PI: **Putnam, Nicole**	Title: The impact of innate immune recognition of Staphylococcus aureus on bone homeostasis and skeletal immunity	
Received: 12/08/2016	FOA: PA16-309	Council: 05/2017
Competition ID: FORMS-D	FOA Title: RUTH L. KIRSCHSTEIN NATIONAL RESEARCH SERVICE AWARD (NRSA) INDIVIDUAL PREDOCTORAL FELLOWSHIP (PARENT F31)	
1 F31 AI133926-01	Dual: AR	Accession Number: 4000839
IPF: 8721001	Organization: VANDERBILT UNIVERSITY	
Former Number:	Department: Pathology and Microbiology Edu	
IRG/SRG: ZRG1 F13-C (20)L	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A)	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Early Stage Investigator:
Senior/Key Personnel:	*Organization:*	*Role Category:*
Nicole Putnam	Vanderbilt University	PD/PI
James Cassat	Vanderbilt University Medical Center	Other (Specify)-Sponsor
Julie Sterling	Vanderbilt University Medical Center	Other (Specify)-Co-Sponsor

[redacted]

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier **TN: Tennessee**

1. TYPE OF SUBMISSION*

○ Pre-application ○ Application ● Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

c. Previous Grants.gov Tracking Number **GRANT12302234**

2. DATE SUBMITTED **2016-12-08**

Application Identifier **U0044255**

5. APPLICANT INFORMATION

Organizational DUNS*: [redacted]

Legal Name*: **Vanderbilt University**
Department: **Pathology and Microbiology Edu**
Division: **School of Medicine**
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application

Prefix: First Name*: **Dorothy** Middle Name: **Janiece** Last Name*: **Harrison** Suffix:

Position/Title: **Director, Sponsored Programs Administration**
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* **O: Private Institution of Higher Education**

Other (Specify):
Small Business Organization Type ○ Women Owned ○ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ○ Resubmission
○ Renewal ○ Continuation ○ Revision

If Revision, mark appropriate box(es).

○ A. Increase Award ○ B. Decrease Award ○ C. Increase Duration
○ D. Decrease Duration ○ E. Other *(specify)* :

Is this application being submitted to other agencies?* ○ Yes ● No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health/Unknown

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE: Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (Parent F31)

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
The impact of innate immune recognition of Staphylococcus aureus on bone homeostasis and skeletal immunity

12. PROPOSED PROJECT

Start Date* **07/01/2017**
Ending Date* **06/30/2019**

13. CONGRESSIONAL DISTRICTS OF APPLICANT
TN-005

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: **Nicole** Middle Name: **E** Last Name*: **Putnam** Suffix:

Position/Title: **Grad Stu Research Asst Monthly**

Organization Name*: **Vanderbilt University**

Department: **Pathology and Microbiology Edu**

Division: **School ofMedicine**

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City*: [redacted]

County:

State*: [redacted]

Province:

Country*: [redacted]

ZIP / Postal Code*:

Phone Number*: [redacted] Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	[redacted]
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	[redacted]
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: **Dorothy** Middle Name: **Janiece** Last Name*: **Harrison** Suffix:

Position/Title*: **Director, Sponsored Programs Administration**

Organization Name*: **Vanderbilt University**

Department: **Sponsored Programs Administrat**

Division:

Street1*: [redacted]

Street2:

City*: [redacted]

County:

State*: [redacted]

Province:

Country*: [redacted]

ZIP / Postal Code*:

Phone Number*: [redacted] Fax Number: Email*: [redacted]

Signature of Authorized Representative* [redacted] **Date Signed†** [redacted]

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:**M-14_RRSF424_Cover_Letter.pdf**

424 R&R and PHS-398 Specific
Table Of Contents

SF 424 R&R Cover Page. 1
Table of Contents. 3
Performance Sites. 4
Research & Related Other Project Information. 5
Project Summary/Abstract(Description). 6
Project Narrative. 7
Bibliography & References Cited. 8
Facilities & Other Resources. 12
Equipment. 16
Research & Related Senior/Key Person. 17
PHS Fellowship Supplemental. 34
Applicant's Background and Goals for Fellowship Training. 38
Specific Aims. 42
Research Strategy. 43
Respective Contributions. 49
Selection of Sponsor and Institution. 50
Training in the Responsible Conduct of Research. 51
Sponsor and Co-Sponsor Statements. 52
Letters of Support from Collaborators, Contributors, and Consultants. 58
Description of Institutional Environment and Commitment to Training. 60
Vertebrate Animals. 62
Resource Sharing Plan. 64

OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: **Vanderbilt University**
Duns Number: [redacted]
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: **TN-005**

Additional Location(s) File Name:

Funding Opportunity Number: PA-16-309. Received Date: 2016-12-08T15:14:00.000-05:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: 1 2 3 4 5 6 If

NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number [redacted]

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	**M-4_Project_Summary.pdf**
8. Project Narrative*	**M-1_Narrative.pdf**
9. Bibliography & References Cited	**M-5_Bibliography.pdf**
10.Facilities & Other Resources	**M-2_Facilities.pdf**
11.Equipment	**M-3_Equipment.pdf**

Funding Opportunity Number: PA-16-309. Received Date: 2016-12-08T15:14:00.000-05:00

PROJECT SUMMARY

Staphylococcus aureus is a ubiquitous human pathogen, resulting in superficial, invasive, and disseminated infections. One of the most common invasive manifestations of *S. aureus* disease is osteomyelitis, a frequently occurring and debilitating infection of bone. Osteomyelitis triggers dramatic alterations in bone architecture, leading to severe complications such as bone destruction, pathologic fractures, and growth defects. An emerging body of literature suggests that both local and systemic inflammation trigger altered interactions between bone-forming osteoblasts and bone-resorbing osteoclasts to impact bone homeostasis. Skeletal cells are known to express innate pattern recognition receptors (PRRs), but the contribution of innate sensing towards bone homeostasis and antibacterial immunity during *S. aureus* osteomyelitis has not yet been explored. The overarching objective of this proposal is to characterize how innate sensing of bacterial pathogens by skeletal cells triggers alterations in bone physiology. In order to define the impact of skeletal cell PRRs on bone homeostasis, we first focused on the critical signaling adaptor protein, MyD88, which is necessary to transduce signals through toll-like receptors (TLRs) and IL-1 receptors (IL-1R). Our preliminary data demonstrate that MyD88 is necessary to control *S. aureus* replication and dissemination *in vivo* and that osteoclast differentiation can be stimulated by bacterial components in a MyD88-dependent manner *in vitro*. Therefore, the central hypothesis of this proposal is that *S. aureus* modulates osteoclast precursor cell biology and bone remodeling through ligation of osteoclast PRRs and the induction of inflammation. To test this hypothesis, I will use a newly developed murine *S. aureus* osteomyelitis model from our laboratory. This model is advantageous compared to other osteomyelitis models because it allows us to utilize genetically modified animals, high-resolution quantitative imaging analysis, and unique histologic techniques for quantifying perturbations in bone remodeling. Experiments proposed in Aim 1 will investigate the roles of TLR and IL-1R signaling on osteoclast differentiation by monitoring osteoclastogenic signaling cascades, transcription factor activity, expression of mature osteoclast markers, and functionality of osteoclasts formed *in vitro.* Aim 2 will explore how MyD88 signaling in skeletal cells impacts clearance of *S. aureus* and bone remodeling. Collectively, these data will define signaling crosstalk between canonical osteoclast differentiation and innate immune pathways to activate osteoclast differentiation and maturation programs. Additionally, these findings will describe how MyD88 signaling in skeletal cells contributes to immune defenses and affects the kinetics of bone remodeling. This proposed work will have broad implications for how innate skeletal cell sensing contributes to the development of an effective immune response and influences bone homeostasis.

PROJECT NARRATIVE

Normal bone remodeling is a tightly regulated process that is dramatically altered by infection and both systemic and local inflammatory conditions. The proposed research will investigate how skeletal cells sense and respond to the human bacterial pathogen *Staphylococcus aureus*, the most common cause of bone infections, and how these cellular responses disrupt normal bone remodeling. This work will therefore describe how bone is altered by the presence of bacterial pathogens and resulting immune responses, providing critical information for the development of therapeutics that may reduce bone pathology triggered by infection or inflammation.

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FACILITIES AND OTHER RESOURCES

The Cassat laboratory studies host-pathogen interactions during invasive bacterial infection, with a special emphasis on understanding how infection and inflammation perturb musculoskeletal cell biology. We have developed a variety of tools to understand how bacteria and inflammation trigger changes in bone biology, including a murine model of *Staphylococcus aureus* osteomyelitis, high-resolution 3D imaging of bone remodeling, and cell culture models of osteoblast and osteoclast differentiation and function. Institutionally, Vanderbilt has supported the acquisition of preliminary data for my research proposal with small pilot funds from the Vanderbilt Center for Microbial Pathogenesis (VCMP) and the Vanderbilt Institute for Clinical and Translational Research (VICTR). Physical resources have been provided primarily from the Vanderbilt Center for Bone Biology (VCBB), Vanderbilt University Institute for Imaging Sciences (VUIIS), and the Vanderbilt Program in Molecular Medicine (VPMM). At the juxtaposition of my project are the fields of bone biology, microbial pathogenesis, infectious diseases, and immunology. Intellectual rapport has been provided by experts in each of these areas for the development on my research proposal, with the following members serving on my thesis committee: Dr. Eric Skaar, Professor of Pathology, Microbiology, and Immunology (PMI) and Director of the Division of Molecular Pathogenesis, serves as the chair to my thesis committee and provides insight on the microbial pathogenesis of *S. aureus*. Expertise in bone biology continues to be provided through close communication with Dr. Julie Sterling, Assistant Professor in Medicine, Clinical Pharmacology, and Cancer Biology, who is co-sponsoring this application. Dr. Isaac Thomsen, Assistant Professor in Pediatric Infectious Diseases, is my clinical mentor through VPMM and he continues to provide advice on the clinical ramifications of invasive musculoskeletal diseases, ensuring that my research questions are both important and relevant. And finally, scientific counsel regarding immunology has been guided by the expertise of Drs. Dan Moore, Assistant Professor in Pediatrics and PMI, and Jeff Rathmell, Professor of Cancer Biology and PMI and Director of the Center for Immunobiology. *In sum, Vanderbilt University has been the ideal environment to study a unique host-pathogen interaction in an invasive infection model, with access to core facilities for state-of-the-art technology that provide assistance in data acquisition and analysis, and successful collaborations between experts in departments and centers at Vanderbilt University Medical Center and Vanderbilt University.*

Institution

Vanderbilt University Medical Center is located between the undergraduate and Peabody campuses and has approximately 7.2 million square feet of building space. The close proximity of Vanderbilt University and Vanderbilt Medical Center promotes interactions, sharing of resources, and collaboration. Biomedical research at Vanderbilt has long been recognized for its contributions to the advancement of medicine. The School of Medicine claims two Nobel Laureates, Earl W. Sutherland Jr., in 1971, for his discover y of the metabolic regulating compound cyclic AMP, and Stanley Cohen, in 1986, for his discovery (with a colleague) of epidermal growth factor. The Medical School's reputation for outstanding research is reflected in the amount of federal and private support it receives. Because of the creativity of the faculty, the School of Medicine ranks among the nation's top 10 medical schools for NIH funding, with VUMC research funding from all sources having more than doubled since 2001. Support for competitive research grants from all external sources has grown continually to more than $675 million as of fiscal year 2015.

Biomedical Research Education and Training (BRET) Office at Vanderbilt

The BRET Office is located on the 3rd floor of Light Hall, just seven floors below the Cassat lab space. Established in 1999, the BRET office coordinates graduate education, postdoctoral training, career development, and training programs. In addition, the BRET Office manages the NIH-funded Broadening Experiences in Scientific Training (BEST) grant awarded in 2013 to fund the Vanderbilt Augmenting Scholar Preparation and Integration with Research-Related Endeavors (ASPIRE) program. The ASPIRE program provides resources to trainees in the form of yearly progress reports through an Individual Development Plan (IDP), "Career Connections" seminar series, an annual career symposium, externship programs, and didactic modules. The BRET Office has been beneficial to me personally by providing the IDP resource to outline my scientific progress and goals as a graduate student through yearly iterations, by providing training through the annual Responsible Conduct of Research (RCR) sessions and the Vanderbilt Program in Molecular Medicine (see description below), and by sponsoring the annual career symposium which I attended in 2016.

Facilities

A. Research Laboratory Space

The Cassat Laboratory (Room 1035) in Medical Research Building IV consists of 850 square feet of laboratory space that encompasses three bays and two tissue culture hood rooms, additional hall space where freezers for biologic and chemical storage and large equipment are located, and adjacent facilities that include access to a cold room, autoclave, and dish washing amenities. The main laboratory contains equipment necessary to conduct all standard cell biology, microbiology, and molecular biology techniques. The Cassat lab has cell culture hoods and incubators that are individually dedicated for primary cell use or bacterial use. There are 4 members in the laboratory (2 Ph.D. students, 1 M.D./Ph.D. student, and 1 research technician), each with expertise in molecular biology of microbial pathogens and host-pathogen interactions.

The Cassat laboratory is designated as a Biohazard Safety Level 2 facility, meaning that all equipment and resources are in place to work with the biohazardous agents described in this application. Furthermore, all members of the Cassat laboratory undergo extensive training prior to working with biohazardous material. I have completed all the necessary training to work in a Biosafety Level 2 laboratory. *These facilities were specifically designed and equipped to support research at the crossroads of microbiology and bone biology, which is the cornerstone of the proposed research.*

B. Individual Space

I have an individual desk and bench space located within the main laboratory in 1035 MRB IV. Dr. Cassat's office is just off of the larger lab area and this close vicinity permits significant daily interactions between Dr. Cassat and myself. My personal bench space is furnished with equipment that allows me to complete experiments efficiently. This includes pipettes, reagents, and tools. The desk space is directly adjacent to my bench and includes a computer, office supplies, and filing cabinets. *These facilities ensure that I will have the necessary space in which to formulate experiments, analyze results, and prepare manuscripts for publication.*

C. Computer

My desk space is equipped with an iMac powered by Mac OS X Yosemite, high-speed internet access, software for image editing and analysis (FIJI), word processing and data analysis (Microsoft Office, GraphPad Prism 6.0), a reference and citation manager (ReadCube), and access to networked printers and a shared server. The shared server allows data generated or edited on any computer within the lab to be immediately accessible with this personal computer. There are two additional shared computers in the laboratory, with one connected to a BioTek plate reader with Gen5.0 software, a UVP GelDoc-It^2 imager with UVP software, and an Olympus microscope and Q-imaging camera with Q-Capture Pro 7 software. *The combination of these information technologies contributes to the potential for success by assuring efficient data management and processing, and optimal communication among members of the research team.*

D. Animal Facility

All proposed activities using vertebrate animals at VUMC and VU are reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). The facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the U.S. Department of Agriculture. The facility has a standing Animal Care Committee, which must review and approve all animal research protocols prior to their initialization. In addition to providing continuing protocol review in accordance with the Animal Welfare Act Regulations, the IACUC also establishes policies (e.g., acceptable mouse cage density, food/water regulation) and reviews, semi-annually, the animal care program and all animal housing and study areas. The Office of Animal Welfare Association (OAWA) provides administrative support to the IACUC with a staff of three Protocol Analysts, two Post Approval Monitors, a Veterinarian Reviewer, and an OAWA Training Coordinator.

All personnel listed on IACUC protocols must complete required training modules and pass the corresponding tests contained within each module. I have completed Animal Biohazard Safety Level 2 (ABSL-2) training, meaning that all equipment, resources, and training are in place to work with biohazardous agents, while protecting yourself and others. To ensure that I remain compliant with any new ABSL-2 or IACUC regulations, I will continue to attend ABSL-2 refresher training courses annually.

The Division of Animal Care (DAC) provides procurement, husbandry and veterinary care services in support of research at VUMC and VU. The DAC's comprehensive preventative medicine and veterinary care program includes daily observation of animals (including weekends and holidays) by animal care, veterinary, and research staffs. Animals identified as ill or injured during daily rounds are given a thorough physical examination. Treatment is prescribed as deemed appropriate by a veterinarian in consultation with researc h personnel. Veterinarians and veterinary technicians communicate directly with each other and with investigative staff regarding diagnostic requests and initiation of, or changes in, treatment. In addition, all treatments are documented in the animal's individual record. Records are maintained by the veterinary/research staff and "closed out" upon resolution.

