficiency to the PI prior to being granted approval to work at the BSL-2 level. All personnel have successfully completed the required training sessions. In addition, procedures and proper handling of agents are carefully reviewed on an annual basis with the Office of Environmental Health Safety and the University of Pittsburgh Biosafety Officer.