Animals for this study will be procured through and housed in a BSL-2 Animal Facility located in Medical Center North. Our laboratory's designated animal space in suite A-8223 includes a mouse breeding facility that remains free of infectious agents, a housing room and a procedure room, both for hazardous agent use, which each include two biohazard cabinets to ensure sterility during surgical procedures and are equipped with anesthesia machines and euthanasia systems. Each room provides sterile food, water and cages for the breeding and infections immunocompromised mice. All animal procedures and harvests will be conducted in the A-8223 suite to ensure safety and sterility. An online reservation system for biosafety cabinets confirms that equipment is available when necessary. *Success of the proposed research is critically dependent on the maintenance and husbandry of mice. ABSL-2 facilities as described above are critical for conducting infections and dissections with sterility and safety in mind.*

Institutional Resources and Cores

A. The Vanderbilt University Institute of Imaging Science (VUIIS)

VUIIS is a University-wide interdisciplinary research initiative to develop new imaging technology based on advances in physics, engineering, and computer science. In addition to high-field MRI and MR spectroscopy, ultrasound, optical and other modalities in human subjects, the VUIIS offers state-of-the-art options for small animal imaging in all modalities. In 2007 Vanderbilt completed a four-floor facility adjacent to Medical Center North to house the VUIIS. The $28 million project ($21 million for construction) provides a 42,000-square-foot facility to integrate current activities in imaging research and provide research space for 42 faculty members and more than 80 graduate students and postdoctoral fellows in biomedical science, engineering, and physics.

The Center for Small Animal Imaging (CSAI) within the VUIIS is dedicated to research studies of small animals for a variety of applications. The CSAI is a comprehensive resource for advanced biomedical imaging instruments spanning a wide range of modalities, including MRI, X-ray/X-ray CT, PET, SPECT, ultrasound, bioluminescence and fluorescence. The center provides training for users on optical imaging, and provides expert support/training for data/image analysis. In particular, I have been trained on the Scanco microCT50, for high resolution 3D X-ray computed tomography of bone, with training and assistance provided by Dr. Dan Perrien (see letter of support). *Support and expertise from VUIIS faculty have been valuable in the development of analysis programs for computed tomography of femurs from our model, providing an exceptional ability to precisely calculate changes in bone remodeling. VUIIS will continue to be essential as we incorporate new models and research strategies.*

B. The Vanderbilt Center for Bone Biology (VCBB)

The VCBB facility was created to investigate diseases of bone and mineral metabolism. Investigators associated with VCBB study embryonic bone development, neuroskeletal biology, biomechanics, fracture repair, osteoporosis, bone infections, and cancers such as breast cancer and prostate cancer, which frequently affect the skeleton. Major goals of the VCBB are to unravel novel biological mechanisms and to develop new treatments and diagnostic tools that can eventually change the quality of life for patients with bone diseases. The VCBB sponsors a weekly seminar in which investigators present preliminary data, describe new techniques, and develop multi-disciplinary collaborations between bioengineering, cancer biology, endocrinology, rheumatology, and infectious disease researchers.

The VCBB facility has multiple high-tech instruments to perform molecular, cellular and biochemical studies, and to precisely quantify changes in bone volume, architecture, biomechanical properties and histology upon gene alterations, growth, aging, disease or pharmacologic treatments. Given that Dr. Cassat is

a primary faculty member in the VCBB, I will have access to and training on this specialized instrumentation. Additionally, the VCBB recruited an expert in bone histology (Josh Johnson) in 2016 as the head of the newly formed Bone Histology core (see letter of support). *The VCBB facility has been a vital resource to our lab emerging in the bone biology field and will provide technical support for completion of studies in the proposal. Additionally, the expertise of Julie Sterling in VCBB and her co-sponsorship for this fellowship is important for our continued progress in acquisition of new skills in the study of bone biology and for accessing necessary equipment to conduct histological analyses.*

C. Vanderbilt Center for Microbial Pathogenesis

The Center for Microbial Pathogenesis within the Department of Pathology, Microbiology, and Immunology was created to address the growing need for new therapeutics to treat infectious diseases. Dr. Eric Skaar is the Director, and his goal for the center is bring together researchers in both basic and translational research to establish a community of investigators interested in discovery and therapeutic development. By combining Vanderbilt's strengths in both basic and clinical science, the center is poised to make numerous exciting discoveries in infection biology, and leverage these advances to develop new drugs to treat a variety of infectious diseases.

The Center for Microbial Pathogenesis presents $2,000 Mini-Sabbatical awards to two Ph.D. students each year to establish collaboration with other Vanderbilt investigators. I was the recip ient for a 2015-2016 award to work with Dr. Julie Sterling in the Vanderbilt Center for Bone Biology and learn bone histology techniques. *The Vanderbilt Center for Microbial Pathogenesis will continue to facilitate our studies on the host-pathogen interface in bone, and we will continue to work closely with Dr. Eric Skaar, who is the head of my Ph.D. Thesis Committee (see letter of reference).*

D. Vanderbilt Institute for Clinical and Translational Research (VICTR)

VICTR provides a Voucher and Pilot Program that offers awards for the generation of preliminary data and pilot work for clinical and translational studies, allowing for rapid acquisition of proof-of-concept initiatives that might justify full-scale investigation. Applications are accepted on a rolling basis for review and all researchers are eligible to apply. Submitted projects are required to meet the following criteria: clinical and translational research, meaning it involves human subjects, human tissue, human cell lines, and/or human information; hypothesis driven with specific research question; and appropriately powered to obtain preliminary data. I received VICTR voucher funding in 2015 as the principal investigator to examine the role of myeloid cell recognition of TLR agonists and subsequent NFκB activation. *We have successfully utilized VICTR as a resource for the funding of preliminary data in experiments utilizing human cells. VICTR vouchers may be used to acquire additional pilot funds to bridge any discoveries made in a mouse model with this research proposal into human cells or samples.*

E. Vanderbilt Program in Molecular Medicine (VPMM)

VPMM is a unique training program available to graduate students and postdoctoral fellows in the Biomedical Sciences at Vanderbilt University, allowing for integration of thesis work with relevant clinical experiences. This program provides a supervised experience in clinical and translational research, including didactic and experiential courses, seminars and individual experiences conducted under the guidance of a clinical mentor and basic science mentor. My application into this program was accepted in 2015, when I was paired with a clinical mentor, Dr. Isaac Thomsen, Assistant Professor and Physician in Pediatric Infectious Diseases (see letter of reference). The VPMM has provided me with real-world observations of patients with severe musculoskeletal infections under supervision in clinic by Dr. Thomsen and observation of orthopedic surgical procedures with Dr. Jonathon Schoenecker at the Monroe Carell Jr. Children's Hospital at Vanderbilt. I have used these experiences to reflect back on my basic science research on osteomyelitis in the Cassat lab. *VPMM has offered me opportunities and training to connect my work as a basic scientist to clinically relevant patient cases at Vanderbilt. My passion for scientific discovery lies in translational research, and this perspective will remain valuable during the research pursuits as outlined in my research strategy.*

F. Additional Core Services

Although I do not anticipate using other Core services for the studies outlined in this application, Vanderbilt is equipped with many additional core facilities. A complete listing can be found at the following link: http://www.vanderbilt.edu/oor/cores/

EQUIPMENT

Cassat Laboratory (1035 MRB IV)

The Cassat laboratory houses all of the necessary equipment to study the molecular biology of microbial pathogens and skeletal cell biology. The equipment that may be utilized in this proposal includes the following: a Thermo Sorvall Lynx 6000 superspeed centrifuge with general purpose and ultraspeed rotors, Thermo Sorvall Legend XTR benchtop centrifuge with 2 rotors; Thermo Legend Micro21 and 21R benchtop microcentrifuges, Locator Jr. Plus Cryo Vessel for liquid nitrogen cell storage, Nuaire Class II Type A2 Biosafety Cabinets (2), Olympus inverted microscope CKX53 with QImaging OQCLR5 digital camera, an AquaSolutions Water Purification System, Eppendorf Nexus Mastercycler Gradient Thermocycler, Mettler Toledo Excellence balance, Thermo Forma series CO_2 incubators (2), New Brunswick Innova Model 44 stackable incubator shaker, Thermo MaxQ4450 tabletop shaking incubator, Fisher Isotemp General Microbiologic Incubators (3), Isotemp bath incubators (3), variable speed rotating shakers (3), hypoxia chambers with ProOx Oxygen controller modules (3) and $ProC0_2$ Carbon Dioxide controller modules (2), a BioTek Hybrid Synergy microplate reader, GeneSys 10S UV-VIS spectrophotometer, Next Advance Bullet Blender for bone homogenization, UVP GelDoc-It^2 Gel Documentation system, Thermo TSU series 600 -80°C freezer, Thermo IsoTemp -20°C freezer, Thermo MR49PA 4°C refrigerator, Mettler Toledo S220 pH meter, protein purification columns and reagents, Thermo Scientific EC 300 XL power supply for gel electrophoresis, BioRad polyacrylamide gel casting equipment and Western transfer apparatus, and a BioRad GenePulser Xcell electroporation apparatus.

Vanderbilt University Institute of Imaging Sciences (VUIIS)

The Center for Small Animal Imaging (CSAI) in the VUIIS contains advanced biomedical imaging instruments spanning a wide range of modalities, including MRI, CT, PET, SPECT, ultrasound, bioluminescence (BLI), fluorescence, and optical imaging. Equipment includes a 4.7T Varian MRI, 9.4T Varian MRI, 15.2T Bruker Biospec MRI, Xenogen IVIS 200 bioluminescent and fluorescent imaging system, Scanco μCT40 and μCT50 microCT scanners for *ex vivo* imaging, Siemens MicroCAT II X-ray microCT and Scanco VivaCT for *in vivo* imaging, 400MHz vertical Bruker Avance III spectrometer for small molecule NMR, Siemens MicroPET Focus 220, Bioscan NanoSPECT SPECT/CT, CRI Maestro optical imaging systems for *in vivo* fluorescence, Visen FMT for quantitative optical tomography, and a VisualSonics high-resolution ultrasound system.

Vanderbilt Center for Bone Biology (VCBB) (1215 MRB IV, Olin Hall)

Shared laboratory space in the VCBB is located two floors above the Cassat laboratory and available for use by our team. The VCBB shared laboratoryincludes the following equipment, General: 4°C refrigerators, -20°C and -80°C freezers, analytical balances, pH meters, laminar-flow hoods, mammalian and bacterial incubators/shakers, gamma-counters, hybridization ovens, ultraturax homogenizer, Bio-Rad DNA gel visualization and documentation system, Beckman and IEC centrifuges, cell culture hoods and incubators, automated Bio-Rad cell counter, luminometer, spectrophotometers, a BioTek plate reader, sonicators (2), FPLC instruments, and cryotanks. Molecular biology: Real-time qPCR instruments (2: ABI and Bio-Rad), Bio-Rad thermal cyclers (2), a Bio-Rad CF x 96 qRT-PCR thermal cycler, DNA and protein gel migration apparatus. Bone histomorphometry: Equipment for soft and calcified tissue histology, a dissecting microscope, an automated paraffin processor, a paraffin embedding station, an explosion proof -20°C freezer for MMA polymerization, a Wehmer plastic embedding grinder, Leica RM2255 microtome (3), analytical balances, pH meters, water baths, chemical hoods dedicated for tissue staining (2), an upright Olympus microscope with fluorescence, an inverted Olympus microscope, and a high-resolution Olympus DP70 camera connected to a computer with BIOQUANT and OsteoMeasure histomorphometry software. Shared laboratory space for VCBB also extends into the Olin Engineering Hall, which is located just across 25th street. This space may be used for alternative approaches necessitating the lyophilization of proteins using the Labconco FreeZone 4.5 benchtop freeze dry system and the loading of compounds into polymer scaffolds with the SeedMixer DAC 150 FVZ.

In sum, access to equipment within our laboratory, the VUIIS, and VCBB will ensure that I am uniquely positioned to study fundamental processes in skeletal cell biology and host-pathogen interactions.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: **First Name*:** Nicole **Middle Name** E **Last Name*:** Putnam **Suffix:**

Position/Title*: Grad Stu Research Asst Monthly
Organization Name*: Vanderbilt University
Department: Pathology and Microbiology Ed
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*:
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number* [redacted] **Fax Number:**

E-Mail* [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI **Other Project Role Category:**

Degree Type: **Degree Year:**

Attach Biographical Sketch*: **File Name:** ID-0122383_BN-1_BIOSKETCH.pdf

Attach Current & Pending Support: File Name:

Funding Opportunity Number: PA-16-309 . Received Date: 2016-12-08T15:14:00.000-05:00

PROFILE - Senior/Key Person

Prefix: **First Name*:** James **Middle Name** E **Last Name*:** Cassat **Suffix:**

Position/Title*: Asst Professor
Organization Name*: Vanderbilt University Medical Center
Department: Pediatrics/Infectious Disease
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*:
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number* [redacted] **Fax Numbe** [redacted]

E-Mail* [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) **Other Project Role Category:** Sponsor

Degree Type: Doctor of Philosophy **Degree Year:** 2006

Attach Biographical Sketch*: **File Name:** ID-0081478_BN-2_BIOSKETCH.pdf

Attach Current & Pending Support: **File Name:**

PROFILE - Senior/Key Person

Prefix: **First Name*:** Julie **Middle Name** A **Last Name*:** Sterling **Suffix:**

Position/Title*: Asst Professor
Organization Name*: Vanderbilt University Medical Center
Department: Medicine/Clinical Pharmacology
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*:
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number* [redacted] **Fax Numbe** [redacted]

E-Mail* [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) **Other Project Role Category:** Co-Sponsor

Degree Type: Doctor of Philosophy **Degree Year:** 2003

Attach Biographical Sketch*: **File Name:** ID-0077342_BN-1_BIOSKETCH.pdf

Attach Current & Pending Support: **File Name:**

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

APPLICANT BIOGRAPHICAL SKETCH

NAME OF APPLICANT: **Nicole Putnam**

eRA COMMONS USER NAME: [redacted]

POSITION TITLE: **Ph.D. candidate**

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	START DATE	END DATE *(or expected end date)*	FIELD OF STUDY
University of Wisconsin-La Crosse La Crosse, WI	B.S.	08/2006	12/2010	Biochemistry, Psychology
Johns Hopkins Bloomberg School of Public Health Baltimore, MD	M.S.	08/2012	06/2014	Molecular Microbiology and Immunology
Vanderbilt University School of Medicine Nashville, TN	Ph.D.	08/2014	05/2019	Microbiology and Immunology

A. Personal Statement

My research goals are focused on studying infectious diseases that have a drastic public health burden. I am seeking a Ph.D. in Microbiology and Immunology with the goal of establishing my own laboratory as an independent translational research scientist. Specifically, I hope to investigate critical immune responses and biological changes induced by pathogens, and contribute to how this knowledge can be leveraged to alleviate the morbidity and mortality associated with infection.

My undergraduate studies provided a strong foundation in biology and chemistry, and I was able to gain additional experience in translational research through an industry internship at Covidien, where I explored the development and application of cancer therapeutics. After identifying microbiology as an area of particular interest, I completed a Master of Science in Molecular Microbiology and Immunology at the Johns Hopkins Bloomberg School of Public Health. My Master's degree thesis work involved the study of immune responses to measles virus in rhesus macaques in the laboratory of Dr. Diane Griffin, a world-renowned virologist. During this time, I developed expertise in immunologic techniques and experimental design, the critical review of primary literature, and scientific communication. These skills allowed me to be extremely productive in my four laboratory rotations as an incoming graduate student at Vanderbilt University. I chose to join Dr. Jim Cassat's laboratory with a focus on osteoimmunologic responses to bacterial pathogens, because of my ongoing interests in the host responses to human pathogens.

Under Dr. Cassat's guidance, I have become well-trained in microbiology and have developed a project that bridges multiple scientific disciplines with a long-term goal to determine the innate sensing capabilities of skeletal cells and how microbial pathogens impact bone remodeling. This project will guide my scientific training at the bench and professionally under the mentorship of Dr. Cassat and with the assistance of the exceptional resources available at Vanderbilt University, including the Vanderbilt Center for Bone Biology (VCBB), the Vanderbilt University Institute for Imaging Sciences (VUIIS), the Vanderbilt Program in Molecular Medicine (VPMM), and outstanding core facilities.

1. **Putnam NE**, Ford C, Wilde AD, Hendrix AS, Allaman M, Cassat JE. Mechanisms of inflammatory bone loss during *Staphylococcus aureus*-induced osteomyelitis. **Abstract:** ***Infection & Immunity Symposium***. 2016. Vanderbilt University, Nashville, TN.
2. **Putnam NE**, Hendrix AS, Cassat JE. The role of innate immune recognition during *S. aureus* osteomyelitis. **Abstract:** ***International Conference on Gram-Positive Pathogens***. 2016. Omaha, NE.

B. Positions and Honors

ACTIVITY/ OCCUPATION	START DATE	END DATE	FIELD	INSTITUTION/ COMPANY	SUPERVISOR/ EMPLOYER
Pharmaceutical Research and Development Intern	06/10	08/10	Organic chemistry, Biology	Covidien	Dr. Raghavan Rajagopalan

ACTIVITY/ OCCUPATION	START DATE	END DATE	FIELD	INSTITUTION/ COMPANY	SUPERVISOR/ EMPLOYER
M.S. Thesis Research	11/12	06/14	Immunology, Infectious disease	Johns Hopkins Bloomberg School of Public Health	Dr. Diane Griffin
Nutrition Teacher	05/13	05/14	Public Health: City Health Initiative	Baltimore City Health Department, Baltimore Medical System	Pam Brown (Baltimore Medical System)
Pre-doctoral student	08/14	Present	Microbiology and Immunology	Vanderbilt University	Dr. Jim Cassat

Academic and Professional Honors

2016 Travel Award, International Conference on Gram Positive Pathogens
2015-2016 Mini-Sabbatical Award from the Center for Microbial Pathogenesis, Vanderbilt University
2015-2016 Vanderbilt Institute for Clinical and Translational Research Voucher Award
2013-2014 MSCI Scholarship
2010 Best Poster Award, Intern Poster Symposium, Covidien
2009-2010 High Honor Award, Psi Chi International Honor Society, UW-La Crosse
2009-2010 Eta Phi Alpha Honors Fraternity, UW-La Crosse

Society Memberships

2014-present Microbial Defense Academic Society, Vanderbilt University
2014-present American Association for the Advancement of Science
2012-2014 American Society for Microbiology
2012-2014 American Chemical Society
2009-2010 Golden Key International Honour Society, UW-La Crosse
2008-2010 Psi Chi International Honor Society, UW-La Crosse

Activities

2016-present Organizing committee for the Southeastern Immunology Symposium 2017
2016 Microbes 101 Guest Lecture, School of Science and Math at Vanderbilt
2016 Essentials of Staphylococcal Genetics Workshop, University of Nebraska Medical Center
2015-present Vanderbilt Program in Molecular Medicine, Vanderbilt University
2015-2016 Microbial Defense Academic Society Officer, Vanderbilt University
2013-2014 Service Outreach Resource Center (SOURCE) Service Scholar, Johns Hopkins University
2009-2010 Laboratory Manager, Visual Sciences Lab with Drs. O'Brien and Van Voorhis, UW-La Crosse
2009 Tutor, Physiological Psychology, UW-La Crosse
2008-2009 Research Assistant, Visual Sciences Lab with Drs. O'Brien and Van Voorhis, UW-La Crosse
2008-2009 Undergraduate Chemistry Research with Dr. Aaron Monte, UW-La Crosse

Students Trained

2015-2016 Caleb Ford (MSTP), Lauren Williamson (IGP), Michael Yarboro (IGP)
2016-present Clare Laut (IGP)

C. Contributions to Science

I. Investigation of bacterial hypoxic responses in the context of invasive infection

As a Ph.D, student in Dr. Jim Cassat's laboratory, my previous expertise in cell culture allowed me to conduct cytotoxicity analyses in human and murine primary cells and cell culture lines. For our *PLoS Pathogens* manuscript in 2015, I provided data showing that decreased oxygen levels during bacterial growth leads to the increased production of bacterial toxins, triggering a dose-dependent increase in cell death among eight cell types. This work was also highlighted in oral presentations at the 2015 *Gordon Research Conference on Staphylococcal Diseases* and the 2016 *Gordon Research Seminar on Microbial Toxins and Pathogenicity*. From these studies and a subsequent publication in *Antimicrobial Agents and Chemotherapy* from the Cassat lab in 2016, I provided assistance with osteomyelitis surgeries, animal monitoring and husbandry, and processing of femurs for CFU enumeration and microCT analysis. These skills obtained in my PhD laboratory have continued to be useful for the development of my research project, as outlined in the Research Strategy.

1. Wilde AD, Snyder DJ, **Putnam NE**, Valentino MD, Hammer ND, Lonergan ZR, Hinger SA, Aysanoa EA, Blanchard C, Dunman PM, Wasserman GA, Chen J, Shopsin B, Gilmore MS, Skaar EP, Cassat JE. Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. 2015. ***PLoS Pathogens***. PMID: 26684646
2. Wilde A, Valentino M, Hammer N, **Putnam N**, Lonergan Z, Hinger S, Perlmutter J, Aysanoa EA, Snyder D, Gilmore MS, Skaar EP, Cassat JE. Transposon sequencing analysis of a murine osteomyelitis model reveals hypoxic responses as key components of the Staphylococcal-host interaction. 2015. **Abstract: *Gordon Research Conference on Staphylococcal Diseases***. Lucca, Italy.
3. Wilde AD, Snyder DJ, **Putnam NE**, Valentino MD, Hammer ND, Lonergan ZR, Hinger SA, Aysanoa EA, Blanchard C, Dunman PM, Wasserman GA, Chen J, Shopsin B, Gilmore MS, Skaar EP, Cassat JE. Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. 2016. **Abstract: *Gordon Research Seminar on Microbial Toxins and Pathogenicity***, Waterville Valley, NH.
4. Hendrix AS, Spoonmore TJ, Wilde AD, **Putnam NE**, Hammer ND, Snyder DJ, Guelcher SA, Skaar EP, Cassat JE. Repurposing the nonsteroidal anti-inflammatory drug diflunisal as an osteoprotective, anti-virulence therapy for *Staphylococcus aureus* osteomyelitis. 2016. ***Antimicrobial Agents and Chemotherapy***. PMID: 27324764

II. Elucidation of adaptive immune responses during measles virus infection

At the Johns Hopkins Bloomberg School of Public Health, I had the opportunity to work with an internationally recognized expert in virology, Dr. Diane Griffin. In my time in the Griffin lab, I completed a long-term measles infection study in rhesus macaques. In this study we had two major aims: (1) characterization of Th17 and Tc17 responses and (2) monitoring of viral RNA persistence. These aims were meant to explore mechanisms that may lead to the extended immunosuppression following measles virus infection.

To define the immune responses to measles virus in non-human primates, we processed and analyzed PBMCs, bone marrow-derived mononuclear cells, and lymph node specimens. Interestingly, we found that after an early lymphopenia, the adaptive immune response to measles virus is elevated in three distinct phases over the course of six months. We measured the measles virus-specific T cells, including CD4 helper T cells producing IFN-γ (Th1) and IL-17 (Th17), and their cytotoxic CD8 effector counterparts (Tc1, Tc17). Additionally, the discovery of persistent viral RNA over the course of infection implies that after clearance of infectious virus, persistent viral RNA is recognized leading to T cell activation.

I submitted a first-authored manuscript titled “Prolonged multiphasic Th17 and Tc17 responses during measles virus infection and RNA clearance” describing my thesis work to *The Journal of Infectious Diseases* in the fall of 2014. The manuscript is currently under revision to include Th1 and Tc1 data. Additionally, my data on the Th17 and Tc17 responses to measles virus infection was presented at the 2015 *Negative Strand Virus Meeting* held in Siena, Italy. Overall, these scientific pursuits have prepared me with the immunological expertise and assay knowledge to transfer these skills into a murine model of bacterial infection, and will allow me to expand on any distinctive immunological findings during this research proposal.

1. Griffin DE, **Putnam NE**, Nelson A, Hauer D, Baxter V, Adams RJ. Prolonged multiphasic Th17 and Tc17 responses during measles virus infection and RNA clearance. 2015. **Abstract: *16th Annual Negative Strand Virus meeting***. Siena, Italy.
2. [redacted]

III. Analysis of regulatory T cells and immune tolerance in autoimmunity

As a rotation student in Dr. Dan Moore’s lab, I was able to optimize an assay that was a major hurdle for his team. Specifically, I worked on developing an *ex vivo* assay for functionally active T regulatory cells. I successfully optimized this assay and obtained consistent results showing suppression of T cell proliferation by T regulatory cells.

I also worked closely with a graduate student in the Moore lab to examine the role of immune tolerance induction in systemic lupus erythematosus (SLE) mice and the role of CD8 T regulatory cells in Type 1 Diabetes in non-obese diabetic (NOD) mice. I assisted with *in vivo* and *ex vivo* experiments, and acquired and analyzed data using flow cytometry. From this project, I am listed as middle author on a 2015 paper in the *American Journal of Transplantation*. The Moore lab provided skillful instruction to begin working with murine models, exposure into designing creative experiments using cell transfer between genetically modified mice, and allowed me to develop familiarity with handling mice and harvesting immune cells.

1. Stocks BT, Wilhelm AJ, Wilson CS, Marshall AF, **Putnam NE**, Major AS, Moore DJ. Lupus-prone mice resist immune regulation and transplant tolerance induction. 2015. ***American Journal of Transplantation***. PMID: 26372909.

IV. Pharmaceutical research and development internship: Development of cancer therapeutics

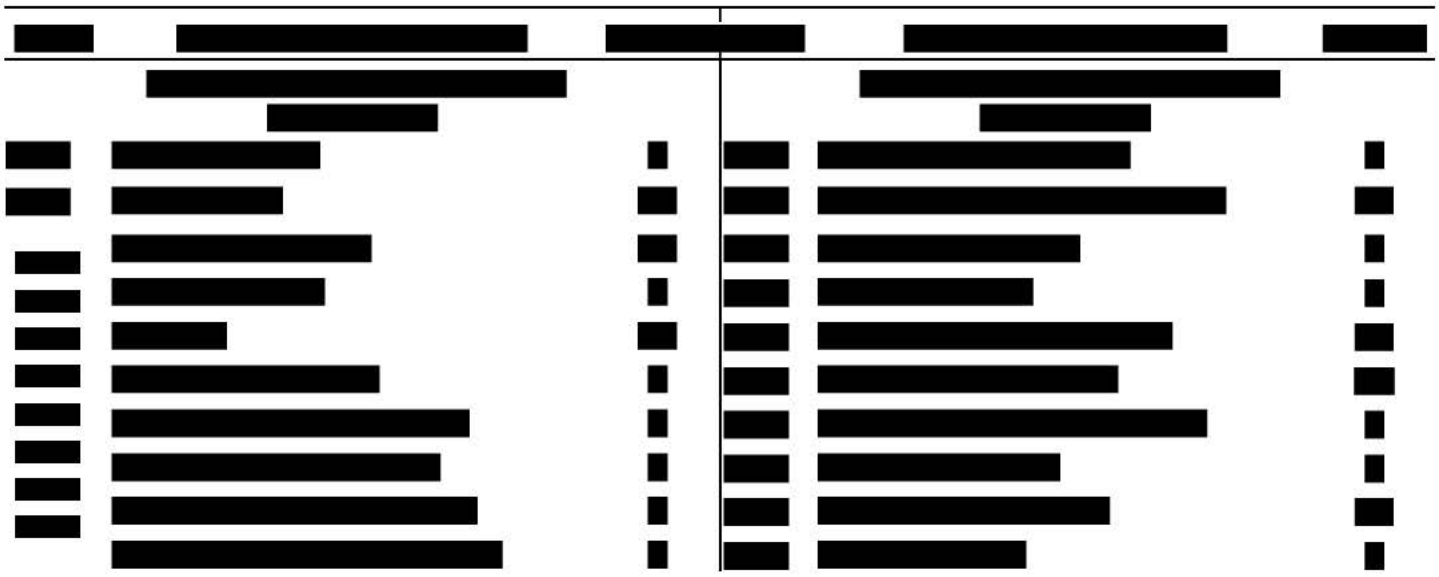
My internship focused on applying the principles of phototherapy to develop cancer therapeutics that target and destroy ovarian and colon cancer cells. Photosensitive compounds are activated by a characteristic wavelength, causing oxygen radicals to form and induce cancer cell death. My project was divided into three parts: (1) synthesis of new photosensitizer bearing a diaza (N-N) bond through organic synthesis and evaluation of free radical formation using electron spin resonance (ESR), (2) conjugation of photosensitizers to bioactive carriers, and (3) *in vitro* cell binding and cell viability assays with the photosensitizers and conjugates.

I successfully generated a new photosensitive compound and confirmed the capacity for radical formation by this and other compounds generated in the laboratory. I accomplished delivery of photosensitizers to cancer cells through conjugation to specific cancer-targeting molecules, followed by selective targeting and internalization confirmation. Finally, I conducted cytotoxicity assays to demonstrate efficacy of photosensitizers *in vitro*. I went on to present my work at the Covidien 2010 Intern Poster Symposium and won the Best Poster Award.

My work on folate receptor targeting and internalization in ovarian cancer was shared in 2011 at the SPiE BiOS: Biomedical Optics symposium. Additionally, my evaluation of compounds for radical formation using ESR was published in *Photodiagnosis and Photodynamic Therapy* and presented at the 13th World Congress of the International Photodynamic Association. My research experiences at Covidien allowed me to directly follow chemical synthesis of each compound with the evaluation of biological effects, which instilled the importance of multidisciplinary research early in my scientific career.

1. **Putnam, N,** Rajagopalan, R, Karwa, A, Nickols, M, and L Chinen. (2010). Targeted photosensitizer bioconjugates for cancer phototherapy. **Covidien Intern Poster Symposium**, Covidien, St. Louis, MO.
2. Rajagopalan R, Poreddy AR, Karwa A, Asmelash B, **Putnam NE**, Chinen L, Nickols M, Shieh JJ, Dorshow RB. Folate receptor targeted Type 1 photosensitizer bioconjugates for tumor visualization and phototherapy. 2011. **Abstract: *Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy XX***, within the SPIE BiOS: Biomedical Optics Symposium. San Francisco, CA.
3. Rajagopalan R, Lin T, Karwa A, Poreddy A, Asmelash B, **Putnam N**, Lin D, Dorshow R. Discovery and development of novel thiaza and thioxa Type 1 photosensitizers. 2011. ***Photodiagnosis and photodynamic therapy.***
4. Rajagopalan, R, Lin, T, Karwa, A, Poreddy, A, Asmelash, B, **Putnam, N**, Lin, D, R Dorshow. Discovery and development of novel thiaza and thioxa type 1 photosensitizers. 2011. **Abstract: *13th World Congress of the International Photodynamic Association***. Innsbruck, Austria.

D. Scholastic Performance



BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Cassat, James Edward	Assistant Professor of Pediatrics and Pathology, Microbiology, and Immunology
eRA COMMONS USER NAME [redacted]	

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	MM/YY	FIELD OF STUDY
University of Arkansas, Fayetteville, AR	B.S.	1996-2000	Microbiology
University of Arkansas for Medical Sciences, Little Rock, AR	M.D.	2000-2008	Medicine
University of Arkansas for Medical Sciences, Little Rock, AR	Ph.D.	2002-2006	Microbiology and Immunology
Vanderbilt University Medical Center, Nashville, TN	Residency	2008-2010	Pediatrics
Vanderbilt University Medical Center, Nashville, TN	Fellowship	2010-2014	Pediatric Infectious Diseases

A. Personal Statement

A major focus of our research is to understand the interaction between host and pathogen during invasive bacterial infections. Specifically, we seek to define the bacterial virulence mechanisms and host defenses employed during osteomyelitis, an invasive infection of bone that occurs commonly in children. Risk factors and protective immune responses for pediatric osteomyelitis are poorly defined, and bone infections are notoriously recalcitrant to antimicrobial therapy due to both pathogen-induced bone destruction and the emergence of multi-drug resistant pathogens. Therefore, we have created new tools to investigate the host-pathogen interface during osteomyelitis. As *Staphylococcus aureus* is by far the most common cause of pediatric and adult osteomyelitis, we have focused our efforts on identifying the mechanisms by which *S. aureus* causes bone infection and resulting bone destruction, as well as the host defenses that protect against staphylococcal infection. Using a combination of bone cell culture models, proteomic analyses of bacterial virulence factors, and a new quantitative *in vivo* model of staphylococcal osteomyelitis, we have uncovered specific bacterial factors that contribute to the pathogenesis of osteomyelitis by triggering osteoblast cell death and bone destruction *in vivo*. We have also identified staphylococcal genes necessary for survival within the bone. By further defining the mechanisms by which *S. aureus* survives within bone and ultimately triggers bone destruction, we hope to identify new therapeutic targets to treat osteomyelitis and to limit the morbidity associated with this invasive pediatric infection. Moreover, by characterizing the host responses to bacterial pathogens in the bone, we seek to define protective correlates of innate immunity in bone and potentially uncover risk factors for the development of osteomyelitis in otherwise healthy children. Finally, by investigating how bacterial pathogens and members of the gut microbiota perturb bone cell physiology, we wish to enhance an understanding of changes in bone remodeling in the face of infectious and inflammatory insults. Collectively, this research environment draws together multiple scientific disciplines to explore how bacterial pathogens and resulting immune responses modulate skeletal homeostasis. This creates an ideal environment for Ms. Putnam to continue her exciting doctoral work on osteo-immunologic crosstalk. In the enclosed F31 proposal, Ms. Putnam will build upon her preliminary studies linking innate immunity to skeletal homeostasis and explore how innate sensing pathways in skeletal cells impact antibacterial host defenses and bone turnover.

1. Wilde AD, Snyder DJ, Putnam NE, Valentino MD, Hammer ND, Lonergan ZR, Hinger SA, Aysanoa EA, Blanchard C, Dunman PM, Wasserman GA, Chen J, Shopsin B, Gilmore MS, Skaar EP, **Cassat JE**. Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. ***PLoS Pathogens***. 2015***.*** December 18; 11(12): e1005341. PMID: 26684646

2. **Cassat JE**, Hammer ND, Campbell JP, Benson MA, Perrien DS, Mrak LN, Smeltzer MS, Torres VJ, and Skaar EP. A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. ***Cell Host and Microbe***. 2013. June 12; 13(6):759-72. PMCID: PMC3721972.

3. Hammer ND*, **Cassat JE***, Noto MJ, Lojek LJ, Chadha AD, Schmitz JE, Creech CB, and Skaar EP. Inter- and Intraspecies Metabolite Exchange Promotes Virulence of Antibiotic-Resistant *Staphylococcus aureus*. ***Cell Host and Microbe*** 2014**.** October 8;16(4):531-7. PMID: 25299336. PMCID: PMC4197139. ***Denotes equal authorship.***

4. Hendrix AS, Spoonmore TJ, Wilde AD, Putnam NE, Hammer ND, Snyder DJ, Guelcher SA, Skaar EP, **Cassat JE**. Repurposing the nonsteroidal anti-inflammatory drug diflunisal as an osteoprotective, anti-virulence therapy for *Staphylococcus aureus* osteomyelitis. ***Antimicrobial Agents and Chemotherapy***. 2016. Epub ahead of print. PMID: 27324764

B. Positions and Honors

Positions

2008-2010	Resident in Pediatrics, Vanderbilt Children's Hospital, Nashville, TN. American Board of Pediatrics Accelerated Research Pathway. State of Tennessee Medical License #47215. Board Certification in Pediatrics, October 2011
2010-2014	Clinical Fellow, Pediatric Infectious Diseases, Vanderbilt Children's Hospital, Nashville, TN
2014-present	Assistant Professor, Department of Pediatrics, Division of Pediatric Infectious Diseases, Vanderbilt Children's Hospital, Nashville, TN

Other Research Experience

1997-2000	Departmental Honors in Research Program, University of Arkansas PI: Dr. D. Mack Ivey
1999	C. Roy Adair Fellowship in Plant Pathology Research, University of Arkansas PI: Dr. Yinong Yang
2001	Tennenbaum Fellowship in Neuroscience and Neurosurgery Supervisor: Dr. Scott Schlesinger

Honors

1999	William M. Harris Memorial Premedical Scholarship
1999	Allan A. Gilbert Premedical Scholarship
1999	George T. Johnson Award for Outstanding Student in Microbiology
2000	Phi Beta Kappa National Honor Society
2000	B.S. awarded *Summa cum laude* with Honors in Research
2000	University of Arkansas Senior Scholar
2000	American Medical Association Foundation Scholarship
2001, 2002	Barton Scholarship
2002	Outstanding Medical Student in Pathology Award
2004	American Heart Association Predoctoral Fellowship
2006-2007	University of Arkansas for Medical Sciences M.D./Ph.D. Scholarship
2007	Alpha Omega Alpha Honor Medical Society
2008	Vanderbilt Garrod Society / Pediatric Scientist Training Program
2010	David Karzon, M.D. Award (Resident Research Award)
2011-2017	NIH Loan Repayment Award for Pediatric Research
2013, 2014	Vanderbilt University Medical Center Research Forum Best Basic Science Poster
2015	Microbiology and Immunology Graduate Program Faculty Teaching Award

Professional Memberships

2008-present	American Academy of Pediatrics
2010-present	Pediatric Infectious Disease Society
2010-present	Infectious Disease Society of America

C. Contribution to Science

1. Osteomyelitis is one of the most common invasive bacterial infections in children. Yet, the immune responses that either protect from, or contribute to, the pathogenesis of osteomyelitis are poorly understood. Moreover, there is a paucity of information regarding how bacterial pathogens successfully colonize and destroy the bone. In order to better characterize host-pathogen interactions during osteomyelitis, we created a new murine model of *S. aureus* osteomyelitis. We adopted high-resolution, three-dimensional micro-computed tomographic imaging to allow for precise quantification of changes in bone remodeling triggered by pathogen and host during osteomyelitis. Additionally, we devised strategies to effectively enumerate bacterial burdens in infected murine bones so that the impact of individual virulence factors or host pathways could be accurately determined. Using these powerful tools, we discovered regulatory loci that are critical for staphylococcal survival in bone, and identified staphylococcal toxins that destroy osteoblasts and osteoclast precursors to contribute to osteomyelitis. These findings enhanced our understanding of osteomyelitis pathogenesis and identified new targets for the design of vaccines and therapeutics against invasive staphylococcal infection. In order to translate some of our research discoveries into potential new treatments for osteomyelitis, we began a collaboration with faculty in the School of Engineering to develop drug-eluting, bioresorbable scaffolds for targeted delivery of antimicrobial, anti-virulence, and immunomodulatory compounds to bone. We demonstrated the efficacy of such an approach in a recent study evaluating the FDA-approved NSAID diflunisal as an anti-virulence, osteoprotective agent for staphylococcal osteomyelitis.

a. **Cassat JE**, Hammer ND, Campbell JP, Benson MA, Perrien DS, Mrak LN, Smeltzer MS, Torres VJ, and Skaar EP. A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. ***Cell Host and Microbe***. 2013. June 12; 13(6):759-72. PMCID: PMC3721972. ***Recommended by Faculty of 1000. Accompanying perspective in Cell Host and Microbe, June 2013***

b. Hendrix AS, Spoonmore TJ, Wilde AD, Putnam NE, Hammer ND, Snyder DJ, Guelcher SA, Skaar EP, **Cassat JE**. Repurposing the nonsteroidal anti-inflammatory drug diflunisal as an osteoprotective, anti-virulence therapy for *Staphylococcus aureus* osteomyelitis. ***Antimicrobial Agents and Chemotherapy***. 2016. Epub ahead of print. PMID: 27324764

c. **Cassat JE**, Skaar EP. Recent Advances in Experimental Models of Osteomyelitis. ***Expert Rev Anti Infect Ther***. 2013 Dec;11(12):1263-5. PMID: 24215241

d. Loughran AJ, Gaddy D, Beenken KE, Meeker DG, Morello R, Zhao H, Byrum SD, Tackett AJ, **Cassat JE**, Smeltzer MS. Impact of *sarA* and phenol-soluble modulins in the pathogenesis of osteomyelitis in diverse clinical isolates of *Staphylococcus aureus*. ***Infection and Immunity.*** 2016 Aug 19; 84(9):2586-94. PMCID: PMC4995912

2. Antimicrobial-resistant pathogens are a major source of morbidity and mortality worldwide. However, the adaptations that enable antimicrobial resistance often pose fitness costs to microorganisms. Resistant pathogens must therefore overcome such fitness decreases to persist within their hosts. Working closely with a colleague in the Skaar laboratory, I discovered that the reduced fitness associated with one resistance-conferring mutation can be offset by community interactions with microorganisms harboring alternative mutations or via interactions with the human microbiota. These fitness enhancements occur through the exchange of metabolites between distinct mutants, leading to enhanced growth, virulence factor production, and pathogenicity in my murine model of osteomyelitis. These findings have profound implications for the study of bacterial pathogenesis and host-pathogen interactions, as well as for the treatment of invasive infection, in that they demonstrate the ability of fitness-impaired pathogens to remain virulent in hosts through community interactions.

a. Hammer ND*, **Cassat JE***, Noto MJ, Lojek LJ, Chadha AD, Schmitz JE, Creech CB, and Skaar EP. Inter- and Intraspecies Metabolite Exchange Promotes Virulence of Antibiotic-Resistant *Staphylococcus aureus*. ***Cell Host and Microbe*** 2014**.** October 8;16(4):531-7. PMID: 25299336 ***Denotes equal authorship. Recommended by Faculty of 1000. Accompanying preview in Cell Host and Microbe, October 2014***

3. The precise *S. aureus* virulence factors required to initiate and sustain osteomyelitis are poorly understood. Previous attempts to define staphylococcal factors that contribute to invasive infection have largely focused on known virulence regulators or toxins. In order to define the *S. aureus* genes essential for colonization and survival in bone, we conducted an unbiased, genome-wide, *in vivo* screen using a technique known as transposon sequencing (TnSeq). TnSeq involves subjecting large pools of bacterial mutants to a particular stress or *in vivo* environment, and measuring the fitness of all non-essential genes in the genome by next-generation sequencing before and after the particular stressor. My lab was the first to successfully employ TnSeq analysis in an animal model of osteomyelitis, and in doing so we identified over 200 *S. aureus* genes that are essential for survival in bone, but not for growth *in vitro*. The majority of these genes had not been previously determined to be important for staphylococcal virulence. Our TnSeq analysis also led to the exciting discovery that oxygen tensions in host tissues profoundly influence the virulence of *S. aureus*.

a. Wilde AD, Snyder DJ, Putnam NE, Valentino MD, Hammer ND, Lonergan ZR, Hinger SA, Aysanoa EA, Blanchard C, Dunman PM, Wasserman GA, Chen J, Shopsin B, Gilmore MS, Skaar EP, **Cassat JE**. Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. ***PLoS Pathogens***. 2015**.** December 18; 11(12): e1005341. PMID: 26684646

4. During my doctoral studies in Mark Smeltzer's laboratory, I sought to determine the genomic and transcriptional differences between commonly studied laboratory strains of *S. aureus*, and more virulent clinical isolates of *S. aureus* obtained from patients suffering from invasive infection. These studies revealed remarkable differences in the transcriptional profiles, virulence, and the potential for biofilm formation between laboratory strains and clinical isolates. Moreover, by comparing laboratory strains with isolates taken from patients suffering from osteomyelitis or septic arthritis, we identified key genomic attributes of virulent staphylococcal strains. These studies prompted a fundamental shift in the field of staphylococcal pathogenesis to studying clinical isolates, and prompted a re-evaluation of the regulatory networks controlling virulence factor production and biofilm formation in *S. aureus*.

a. **Cassat JE,** Dunman PM, McAleese F, Murphy E, Projan SJ, Smeltzer MS**.** Comparative genomics of *Staphylococcus aureus* musculoskeletal isolates. ***Journal of Bacteriology***. 2005, Jan; 187(2):576-592. PMCID: PMC543526

b. **Cassat JE,** Dunman PM, Murphy E, Projan SJ, Beenken KE, Palm KJ, Yang SJ, Rice KC, Bayles KW, Smeltzer MS**.** Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. ***Microbiology***. 2006, Oct; 152(10):3075-3090. PMID: 17005987

c. Rice KC, Mann EE, Endres JL, Weiss EC, **Cassat JE**, Smeltzer MS, Bayles KW. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. ***Proceedings of the National Academy of Sciences of the United States of America***. 2007, May; 104(19):8113-8118. PMCID: PMC1876580

d. Tsang LH*****, **Cassat JE***, Shaw LN, Beenken KE, Smeltzer MS. Factors contributing to the biofilm-deficient phenotype of *Staphylococcus aureus sarA* mutants. ***PLoS One***. 2008, Oct; 3(10): e3361. PMCID: PMC2556392 ****Denotes equal authorship***

5. During my clinical fellowship, I worked in the laboratory of Dr. Eric Skaar. In addition to my main project aimed at creating new tools for the study of staphylococcal osteomyelitis and osteoimmunology, Dr. Skaar and I led a project aimed at developing a new multi-modality imaging analysis of host-pathogen interactions during invasive infection. In order to identify both bacterial and host proteins at the host pathogen interface, we used a

combination of magnetic resonance imaging, imaging mass spectrometry, blockface histology, and bioluminescence in a murine model of invasive staphylococcal infection. Because a major focus of the Skaar lab is understanding how bacteria obtain essential metals from the host, and how the host sequesters these metals, we also performed elemental imaging with inductively-coupled plasma mass spectrometry, and co-registered all five imaging modalities together to create an unprecedented three-dimensional map of the host-pathogen interface in infected tissues. We anticipate that this approach will identify new host and bacterial proteins that protect from, or contribute to, invasive infection. A manuscript describing our technique and findings is currently in preparation. Additionally, we previously used a subset of these techniques to identify host proteins in staphylococcal abscesses, as described in the manuscripts below.

a. Attia AA*, **Cassat JE***, Aranmolate O, Zimmerman LJ, Boyd KL, and Skaar EP. Analysis of the *Staphylococcus aureus* abscess proteome identifies antimicrobial host proteins and bacterial stress responses at the host-pathogen interface. ***Pathogens and Disease***. July 2013. PMCID: PMC3877740
****Denotes equal authorship***

b. **Cassat JE**, Skaar EP. Metal ion acquisition in *Staphylococcus aureus*: overcoming nutritional immunity. ***Seminars in Immunopathology***. 2012. Mar;34(2):215-35. PMCID: PMC3796439.

c. **Cassat JE**, Skaar EP. Iron in infection and immunity. ***Cell Host and Microbe***. 2013. May 15; 13(5):509-19. PMCID: PMC3676888. ***Recommended by Faculty of 1000***

Complete list of published work in MyNCBI (20 publications total, including 12 in the last 3 years): https://www.ncbi.nlm.nih.gov/sites/myncbi/james.cassat.1/bibliography/44325575/public/?sort=date&direction=descending

D. Research Support

Ongoing Research Support

1 K08 AI113107-01 **Cassat (PI)** **07/01/2014-06/30/2019**
NIH/NIAID
Host Pathogen Interactions During Osteomyelitis

The Specific Aims of this project are to (i) investigate the mechanisms by which *S. aureus* exoproteins perturb osteoblast physiology, (ii) define host responses that impact bone remodeling and bacterial clearance during osteomyelitis, focusing specifically on the role of pattern recognition receptors, and (iii) to identify *S. aureus* factors required for survival within bone by transposon sequencing. We have completed Aim 3, which provides the preliminary data for this R01 application. The remaining Aims have no overlap with this proposal.

Role: PI

[redacted]

Pending Research Support

[redacted]

OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

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

NAME: Julie A. Sterling

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

POSITION TITLE: Assistant Professor

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
Bowling Green State University, Bowling Green OH	BS	1994-1998	Microbiology
University of Toledo, Medical Branch (formerly Medical College of Ohio), Toledo OH	PhD	1998-2003	Molecular and Cellular Biology
University of Texas Health Science Center at San Antonio, San Antonio TX	Postdoctoral Fellow	2003-2006	Bone Biology and Cancer Metastasis to Bone
Vanderbilt Medical Center, Nashville TN	Postdoctoral Fellow	2006-2008	Cancer Metastasis to Bone

NOTE: The Biographical Sketch may not exceed five pages. Follow the formats and instructions below.

A. Personal Statement

My research focuses on studying the interactions between tumors and the bone microenvironment and the molecular mechanisms that drive tumor-induced bone disease with the goal of developing better therapeutics for inhibiting tumor-induced bone disease. In order to explore many different aspects of the tumor micro-environment we blend molecular biology, engineering, and pre-clinical approaches to better understand tumor behavior in bone. Currently, a major focus of my group involves studying how the physical and cellular components of the bone microenvironment alter tumor cell gene expression and signaling. For these studies, in collaboration with Scott Guelcher's laboratory in Chemical and Biomolecular Engineering at Vanderbilt, we have developed 3D scaffolds that mimic the bone microenvironment. These studies have further refined our understanding of signaling pathways that regulate tumor-induced bone disease, and we are currently exploring targeting approaches to deliver therapeutics against proteins overexpressed when tumors metastasize to bone. I am pleased to support this research proposal by Nicole Putnam. Many parallels can be drawn between my research on tumor-induced bone disease and the proposed experimental plan to define how bacterial recognition by skeletal cells leads to altered bone remodeling. As a co-sponsor for this application, I am happy to lend expertise on cellular signaling and quantification of bone destruction.

My 4 most relevant publications to this grant include:

1. **Sterling JA**, Oyajobi BO, Grubbs BG, Padalecki SS, Gupta A, Story B, S Munoz, Zhao M, and Mundy GR. The Hedgehog Signaling Molecule Gli2 Induces PTHrP Expression and Osteolysis In Metastatic Human Breast Cancer Cells. Cancer Research 66 (2006).
2. Johnson RW, Nguyen MP, Padalecki SS, Grubbs BG, Merkel AR, Oyajobi BO, Matrisian LM, Mundy GR, **Sterling JA**. TGF-β promotion of Gli2 induced PTHrP expression is independent of canonical Hedgehog signaling. Cancer Research 71 (2011). PMCID: PMC3077118.
3. Guo R, Lu S, Page JM, Merkel AR, Basu S, **Sterling JA**, Guelcher SA. "Fabrication of 3D Scaffolds with Precisely Controlled Substrate Modulus and Pore Size by Templated-Fused Deposition Modeling to Direct Osteogenic Differentiation." Adv Healthc Mater. (2015). PMCID: PMC4558627.
4. Page JM, Merkel AR, Ruppender NS, Guo R, Dadwal UC, Cannonier SA, Basu S, Guelcher SA, **Sterling JA**. "Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II." *Biomaterials. (2015).*

B. Positions and Honors

Positions and Employment

2003-2006 Postdoctoral Fellow, Department of Cellular & Structural Biology, UTHSC, San Antonio, TX. Mentor: Gregory Mundy, MD.
2006-2008 Postdoctoral Fellow, Department of Medicine, Clinical Pharmacology, Center for Bone Biology, Vanderbilt University Medical Center. Mentor: Gregory Mundy, MD
2008-2010 Research Instructor. Department of Cancer Biology. Vanderbilt University Medical Center.
2010- 2012 Research Assistant Professor. Department of Cancer Biology. Vanderbilt University Medical Center.
2010-present Research Microbiologist. Department of Veterans Affairs. Tennessee Valley Health Care.
2012- present Assistant Professor. Department of Medicine, Division of Clinical Pharmacology. Vanderbilt University Medical Center.

Other Experience and Professional Memberships

1998-2003- American Association of Cancer Research
2000-2001- Secretary/Treasurer of MCO Graduate Student Council
2003-present American Society for Bone and Mineral Research
2004- present International Bone and Mineral Society (IBMS)
2011- present IBMS Cancer and Bone Disease Advisory Board Member
2012- present Co-organizer of the Vanderbilt Center for Bone Biology Seminar Series
2013 Organizing committee for the Cancer Induced Bone Disease Young Investigators Carnival
2013- present Associate member of the IBMS Young Investigators Committee
2014- present Editorial Board of Journal of Clinical Oncology
2013- 2014 Organizing Committee for 9th International Conference of Anticancer Research
2015-pres Editorial board of the Journal of Bone Oncology
2015-2016 Co-Chair of the IBMS Young Investigator Committee
2016-pres Secretary Treasurer of the Cancer and Bone Society

Reviewed for: BioMed Central, British Journal of Pharmacology, British Journal of Cancer, Bone, Cancer Cell, Cancer Control: Journal of the Moffitt Cancer Center Cancer Research, Cancer Discovery, Clinical Breast Cancer, Clinical Cancer Research, Clinical Interventions in Aging, FEBS Letters, International Journal of Cancer Research, Journal of Bone and Mineral Research, Journal of Clinical Investigation, Journal of the National Cancer Institute, Journal of Orthopedic Research, JoVE, Molecular Biosystems, Molecular Cancer Research, Molecular Oncology, PLoSOne, Recent Patents on Cardiovascular Drug Discovery

C. Contribution to Science

1. Identifying Gli2 as a major regulator of tumor-induced bone disease. When I first began my postdoctoral research we knew that parathyroid hormone related protein (PTHrP) was a critical factor for regulating tumor-induced bone disease; however, while inhibiting PTHrP expression in animal studies showed some success, no therapeutic inhibitors had been successful. It was critical at the time to understand what regulated PTHrP in order to develop therapies. My early work focused on finding regulators of PTHrP, including investigating the regulation of the PTHrP promoter. Through this work we discovered that a Hedgehog transcription factor, Gli2 regulated PTHrP expression. This early work has continued as a focus in my career and my laboratory is actively testing Gli inhibitors in animal models as potential therapies for patients with bone metastases. The 4 major publications that came from this work include:
    a. **Sterling JA**, Oyajobi BO, Grubbs BG, Padalecki SS, Gupta A, Story B, S Munoz, Zhao M, and Mundy GR. The Hedgehog Signaling Molecule Gli2 Induces PTHrP Expression and Osteolysis In Metastatic Human Breast Cancer Cells. *Cancer Research* **66** (2006).
    b. Johnson, R.W., Merkel, A.R., Danilin, S., Nguyen, M.P., Mundy, G.R., and **Sterling, J.A.,** 6-Thioguanine Inhibition of Parathyroid Hormone-related Protein Expression is Mediated by GLI2. *Anticancer Research* **31** (2011). PMCID: PMC3430074.
    c. **Sterling JA,** Edwards JR, Martin TJ, Mundy GR. Advances in the biology of bone metastasis: How the skeleton affects tumor behavior. *Bone* **48** (2011). 6-15. PMCID: PMC2974944.

d. Johnson RW, Nguyen MP, Padalecki SS, Grubbs BG, Merkel AR, Oyajobi BO, Matrisian LM, Mundy GR, **Sterling JA**. TGF-β promotion of Gli2 induced PTHrP expression is independent of canonical Hedgehog signaling. *Cancer Research* **71** (2011). PMCID: PMC3077118

2. Investigating signaling factors that regulate prostate cancer metastasis to bone. Despite the fact that prostate cancer routinely metastasizes to bone little is known about why some tumors metastasize to bone and others do not. Therefore, my collaborators and I set out to 1) develop better approaches for imaging prostate cancer in bone. 2) Develop consistent models for studying bone metastasis. 3) Investigate the molecular mechanisms that regulate prostate cancer metastasis. These studies were performed with both imaging groups and urology groups in highly collaborative interactions. My main role in these studies was to design, perform, and analyze animal models of bone metastases, which were a large part of each of these studies. To highlight my significant contributions to these studies, I am either 2nd or 2nd to last author on these manuscripts.
    a. Li X, **Sterling JA**, Fan KH, Vessella RL, Shyr Y, Hayward SW, Matrisian LM, Bhowmick NA. Loss of TGF-β Responsiveness in Prostate Stromal Cells Alters Chemokine Levels and Facilitates the Development of Mixed Osteoblastic/Osteolytic Bone Lesions. *Mol Cancer Res.* ***10*** *(*2012). PMCID: PMC3900026.
    b. Jin R, **Sterling JA**, Edwards JR, Degraff DJ, Lee C, Park SI, Matusik RJ. "Activation of NF-kappa B Signaling Promotes Growth of Prostate Cancer Cells in Bone." PLoS One **8** (2013). PMCID: PMC3618119.
    c. Bi X, **Sterling JA**, Merkel AR, Perrien DS, Nyman JS, Mahadevan-Jansen A. "Prostate cancer metastases alter bone mineral and matrix composition independent of effects on bone architecture in mice- A quantitative study using microCT and Raman spectroscopy." Bone **56** (2013). PMCID: PMC3799839.
    d. Hansen AG, Arnold SA, Jiang M, Palmer TD, Ketova T, Merkel A, Pickup M, Samaras S, Shyr Y, Moses HL, Hayward SW, **Sterling JA**, Zijlstra A. "ALCAM/CD166 is a TGFβ responsive marker and functional regulator of prostate cancer metastasis to bone." *Cancer Res*. ***74*** (2014). PMCID: PMC4149913.

3. Improving imaging approaches for identifying tumors in bone at early stages. When my laboratory first began studying the interactions between tumor cells and their micro-environment, it became more important to detect tumors in animals at earlier stages. However, at the time the earliest we could detect a tumor was approximately 14 days after tumor cell injection by x-ray or fluorescence analysis, and this was often unreliable. In order to develop more quantitative and translational approaches we collaborated with the Vanderbilt University Institute for Imaging Science to develop live animal (and ex vivo) approaches for detecting and quantifying tumors in bone or bone destruction. This resulted in improved μCT, and a push to develop techniques to fuse multi-modality imaging techniques, including a mix of in vivo and ex vivo techniques. These focused on micro PET, microCT, MALDI_IMS, and Raman Spectroscopy. Many of these studies are still ongoing, but the most impactful papers from this research to date include:
    a. Johnson LC*, Johnson RW*, Munoz SA, Mundy GR, Peterson TE, **Sterling JA**. Longitudinal live animal microCT allows for quantitative analysis of tumor-induced bone destruction. Bone 48 (2011). 141-151. PMCID: PMC2974944
    b. Ding H, Nyman JS, **Sterling JA**, Perrien DS, Mahadevan-Jansen A, Bi X. "Development of Raman spectral markers to assess metastatic bone in breast cancer." J Biomed Opt. 19 (2014).
    c. Seeley E, Wilson KJ, Yankeelov TE, Johnson RW, Gore J, Caprioli RM, Matrisian LM, **Sterling JA.** "Co-registration of multi-modality imaging allows for comprehensive analysis of tumor-induced bone disease." *Bone* ***61*** (2014). PMCID: PMC4005328.

4. The role of the tumor micro-environment in the regulation of bone disease. Currently, the primary focus of our research centers on investigating the role of the micro-environment on tumor-induced bone disease. Early studies in soft-tissue tumors began to explore this, but until recently few laboratories have investigated how the vast array of cell types in the bone marrow influence bone metastases and tumor growth in bone. As part of the Vanderbilt University Tumor Micro-environment Network group (lead by Lynn Matrisian), we began to focus on determining the role of multiple cell-types in bone. One aspect of these studies was to improve imaging approaches as describe above and a major success

that came out of these studies was the Seeley et al., paper, in which I am the senior author, published in *Bone* this year. Other important papers that has resulted from these ongoing studies include:

a. Campbell JP, Karolak M, Ma Y, Perrien DS, S., Masood-Campbell SK, Penner NL, Munoz SA, Zijlstra A, Yang X, **Sterling JA**, Elefteriou F. Stimulation of Host Bone Marrow Stromal Cells by Sympathetic Nerves Promotes Breast Cancer Bone Metastasis in Mice. *PLoSBiology* **10** (2012). PMCID: PMC3398959.
b. Danilin S, Merkel AR, Johnson JR, Johnson RW, Edwards JR, **Sterling JA**. "Myeloid-derived suppressor cells expand during breast cancer progression and promote tumor-induced bone destruction". *OncoImmunology* **1** (2012). PMCID: PMC3525604.
c. Buenrostro D, Park SI, **Sterling JA.** "Dissecting the Role of Bone Marrow Stromal Cells on Bone Metastases." BioMed Research International (2014). PMCID: PMC4099112.
d. Johnson RW, Merkel AR, Page JM, Ruppender NS, Guelcher SA, Sterling JA. "Wnt signaling induces gene expression of factors associated with bone destruction in lung and breast cancer." Clinical and Experimental Metastasis. (2014). PMCID: PMC4258192.

5. <u>The role of rigidity on the regulation of tumor-induced bone disease.</u> In addition to the cellular components of the micro-environment, physical components of the micro-environment can influence tumor cell gene expression and behavior. To investigate these aspects, we work closely with Dr. Scott Guelcher's group in Chemical and Biomolecular Engineering at Vanderbilt. Through these studies we have developed many novel resources including 2D polyurethane (PUR) films and 3D PUR scaffolds of varying rigidities. These studies have demonstrated that the rigidity of bone (independently of other factors) alters tumor cell gene expression. While these studies are actively underway, the following are important manuscripts that have come from this work:

a. Ruppender NS, Merkel AR, Martin TJ, Mundy GR, **Sterling JA**, Guelcher SA. Matrix Rigidity Induces Osteolytic Gene Expression of Metastatic Breast Cancer Cells. *PLoSONE* **5** (2010). PMCID: PMC2981576.
b. Johnson RW, Merkel AR, Page JM, Ruppender NS, Guelcher SA, **Sterling JA**. "Wnt signaling induces gene expression of factors associated with bone destruction in lung and breast cancer." *Clinical and Experimental Metastasis*. In Press (2014).
c. Page JM, Merkel AR, Ruppender NS, Guo R, Dadwal UC, Cannonier SA, Basu S, Guelcher SA, **Sterling JA.** "Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II." Biomaterials. (2015).
d. Guo R, Lu S, Page JM, Merkel AR, Basu S, **Sterling JA**, Guelcher SA. "Fabrication of 3D Scaffolds with Precisely Controlled Substrate Modulus and Pore Size by Templated-Fused Deposition Modeling to Direct Osteogenic Differentiation." Adv Healthc Mater. (2015). PMCID: PMC4558627.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/julie.sterling.1/bibliography/44660846/public/?sort=date&direction=ascending

D. Research Support

ACTIVE

W81XWH-15-1-0622 (Sterling/Duvall)
09/01/2015-8/31/18
DOD-CDMRP- Breast Cancer Breakthrough Award
Targeted Drug Nanocarriers for Inhibiting Bone Metastatic Breast Cancer
This application is developing nanoparticle carriers for the treatment of bone metastatic breast cancer. Specifically, it investigates targeting the Gli inhibitor GANT58 or siRNA to the bone using novel bone targeted nanoparticles.

1I01BX001957-01 (Sterling)
07/01/2013-06/30/2017
VA Merit
Strategies for early treatment of bone metastases.

This application seeks to investigate the role of TGF-β in the regulation of early breast cancer establishment in bone. Specifically, it investigates the role of cells in the bone marrow micro-environment and tests several inhibitors targeted to bone to test their role in vivo and in vitro in the inhibition of tumor establishment.
Role: PI

1R01CA163499-01A1 (Guelcher/Sterling)
10/01/2012 - 09/30/2017
NIH/NCI
The Role of Mechanotransduction in Progression of Tumor-induced Bone Disease
Once established in bone, tumor cells begin to produce factors that cause changes in normal bone remodeling, such as parathyroid hormone-related protein (PTHrP), which in human patients is detectable in most bone metastases from breast cancer, but only in relatively few soft tissue metastases. However, the reasons for the differential expression of PTHrP in the bone microenvironment are not known.
Role: Co-PI

COMPLETED

5P01 CA40035- 22 (Elefteriou) 09/01/2006-08/31/2013 (NCE)
NIH/NCI
Effects of Tumors on the Skeleton
Role: Project Leader (Project 1)

In Vivo Cellular and Molecular Imaging Center Pilot Grant (Sterling) 10/01/2011-9/30/2013
Parent Grant: **5P50CA128323-04** (Gore) 09/22/08 – 08/31/13
NCI/NIH
Multi-modality molecular imaging to assess early stages of bone metastases.
Role: Principal Investigator

Veterans Affairs Career Development Award (Sterling) 08/01/2010-7/31/2013
The Effect of the Tumor Microenvironment on Cancer Metastasis to Bone.
Role: Principle Investigator

5U54 CA 126505-02 (Matrisian) 09/25/2006-08/21/2011
NIH/NCI
Paracrine TGF-Beta signaling in Tumor Initiation and Progression
Role: Co-investigator

5U54 CA 126505-02 (Matrisian) Supplement 09/01/2008-08/31/2010
NIH/NCI
Paracrine TGF-Beta signaling in Tumor Initiation and Progression
Role: Co-investigator

2P50 CA098131-06 (Arteaga) 07/01/2008-06/30/2013
NIH/NCI
SPORE in Breast Cancer
Role: Co-investigator

PHS Fellowship Supplemental Form

OMB Number: 0925-0001
Expiration Date: 10/31/2018

Introduction

1. Introduction
(RESUBMISSION)

Fellowship Applicant Section

2. Applicant's Background and Goals for Fellowship Training* **M-10_PHS_Fellow_Goals_FellowshipTrainingCareer.pdf**

Research Training Plan Section

3. Specific Aims* **M-11_PHS_Fellow_SpecificAims.pdf**

4. Research Strategy* **M-13_PHS_Fellow_ResearchStrategy.pdf**

5. Respective Contributions* **M-7_PHS_Fellow_RespectiveContributions.pdf**

6. Selection of Sponsor and Institution* **M-8_PHS_Fellow_SelectionSponsorInstitution.pdf**

7. Progress Report Publication List
(RENEWAL)

8. Training in the Responsible Conduct of Research* **M-9_PHS_Fellow_ResponsibleConductResearch.pdf**

Sponsor(s), Collaborator(s) and Consultant(s) Section

9. Sponsor and Co-Sponsor Statements **M-15_PHS_Fellow_Sponsor_CoSponsor_Info.pdf**

10. Letters of Support from Collaborators, Contributors and Consultants **M-16_PHS_Fellow_LettersOfSupport.pdf**

Institutional Environment and Commitment to Training Section

11. Description of Institutional Environment and Commitment to Training **M-17_PHS_Fellow_Inst_Environment_Commitment.pdf**

Other Research Training Plan Section

Human Subjects

Please note. The following item is taken from the Research & Related Other Project Information form. The response provided on that page, regarding the involvement of human subjects, is repeated here for your reference as you provide related responses for this Fellowship application. If you wish to change the answer to the item shown below, please do so on the Research & Related Other Project Information form; you will not be able to edit the response here.

Are Human Subjects Involved? ☐ Yes ☑ No

12. Human Subjects Involvement Indefinite?

13. Clinical Trial? ☐ Yes ☑ No

14. Agency-Defined Phase III Clinical Trial?

15. Protection of Human Subjects

16. Data Safety Monitoring Plan

17. Inclusion of Women and Minorities

18. Inclusion of Children

Vertebrate Animals

The following item is taken from the Research & Related Other Project Information form and repeated here for your reference. Any change to this item must be made on the Research & Related Other Project Information form.

Are Vertebrate Animals Used? ☑ Yes ☐ No

19. Vertebrate Animals Use Indefinite? ☐ Yes ☑ No

Funding Opportunity Number: PA-16-309. Received Date: 2016-12-08T15:14:00.000-05:00

PHS Fellowship Supplemental Form

20. Are vertebrate animals euthanized?	☑ Yes ☐ No
If "Yes" to euthanasia Is method consistent with American Veterinary Medical Association (AVMA) guidelines?	☑ Yes ☐ No
If "No" to AVMA guidelines, describe method and provide scientific justification	
21. Vertebrate Animals	**M-6_PHS_Fellow_VertebrateAnimals.pdf**

Other Research Training Plan Information

22. Select Agent Research	
23. Resource Sharing Plan	M-12_PHS_Fellow_ResourceSharingPlan.pdf
24. Authentication of Key Biological and/or Chemical Resources	

PHS Fellowship Supplemental Form

Additional Information Section

25. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ☐ Yes ☑ No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s), using the registry information provided within the agency instructions. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s):

26. Alternate Phone Number:

27. Degree Sought During Proposed Award:

Degree:	If "other", please indicate degree type:	Expected Completion Date (month/year):
PHD: Doctor of Philosophy		**05/2019**

28. Field of Training for Current Proposal*: 157 Microbiology

29. Current Or Prior Kirschstein-NRSA Support?* ☐ Yes ☑ No

If yes, please identify current and prior Kirschstein-NRSA support below:

Level*	Type*	Start Date (if known)	End Date (if known)	Grant Number (if known)

30. Applications for Concurrent Support?* ☐ Yes ☑ No

If yes, please describe in an attached file:

31. Citizenship*

U.S. Citizen — U.S. Citizen or Non-Citizen National? ☑ Yes ☐ No

Non-U.S. Citizen
- ☐ With a Permanent U.S. Resident Visa
- ☐ With a Temporary U.S. Visa

If you are a non-U.S. citizen with a temporary visa who has applied for permanent resident status and expect to hold a permanent resident visa by the earliest possible start date of the award, please also check here. ☐

Name of Former Institution:*

32. Change of Sponsoring Institution

PHS Fellowship Supplemental Form

Budget Section

All Fellowship Applicants:

1. Tuition and Fees*:

☐ None Requested ☑ Funds Requested

Year 1	[redacted]
Year 2	[redacted]
Year 3	$0.00
Year 4	$0.00
Year 5	$0.00
Year 6 (when applicable)	$0.00
Total Funds Requested:	[redacted]

Senior Fellowship Applicants Only:

2. Present Institutional Base Salary: Amount | Academic Period | Number of Months

3. Stipends/Salary During First Year of Proposed Fellowship:

a. Federal Stipend Requested: Amount | Number of Months

b. Supplementation from other sources: Amount | Number of Months

Type (sabbatical leave, salary, etc.)

Source

Appendix

Tracking Number: GRANT12302470 Funding Opportunity Number: PA-16-309. Received Date: 2016-12-08T15:14:00.000-05:00

BACKGROUND AND GOALS FOR FELLOWSHIP TRAINING

Doctoral Dissertation and Research Experience

Undergraduate Research

I began to participate in independent research during my sophomore year at the University of Wisconsin-La Crosse, while pursuing degrees in Biochemistry and Psychology. I served as a research assistant and laboratory manager in the Visual Sciences Research Laboratory for Drs. Alex O'Brien and Bart Van Voorhis for two and a half years, during which time my critical thinking and scientific interpretation skills were greatly enhanced. Our main areas of study were object recognition and synesthesia, which we tested through the creation of programs to measure reaction time from our participants. Also during my sophomore year, I began to pursue research in organic chemistry with the chair of the Chemistry Department, Dr. Aaron Monte, in the synthesis of the beta-alkaloid compound tetrahydroharmine. These research experiences had a profound impact on my desire to pursue research as a career.

Research and Development Internship

The summer before my December 2010 graduation, I attained a position as a cancer research and development intern with the global health care company, Covidien. My research skills matured significantly under the mentorship of Dr. Raghavan Rajagopalan. In this internship, I focused on the creation and therapeutic application of photosynthetic compounds targeted for use in colon and ovarian cancers. The premise of this research was to localize a compound that alone is nontoxic, but can be subsequently activated by light to form radicals, inducing death of surrounding cancerous cells. I designed and synthesized a novel photosensitive compound, conjugated this compound to carrier molecules specific for ovarian and colon cancer cells, and performed cytotoxicity assays to determine its efficacy. In this internship, I further developed skills in cellular physiology and organic chemistry. I was able to demonstrate that the compounds in conjunction with light were cytotoxic and could selectively target the desired cancer cells, and was awarded the Best Poster Award among summer interns at the *Covidien Intern Poster Symposium*. These data were presented at the 2010 *SPiE BiOS: Biomedical Optics Symposium* and the 2011 *World Congress of the International Photodynamic Association*, and were published in *Photodiagnosis and Photodynamic Therapy* in 2011.

Interest in Microbiology and Immunology

As I prepared to embark upon training towards an advanced degree, I felt called to explore other scientific disciplines. I first became drawn to the field of microbiology and immunology during the Capstone Seminar of my final semester at University of Wisconsin-La Crosse. The seminar I gave focused on the emergence of infectious diseases into new populations and spread to previously uninfected areas. I became captivated with the study of infectious diseases while preparing this presentation. So much so, that I chose to apply for and complete a microbiology course after graduating with my degrees in Biochemistry and Psychology. After these experiences I was committed to applying to graduate degree programs in Microbiology and Immunology.

Master's Degree Thesis Research

I went on to complete a Master of Science in Molecular Microbiology and Immunology at the Johns Hopkins Bloomberg School of Public Health. My Master's degree thesis work involved the study of immune responses to measles virus in rhesus macaques in the laboratory of Dr. Diane Griffin, a world-renowned virologist. During this time, I developed expertise in immunologic techniques and experimental design, critical review of the primary literature, and scientific writing. My projects in the Griffin laboratory focused on (1) the characterization of Th17 (CD4) and Tc17 (CD8) T cell responses to wild-type measles in a rhesus macaque model, (2) monitoring viral RNA persistence, and (3) determination of the immunological effects of the measles virus C and V proteins between wild-type and vaccine strains. I became proficient in the analysis of primary samples, including peripheral blood mononuclear cells (PBMCs) from human and non-human primates, as well as bone marrow, skin, and lymph node biopsies. My research found three distinct activation periods of Th17 and Tc17 cells over the course of six months post-infection, a consequence of persistent, noninfectious measles virus RNA. Additionally, I found that the C and V proteins limited the human type I interferon response and were necessary for optimal viral replication. My research in the Griffin laboratory resulted in a middle authorship publication in the *Journal of Virology* in 2014, presentation of my research at the 2015 *International Conference on Negative Strand Viruses*, and the recent resubmission of a first author paper to *The Journal of Infectious Diseases* in December 2016.

Ph.D. Laboratory Rotations

The skills obtained from my undergraduate, internship, and master's degree research allowed me to be extremely productive in my four laboratory rotations and as an incoming graduate student at Vanderbilt University. In these rotations, I was able to explore cell death mechanisms induced in human osteoblasts in Dr. Jim Cassat's laboratory, design a mass cytometry (CyTOF) panel to phenotype T lymphocyte populations in the laboratory of Dr. Marco Davila, optimize an *ex vivo* T regulatory cell suppression assay and contribute to analyses focusing on immune tolerance in Type 1 Diabetes and Systemic Lupus Erythematosus (SLE) with Dr. Dan Moore's research group, and provide preliminary data to support *Helicobacter pylori* toxin-mediated T cell suppression in conjunction with Drs. Tim Cover and Spyros Kalams. My rotation in the Moore laboratory resulted in a middle author publication in the *American Journal of Transplantation* in 2015.

Ph.D. Thesis Laboratory

My interests in the host responses to human pathogens led me to join Dr. Cassat's laboratory to study osteoimmunologic responses to bacterial pathogens. Under Dr. Cassat's guidance, I have become well-trained in microbiology and have developed a project that bridges multiple scientific disciplines with the long-term goal of determining the innate sensing capabilities of bone cells and how microbial pathogens impact skeletal remodeling. This project will guide my scientific training, both at the bench and professionally, under the sponsorship of Dr. Cassat and Dr. Julie Sterling of the Vanderbilt Center for Bone Biology. In the proposed research, I will explore how skeletal cells sense and respond to the human bacterial pathogen *Staphylococcus aureus*, the most common cause of osteomyelitis, and how these responses disrupt normal bone remodeling processes.

Training Goals and Objectives

My research ambitions are focused on studying infectious diseases that have a substantial public health burden. I am seeking a Ph.D. in Microbiology and Immunology within the Department of Pathology, Microbiology and Immunology (PMI) with the goal of establishing my own laboratory as an independent scientist studying translational research. Specifically, I hope to investigate critical immune responses and biological changes induced by pathogens, and contribute to how this knowledge can be leveraged to alleviate the morbidity and mortality associated with infection. In this Ph.D. program I aim to develop independent thinking and the experimental skills necessary to investigate translational research problems. I will continue to become proficient in project development and hypothesis-driven research. I hope to expand my scientific skills in critical thinking, experimental design, data interpretation, and statistical analysis.

Vanderbilt University is an exceptional environment to gain a comprehensive knowledge of host-pathogen interactions. To expand on previous didactic coursework in bacteriology, virology, parasitology, and immunology at JHSPH and VU, there are several ongoing seminars and journal clubs to fortify knowledge in microbiology and immunology in order to pursue a scientific career in the field of infectious disease. I have carefully chosen seminar series to enhance my didactic coursework in conjunction with my mentor and thesis committee. Specifically, I will attend weekly seminars from PMI and the Vanderbilt Center for Bone Biology (VCBB), a weekly Research in Progress seminar for student presentations, a monthly Infection, Inflammation, and Immunity Frontiers seminar featuring luminaries in microbiology and immunology, a bi-monthly Microbial-Host Interaction meeting, a bi-monthly Host-Pathogen Interaction journal club, and the annual Vanderbilt Symposium on Infection and Immunity.

In keeping with my desire to conduct translational research, I applied to and was subsequently selected to join the Vanderbilt Program in Molecular Medicine (VPMM). The VPMM is a unique training program established in 2010 with the goal to integrate thesis work with clinical experiences, didactic courses, and seminars under the guidance of my primary mentor, Dr. Cassat, and a clinical mentor, Dr. Isaac Thomsen in Pediatric Infectious Diseases. The VPMM provides valuable clinical experience that ensures that my research project will reflect the most important challenges posed by bone infections. In addition to observational experiences in clinic, the VPMM provides essential information on translational research through an Introduction to Clinical and Translational Research and VPMM Rounds, which brings a panel of clinicians, basic scientists, and patients into a classroom to discuss the different perspectives on human disease. Additionally, the VPMM supports attendance at Pediatric Infectious Diseases research conferences, rounds, and clinical case conferences, as well as the bi-monthly seminar series, and an annual VPMM Spring Retreat to share our thesis research. I expect to complete the VPMM training program by the summer of 2018.

Vanderbilt University also offers elective Modules through the Augmenting Scholar Preparation and Integration with Research-Related Endeavors (ASPIRE) Program, founded in 2013 under support of the NIH-

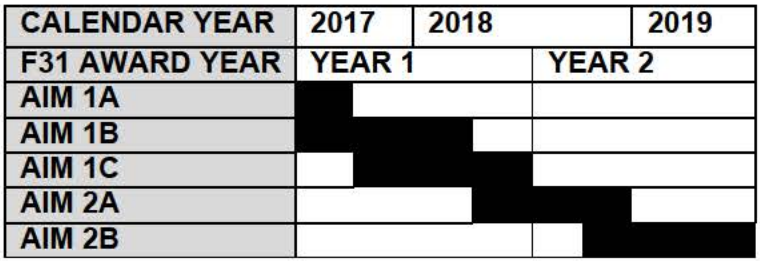
funded BEST grant. I have recently been accepted into the ASPIRE Module for Clinical Laboratory Medicine, which is a training experience to provide exposure to clinical research and laboratories. Due to the small number of graduate students and post-doctoral fellows accepted into the Clinical Laboratory Medicine Module each year, training can be personalized to emphasize a particular clinical sub-discipline. The 2017 Clinical Laboratory Medicine Module will tailor my experiences to highlight training in Clinical Microbiology with Dr. Jonathan Schmitz, Associate Director of the Fellowship Training Program in Medical and Public Health Laboratory Microbiology and Instructor in PMI. This experience will provide meaningful preparation for the continued study of human infectious pathogens.

Over the course of my Ph.D. training program, I will gain experience in mentoring and teaching. For example, in just the last year and a half, I have had the opportunity to train 4 rotation students coming in through the Medical Scientist Training Program and the Interdisciplinary Graduate Program for Ph.D. students. In the future, I hope to foster the ongoing training of an undergraduate student over the course of a few years to understand cellular differentiation and/or advanced imaging and quantification techniques. Furthermore, I have facilitated communication with the School for Science and Math at Vanderbilt to teach periodic guest lectures to high school students, of which I have taught a "Microbes 101" course in the fall of 2016. Further teaching experience is incorporated into the Microbiology and Immunology Graduate Program curriculum, in which graduate students serve as teaching assistants (TAs) to the first year medical students for the laboratory component of the Medical School Microbiology and Immunology course.

I will continue to hone my skills in scientific communication through presentation of my research at laboratory meetings every one to two months, to my thesis committee twice a year, and to other graduate students, post-doctoral fellows, and faculty at the Research in Progress, Microbial-Host Interaction, VCBB, and VPMM seminars. Additionally, I will present my research at national and international meetings. To this end, I have presented my research at the 2016 *Vanderbilt Symposium on Infection and Immunity* and the 2016 *International Conference on Gram Positive Pathogens*. Next year, I plan to attend the *St. Jude's Invited Graduate Student Symposium* and the *Southeastern Immunology Symposium*. My written communication will be enhanced by assisting Dr. Cassat in the peer review of manuscripts, by the review of other manuscripts from the Cassat laboratory, and through submission of my own manuscripts and grant applications.

Activities Planned Under this Award

I have applied for two years of NRSA support. I will dedicate 100% of my effort to research, training, and career development activities detailed below.



Year 1: July 2017-June 2018

Research: 80%

- Meetings with Dr. Cassat (weekly)
- Meetings with Thesis Committee (bi-annually)
- Submit first lead-author publication, detailing the impact of staphylococcal coagulases and *S. aureus* strain differences in osteomyelitis
- Submit second first-author publication, detailing findings in Aim 1

Seminars and Presentations: 10%

- Present research and participate at events at Vanderbilt University Medical Center (VUMC):
  - Pathology, Microbiology, and Immunology seminar (weekly)
  - Microbiology and Immunology Research in Progress seminar (weekly)
  - Microbial-Host Interaction seminar (bi-monthly)
  - Host-Pathogen Interaction journal club (bi-monthly)
  - Infection, Inflammation & Immunity Frontiers seminar (monthly)
  - Vanderbilt Symposium of Infection and Immunity (annually)
  - Bone Center Seminar Series (weekly)
- Organize/Attend Southeastern Immunology Symposium 2017
- Attend St. Jude's Invited Graduate Student Symposium 2017

Career Development/Mentorship: 10%

- Mentor Vanderbilt undergraduate (1) and graduate students (3) rotating through the laboratory
- Participate in Vanderbilt Program in Molecular Medicine Training Program Activities:
  - Vanderbilt Program in Molecular Medicine seminar (bi-monthly)
  - Vanderbilt Program in Molecular Medicine Retreat (annually)
  - Observational experiences
  - Clinical Rounds, Case Conferences, and Seminars
- Participate in activities sponsored by the VUMC Office of Career Development
  - ASPIRE Program, *Explore Phase*: Designed to highlight career options and networking
    - ASPIRE Module Clinical Laboratory Sciences
    - BRET Career Connections (monthly)
    - BRET Career Symposium (annual)
    - Complete Individual Development Plan (IDP) with Dr. Cassat
- Laboratory teaching assistant (TA) for the Medical School Microbiology and Immunology course

<u>Year 2: July 2018- June 2019</u>

Research: 80%

- Meetings with Dr. Cassat (weekly)
- Meetings with Thesis Committee (bi-annually)
- Submit third first-author publication, detailing findings from Aim 2

Seminars and Presentations: 10%

- Present and participate in VUMC seminar series (see above)
- Present research at national and/or international meeting
- Mentor Vanderbilt graduate students rotating through the laboratory/undergraduate

Career Development/Mentorship: 10%

- Mentor Vanderbilt undergraduate (1) and graduate students (3) rotating through the laboratory
- Participate in activities sponsored by VUMC Office of Career Development
  - ASPIRE Program, *Enhance Phase*: Communication module designed to improve oral communication skills
    - BRET Career Connections (monthly)
    - BRET Career Symposium (annual)
    - Complete Individual Development Plan (IDP) with Dr. Cassat
- Interview for a postdoctoral fellowship
- Write thesis and defend PhD dissertation

SPECIFIC AIMS

The impact of innate immune recognition of *Staphylococcus aureus* on bone homeostasis and skeletal immunity

Bone is constantly remodeled through the coordinated efforts of bone-forming osteoblasts (OBs) and bone-resorbing osteoclasts (OCs). This process is referred to as bone homeostasis and is tightly regulated by local and systemic factors, including cytokines, hormones, and growth factors. *Staphylococcus aureus* is the leading cause of invasive bone infection (osteomyelitis), during which inflammation leads to altered interactions between skeletal cells. Dysregulation in bone homeostasis triggers aberrant bone formation and bone destruction, which may result from changes in skeletal cell physiology during osteomyelitis that are distinct from cell death. Our preliminary data show that bacterial components modulate the differentiation of OCs (osteoclastogenesis) from myeloid cells with and without the canonical OC differentiation factor, receptor activator of nuclear factor κB-ligand (RANKL). Specifically, BM treatment with *S. aureus* supernatants induces OC differentiation without canonical RANKL signaling, and limits OC formation when pretreated with RANKL. The primary objective of this proposal is to define the mechanisms by which bacterial pathogens alter osteoclastogenesis to impact bone homeostasis and skeletal immunity.

Skeletal cells are known to express innate pattern recognition receptors (PRRs), but the contribution of innate sensing by OC PRRs, such as Toll-like receptors (TLRs) towards pathogen clearance and bone remodeling during *S. aureus* osteomyelitis has not yet been explored. In order to further define the contribution of skeletal cell PRRs to altered bone homeostasis and antibacterial immunity during osteomyelitis, we focused on the critical PRR signaling adaptor MyD88, which is required for TLR and IL-1 family cytokine signaling. In preliminary studies, data support a MyD88-mediated mechanism by which bacteria perturb OC differentiation, emphasizing the importance of innate signaling in modulating osteoclastogenesis. Overall, I hypothesize that *S. aureus* modulates OC precursor (pre-OC) cell biology and bone remodeling through ligation of OC PRRs and the induction of inflammation. To test this hypothesis, we propose two integrated Aims that will define how *S. aureus* perturbs the differentiation and functional ability of OC-like cells to resorb bone, and determine how innate activation of skeletal cells affects bacterial clearance and bone homeostasis in a powerful new osteomyelitis murine model that is capable of precise quantification of pathogen-induced changes in bone turnover. The Aims will elucidate bacterial-induced mechanisms of altered bone remodeling and further define the ability of skeletal cells to respond to *S. aureus*. These studies have the potential to significantly impact human health by identifying therapeutic targets to limit bone destruction during osteomyelitis. The Aims are:

Aim 1: Define the role of TLRs and IL-1R in *S. aureus*-mediated perturbation of osteoclastogenesis.

Based on preliminary studies that suggest a MyD88-mediated mechanism of OC perturbation by bacterial components *in vitro,* I hypothesize that *S. aureus* modulates pre-OC cell biology through TLR recognition or IL-1R signaling upstream of MyD88. To test this hypothesis, we will perform osteoclastogenesis assays on bone marrow (BM) cultures from wild-type and immune-deficient mouse strains, including TLR2, TLR9, and IL-1R-deficient mice, with and without RANKL stimulation, components of *S. aureus*, TLR agonists, or recombinant IL-1 to (i) identify changes in expression of TLRs and factors known to modulate osteoclastogenesis, (ii) define the activation status of intracellular signaling cascades and transcription factors, and (iii) investigate the functionality of OCs induced by bacterial components with bone resorption assays. Taken together, these data will detail how bacterial stimulation modulates OC differentiation and function through TLR and IL-1 signaling.

Aim 2: Elucidate the role of skeletal cell-specific MyD88 signaling on pathogen clearance and bone remodeling during *S. aureus* osteomyelitis.

Aim 1 will identify *in vitro* changes caused by *S. aureus* during osteoclast differentiation, including alterations in OC signaling and function. Our *in vitro* assays demonstrate that MyD88 in skeletal cell precursors could be responsible for downstream changes following *S. aureus* stimulation. Interestingly, preliminary data obtained in our *S. aureus* osteomyelitis model shows that MyD88 is also necessary to limit bacterial replication and dissemination to other organs. Based on these data, I hypothesize that innate sensing of *S. aureus* by skeletal cells *in vivo* impacts bacterial clearance and alters bone remodeling during osteomyelitis. To test this hypothesis we will induce osteomyelitis in wild-type mice and mice with skeletal cell-specific MyD88 deletion to (i) differentiate the kinetics of pathogen clearance from bone and bacterial dissemination to other organs, (ii) investigate bone remodeling alterations in cortical and trabecular bone using micro-computed tomography (microCT) analysis, and (iii) quantify osteoclast differentiation *in vivo* through histological assessment. Collectively, these Aims will investigate how innate immune activation of skeletal cells alters bone homeostasis, thereby elucidating fundamental mechanisms of osteo-immunologic crosstalk.

RESEARCH STRATEGY

A. SIGNIFICANCE

A1. *Staphylococcus aureus* is the most common cause of bacterial bone infection (osteomyelitis).

S. aureus is the leading cause of healthcare-associated infections, and in otherwise healthy individuals, infections have increased with the spread of community-acquired *S. aureus* strains since the early 2000s (1-4). *S. aureus* is responsible for approximately 80% of all osteomyelitis cases in humans (5). Colonization of bone with bacteria occurs by spread from a contiguous soft tissue infection or contamination of the bone following trauma (6). Additionally, children are more likely to establish bone infections via hematogenous spread of bacteria through blood without known risk factors. Osteomyelitis can lead to serious complications resultant to alterations in bone remodeling, forming large areas of bone destruction, aberrant bone formation, and local vasculature damage. Treatment requires drastic measures to eradicate the infection, and antibiotic resistance complicates this already difficult-to-treat invasive infection. The current standard of treatment for osteomyelitis entails surgical debridement of the infected bone and prolonged courses of antibiotics (6). Unfortunately, treatment does not always prevent complications, including pathologic fractures, thomboses, septic arthritis, and growth defects (6). Altered bone remodeling can also be induced by other inflammatory disorders, and understanding how bacterial-induced inflammation impacts bone remodeling will lead to an enhanced understanding of how systemic inflammatory disorders or alterations in the microbiota affect bone health.

A2. OC-mediated bone loss occurs during inflammatory disease states.

Many disease states significantly alter bone homeostasis and lead to bone loss by perturbing the equilibrium between OBs that deposit new bone and OCs that resorb bone (7,8). Bone loss occurs when OC differentiation is favored leading to excessive bone resorption (7), which can be seen during systemic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (8), as well as locally due to periodontal disease and joint inflammation (9-11). Notably, dramatic local inflammation of bone occurs subsequent to the establishment of *S. aureus* osteomyelitis (12).

A3. *S. aureus* alters normal bone remodeling.

The presence of *S. aureus* in bone disrupts normal, homeostatic bone remodeling (13-15). Specifically, a class of staphylococcal toxins, alpha-type phenol soluble modulins (α-PSMs) are both necessary and sufficient to induce direct cell death of OBs *in vitro* (12). However, preliminary *in vivo* experiments have shown that toxin-deficient *S. aureus* strains still induce dramatic alterations in bone physiology, indicating that other mechanisms also lead to changes in bone during *S. aureus* osteomyelitis **(Figure 1)**. OCs are able to resorb bone by forming a localized resorption compartment on bone with a low pH and secreted enzymes to mobilize mineral and digest the organic matrix (16-18). We have observed OC-like cell formation induced by *S. aureus* supernatants *in vitro* (see Preliminary Studies), which implicate an OC-mediated mechanism of bone loss during osteomyelitis.

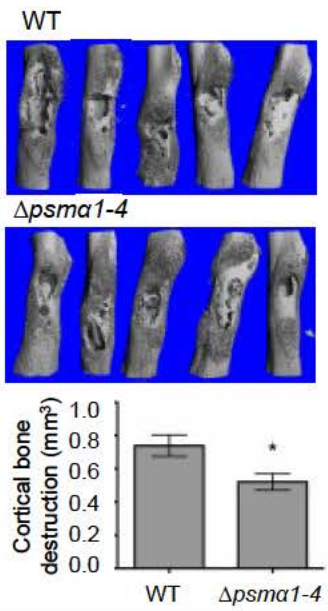


Figure 1. Toxin-deficient *S. aureus* induces dramatic bone remodeling. MicroCT scans of infected femurs on day 14 post-infection with WT *S. aureus* or Δ*psmα1-4* strains were used for 3D reconstruction and quantification of cortical bone destruction.

A4. RANKL/OPG axis mediates classic OC differentiation.

Classically, the cytokine RANKL signals through the RANK receptor of myeloid cells to drive OC differentiation *in vivo* (19). Mice deficient in RANK or RANKL do not have OCs, resulting in very dense bones, or severe osteopetrosis (20-22). The main source of RANKL necessary to initiate bone resorption *in vivo* is produced by OBs, although RANKL is also expressed by activated lymphocytes and osteocytes (23-26). Tight regulation of osteoclastogenesis is imposed by OBs, as they also produce the soluble decoy receptor, osteoprotegerin (OPG). OPG is an important physiologic inhibitor, in that OPG-deficient mice exhibit osteoporosis (27). To induce osteoclastogenesis RANK signaling must occur on a myeloid lineage cell in the presence of M-CSF, and with co-stimulation through immunoglobulin-like receptors TREM2 or OSCAR. Complex signaling pathways during OC differentiation lead to the activation of transcription factors NFκB, AP-1, and NFATc1 to induce OC-specific genes **(Figure 2)**.

A5. Innate recognition of *S. aureus* leads to crosstalk with osteoclastogenesis pathways.

S. aureus contains conserved molecular patterns that are recognized by PRRs, to initiate innate immune responses during infection. OB- and OC-lineage cells are known to express PRRs, although their contribution

to changes in bone physiology and pathogen clearance have not been defined during osteomyelitis. Innate sensing of bacteria and PRR activation leads to activation of transcription factors that are also involved in OC differentiation. Proinflammatory cytokine production downstream of PRRs has also been implicated in non-canonical OC differentiation (36-38). In regards to *S. aureus*, reports on osteoclastogenic effects are unclear due to the use of various pre-OC cultures and stimulation methods (39-42). The field would benefit from the clarity of a mechanism using primary skeletal cells and a clinically relevant strain of *S. aureus*. To address these concerns, we are using an isolate of *S. aureus* (LAC, a USA300-type strain) that represents the most common lineage causing bone infections, which may help clarify previous studies using laboratory or regional strains.

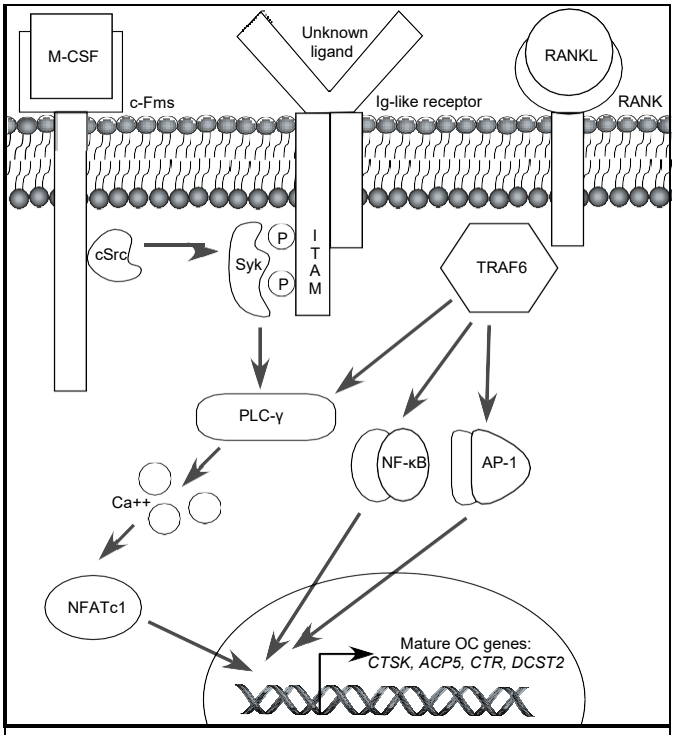


Figure 2. OC differentiation requires RANKL signaling in the presence of M-CSF and co-stimulation from an Ig-like receptor. M-CSF signaling through its receptor, c-Fms, leads to activation of cSrc kinase to phosphorylate ITAMs on a co-stimulatory Ig-like receptor, allowing for Syk kinase docking. Syk kinase and TRAF6-mediated signaling activate phospholipase C (PLC)-γ to cleave the substrate PIP_2. This reaction leads to cytoplasmic mobilization of calcium and activation of the canonical OC transcription factor (TF), NFATc1. RANK signaling through TRAF6 activates additional TFs necessary for osteoclastogenesis, including canonical and non-canonical NFκB and AP-1. In the nucleus, these TFs work together to induce OC-specific genes, *CTSK* (cathepsin K), *TRAP* (tartrate-resistant acid phosphatase), *CTR* (calcitonin receptor), and *DCST2* (DC-STAMP) (28-35).

A6. OC differentiation is also regulated by cytokine signaling and transcription factor activation.

Positive regulation of osteoclastogenesis occurs through indirect alteration of OB function or direct interaction with pre-OCs (11). Indirectly, cytokines IL-1 and TNFα favor OC differentiation through an increase of the RANKL/OPG ratio (11, 43, 44). Directly, alterations in osteoclastogenic potential of pre-OCs result from enhanced ability to undergo differentiation (45, 46), cell-cell fusion (38), increased survival (38, 41), intracellular signaling (TRAF6, PLC-γ) (47), activation of transcription factors (NFATc1), and receptor expression (RANK, c-Fms). Negative regulation of osteoclastogenesis occurs through decreased receptor expression (RANK, c-Fms, OSCAR, TREM2), decreased RANKL and M-CSF production, suppression of intracellular signaling, increased anti-OC transcription factors, or increased OPG levels (48). The proposed experiments will explore how *S. aureus* alters pre-OC cell biology via TLR/IL-1R signaling through transcription factor activation, gene expression changes, and functional OC differentiation **(Aim 1)**, and how innate recognition of *S. aureus* through MyD88-dependent receptors on skeletal cells induces physiological changes in bone and impacts pathogen clearance during osteomyelitis **(Aim 2)**.

B. INNOVATION

B1. The effects of *S. aureus* on bone at the cellular and organ level could describe common processes shared between sensing of the microbiota, infection, and inflammatory states.

Fundamentally, these Aims will determine how skeletal cells respond to microbes and how this leads to drastic changes in immune defenses and bone remodeling. Though RANKL-dependent osteoclastogenesis is well defined as the canonical OC differentiation signal, it has been shown that immunological crosstalk between parallel signaling pathways and transcription factors promote osteoclastogenesis (14, 49). Alterations in cytokines and transcription factor activity have been defined as potential mechanisms for non-canonical (RANKL-independent) osteoclastogenesis (15, 47). We plan to use primary cell culture models of both whole bone marrow (WBM) and bone marrow macrophages (BMMs) to explore differences in cellular stimulation, rather than using cell culture lines or limiting our scope to only one BM culture condition. Disparities in osteoclastogenic changes between WBM and BMM cultures will be revealed at the level of gene and protein expression. Furthermore, though phenotypic analyses are prevalent in the field, functional readouts are lacking in many studies. The proposed experiments aim to elucidate how *S. aureus* alters OC differentiation and function, contributing to the fundamental knowledge of bone biology and improved understanding of innate

immune signaling during osteomyelitis. Information uncovered in these experiments may identify critical bacterial targets to limit bone destruction during *S. aureus* osteomyelitis.

B2. Novel genetic mouse models will define the effect of innate sensing by skeletal cells in bacterial clearance and bone remodeling.

In 2013, our laboratory developed a murine model of post-traumatic bacterial osteomyelitis (12). Contrary to larger animal and fracture models of osteomyelitis, this infection model is advantageous for several reasons: we use a genetically tractable animal, we can precisely calculate colony-forming units (CFUs), we do not implant foreign bodies, and we have generated imaging analyses to accurately quantify bone remodeling. To date, many immune mediators hypothesized to alter OC differentiation have not yet been explored in an animal model of osteomyelitis. In addition to testing MyD88-null mice during preliminary studies, this proposal will result in the creation of novel mouse strains with skeletal cell-specific deletion of MyD88. Bone remodeling analyses will be important to determine the function of MyD88 in skeletal cells in the absence and presence of infection. These mice will be powerful new tools for the osteoimmunology field, which investigates the intersection of bone biology and immunology.

C. APPROACH

C1. Preliminary Studies

C1A. *S. aureus* supernatants modulate OC differentiation.

Our preliminary data demonstrate that *S. aureus* supernatants modulate differentiation of OCs with and without the canonical OC differentiation factor RANKL, in cultures of whole bone marrow (WBM), containing myeloid, lymphoid, and stromal cells, and in bone marrow macrophages (BMMs). BM cultures were stimulated with *psm*-deficient *S. aureus* supernatants to prevent cytotoxicity resulting from the α-PSMs in *S. aureus* wild-type supernatants. We observed that *S. aureus* supernatants drive BMM differentiation to OC-like cells without the addition of exogenous RANKL **(Figure 3A).** These cells are TRAP-positive, but differ in size and cell fusion from RANKL-treated BMMs. Alternatively, *S. aureus* supernatants inhibit osteoclastogenesis from BMMs following RANKL treatment by limiting the size (data not shown). These data support the notion that in pre-OC cultures, *S. aureus* induces RANKL-independent osteoclastogenesis and inhibits RANKL-induced osteoclastogenesis.

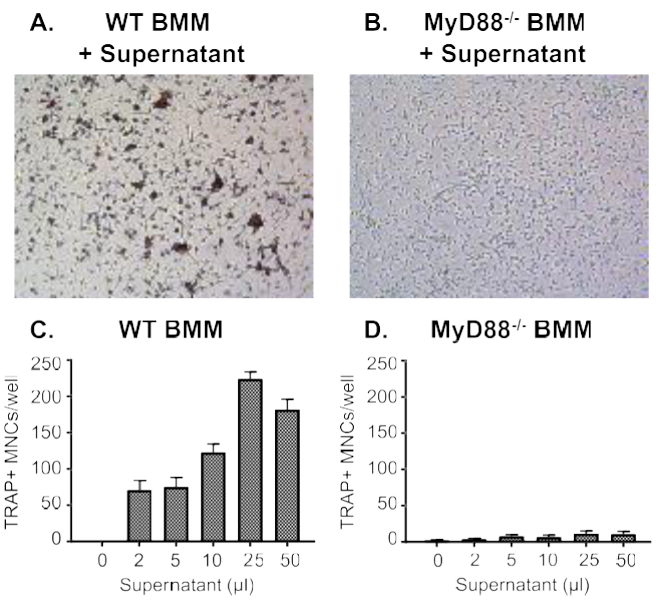


Figure 3. *S. aureus* supernatants are able to induce OCs from WT, but not MyD88-deficient BMMs. BMMs were stimulated for 7 days with M-CSF and concentrated *S. aureus* supernatants (50µl). WT (A) and MyD88 (B) are shown following fixation, TRAP stain, and 20X imaging. TRAP-positive multinucleated cells were quantified manually in triplicate from WT (A) and MyD88-deficient (B) BMM cultures with varying amounts of supernatants.

C1B. *S. aureus* supernatants do not modulate OC phenotypes in MyD88-deficient BMM cultures.

The innate immune response to bacteria is often mediated by TLRs and IL-1R, and all TLRs known to recognize *S. aureus* and IL-1 family receptors must signal through the MyD88 adaptor protein. In order to determine if TLR activation or IL-1 family receptor signaling are involved in *S. aureus*-induced changes on pre-OC differentiation, MyD88-deficient BM cells were used for osteoclastogenesis assays. Importantly, RANKL is similarly able to induce OC differentiation in both wild-type C57Bl/6 (WT) and MyD88-deficient BM cells (data not shown). In MyD88-deficient BMMs, *S. aureus* did not induce RANKL-independent differentiation of OC-like cells **(Figure 3B)**, nor did *S. aureus* diminish RANKL-dependent OC differentiation as observed in WT BMM cultures (data not shown). A quantitative analysis shows that *S. aureus* induces a robust, dose-dependent formation of OC-like cells from WT BMM **(Figure 3C)**, but not in MyD88-deficient BMM cultures **(Figure 3D)**. These data support a MyD88-mediated mechanism by which *S. aureus* perturbs OC differentiation, emphasizing the importance of innate sensing and signaling through MyD88 in modulating osteoclastogenesis.

C1C. MyD88 is critical for control of *S. aureus* replication and dissemination during bone infection.

WT mice show dramatic bone remodeling changes in our murine osteomyelitis model by day 14 post-infection **(Figure 1)**, but they are able to control bacterial replication and do not develop disseminated disease. To assess the role of MyD88 *in vivo* during osteomyelitis, MyD88-null mice were infected using various doses

of *S. aureus*. Our data reveal that MyD88-deficient mice are unable to control *S. aureus* replication, leading to higher bacterial burdens in the infected femur, and increased dissemination to other *S. aureus*-susceptible organs, causing some mice to succumb to disseminated *S. aureus* infection **(Figure 4)**. These data indicate that MyD88 is involved in the initiation of an immune response to *S. aureus* in bone to limit bacterial survival and dissemination.

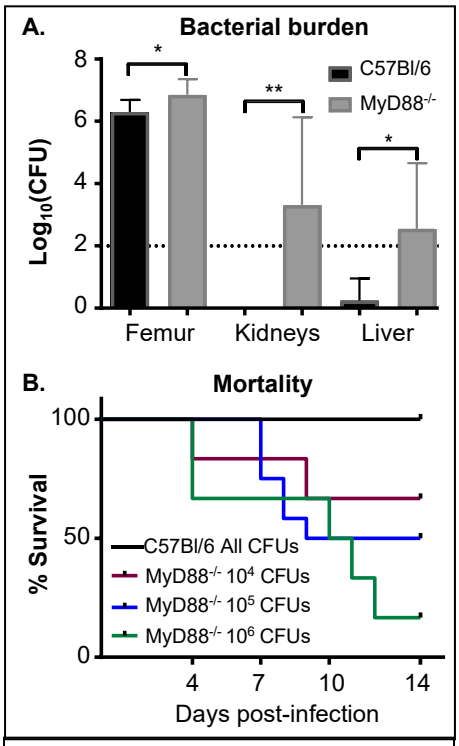


Figure 4. MyD88-deficient mice are unable to control *S. aureus* replication following osteomyelitis, resulting in increased bacterial burdens and death. WT C57Bl/6 and MyD88-deficient mice were infected with 10^5 CFUs. At day 14, CFUs were enumerated from the inoculated femur, kidneys, and liver (A). A mortality curve shows enhanced death in MyD88-deficient mice even if the infectious dose was dramatically reduced (B). * $p < 0.05$, ** $p < 0.01$.

C2. Specific Aims

Specific Aim 1: Define the role of TLRs and IL-1R in *S. aureus*-mediated perturbation of osteoclastogenesis.

Rationale and Hypothesis: Preliminary data show that bacterial components modulate OC differentiation from myeloid cells with and without the canonical OC differentiation factor RANKL by a MyD88-mediated mechanism. Specific TLRs and IL-1 family cytokines require the critical adaptor protein MyD88 to transduce their intracellular signals, emphasizing the importance of innate recognition in modulating osteoclastogenesis. Of unique interest are the TLRs 1, 2, 6, and 9 because they have been implicated in OB sensing of *S. aureus* and signal through MyD88 (7). Extracellular TLR1/2 and TLR2/6 heterodimers are known to engage *S. aureus* lipoproteins (50, 51) and *S. aureus* CpG DNA stimulates TLR9 during bacterial replication in the endosome (52). I hypothesize that *S. aureus* modulates pre-OC cell biology through TLR recognition or IL-1R signaling upstream of MyD88. The proposed experiments will characterize the potential of *S. aureus*-specific TLRs and IL-1R in modulating OC differentiation and the inflammatory environment.

Aim 1A: Determine effects of bacterial stimulation, TLR ligation, and IL-1 signaling on osteoclastogenesis in primary BM cultures.

Experimental Design: Osteoclastogenesis assays will be performed in primary murine WBM or BMM cultures from 8-12 week old, male C57Bl/6 mice and mice deficient in TLR2, TLR9, or IL-1R. Mice deficient in TLR2 will be unable to signal through TLR1 and TLR6, as they require TLR2 to signal. Preliminary data show no difference in OC differentiation in male versus female BM (data not shown). BM cultures will be supplemented with M-CSF and with or without RANKL (46). To determine the effects of bacterial and TLR/IL-1R stimulation between RANKL conditions, BM cultures will be stimulated the following day with bacterial supernatants, TLR agonists, recombinant IL-1, or vehicle controls. BM cultures treated with M-CSF (negative control) will monitor baseline osteoclastogenic phenotypes, whereas BM cultures stimulated with M-CSF and RANKL (positive control) will monitor normal OC differentiation *in vitro.*

After seven days, cultures will be fixed and stained for expression of tartrate-resistant acid phosphatase (TRAP). TRAP is an enzyme dramatically upregulated during OC differentiation, which makes it a widely accepted histochemical marker for identification of mature OCs (53). Quantification of OCs from these assays will be completed using the OsteoMeasure software in the Vanderbilt Center for Bone Biology (VCBB) facility, with OCs defined as TRAP-positive cells with 3 or more nuclei present **(Figure 3)**. Finally, the functional ability of TRAP-positive, multinucleated cells to resorb bone will be assessed by differentiating BM cultures on dentin chips, which are small sections of hydroxyapatite-mineralized organic matrix similar to bone. Functional OCs will be defined by their ability release soluble cross-linked type I collagen peptides (Ctx-1) and form resorption pits on the dentin surface. Ctx-1 will be quantified using an ELISA and dentin will be stained with 0.5% toluidine blue to visualize resorption pits (28). These experiments will provide phenotypic and quantitative data of how bacterial, TLR, and IL-1 stimulation alter differentiation of BM-derived OC-like cells, and more importantly, if these conditions induce functionally resorbing OCs.

Anticipated Results, Potential Problems, and Alternative Approaches: Modulation of OC differentiation has been published elsewhere to occur by divergent mechanisms, where non-canonical OC-specific signaling has induced TRAP-positive *multinucleated* cells with dramatically reduced ability to resorb bone, as well as TRAP-positive *mononuclear* cells that are unable to fuse but are still functionally active (15). Based on the supporting data for this aim, both outcomes are plausible. We expect that if our IL-1R-deficient BMMs differ

from WT during OC differentiation, this may reflect IL-1 produced by cells recognizing pathogens through TLRs, or inflammasome activation through NLRs. We will test this by adding recombinant IL-1R antagonist to cultures and testing cells from mice deficient in critical components of the inflammasome.

It is possible that we will see no bone resorption over the course of a 7-day assay, in which case we will lengthen our assays, as other groups perform culture resorption assays over the course of 14 to 21 days. Additionally, if select TLR- or IL-1R-deficient mice do not express robust phenotypes, we will test BM cultures from the NOD intracellular receptors capable of recognizing cytoplasmic *S. aureus* (54, 55), or other IL-1 family cytokine-deficient mice. In total, Aim 1A will provide quantification and functional analysis of OC-like cells to characterize the extent of differentiation down the OC lineage due to *S. aureus* and TLR stimulation *in vitro*.

Aim 1B: Define expression of TLRs and OC-modulating receptors on pre-OCs throughout osteoclastogenesis.

Experimental Design: In order to determine changes in expression of receptors, TLRs, and other known factors that modulate OC differentiation, we will isolate RNA over the course of our osteoclastogenesis assays, and use quantitative RT-PCR (qRT-PCR) to measure transcripts of the corresponding genes. Specifically, we will monitor RANKL and OPG transcription, which balance the main axis that controls bone homeostasis *in vivo* (14, 37). We will also measure transcripts from receptors with the ability to alter osteoclastogenic potential of cells, such as RANK, c-Fms, OSCAR, or TREM2 necessary for pre-OC signaling (22, 56), and the newly described RANKL-binding, inhibitory receptor LGR4 (57). Importantly, Aim 1B will define the TLR repertoire expressed throughout the course of differentiation to determine how OC differentiation impacts TLR expression. These data will describe the RANKL/OPG axis *in vitro*, and define which receptors may be necessary for the promotion or inhibition of osteoclastogenesis.

Anticipated Results, Potential Problems, and Alternative Approaches: We expect that RANKL and OPG levels will not be dramatically altered in BMMs, as myeloid cells are not described as a major source of either *in vivo*. However, WBM cultures may allow for production of these signaling molecules. We expect that receptor expression of RANK, OSCAR, TREM2, LGR4, or TLRs varies over the course of differentiation. These experiments will provide potential upstream mechanisms in pre-OCs that lead to altered OC differentiation upon exposure to *S. aureus*. We may find that these receptors remain constant at the mRNA level, in which case protein levels will be monitored using Western blots.

Aim 1C: Measure how *S. aureus* alters osteoclastogenic signaling pathways and transcription factors.

Experimental Design: Osteoclastogenic signaling is complex, and many components crosstalk with other immune signaling pathways. To determine if *S. aureus* modulation of OC differentiation is due to signaling changes, qRT-PCR will be performed on the transcription factors NFATc1 and Blimp1, which require an increased expression to induce OC differentiation. Activation of signaling components will be analyzed using Western blot detection of activated proteins (PLC-γ, p38, IκBα, cFos) using phospho-specific antibodies (58), and preparation of the nuclear fraction to detect translocated NFATc1 and c-Jun using subcellular fractionation. Additionally, mature OC marker expression will be measured throughout stimulation, including DC-STAMP, cathepsin K and the calcitonin receptor (22, 56). These experiments will determine perturbations of normal RANKL-mediated osteoclastogenesis and define the signaling elements activated by *S. aureus* over the course of differentiation.

Anticipated Results, Potential Problems, and Alternative Approaches: We expect that functionally active OC-like cells phenotyped in Aim 1A will display typical OC signaling activity throughout differentiation, such as the upregulation of NFATc1 and Blimp1 and expression of mature OC markers such as cathepsin K and the calcitonin receptor, regardless of RANKL-stimulation. Furthermore, we expect that inhibition of RANKL-stimulated osteoclastogenesis will cause a decrease in osteoclastogenic signaling, relative to RANKL treatment alone. To rule out differentiation toward other myeloid cell lineages, we will test for M1 and M2 macrophage differentiation, by measuring mRNA expression of *nos2* and *arg1*, respectively. If issues arise with detection of phosphorylated signaling molecules, signaling changes that occur before cell-cell fusion will be analyzed using intracellular flow cytometry after fixation, permeablization and incubation with fluorescently labeled phospho-specific antibodies. Taken together, these data will detail how bacterial stimulation specifically modulates OC differentiation and function through TLR sensing and IL-1 signaling.

Specific Aim 2: Elucidate the role of skeletal cell-specific MyD88 signaling on pathogen clearance and bone remodeling during *S. aureus* osteomyelitis.

Rationale and Hypothesis: Aim 1 will identify changes in OC differentiation and function induced by *S. aureus*, and will define the roles of innate immune sensors in these processes *in vitro.* Supporting data show that mice completely lacking MyD88 are more susceptible to *S. aureus* osteomyelitis, leading to increased

bacterial burdens in bone, systemic dissemination to liver and kidneys, and death **(Figure 4)**. I hypothesize that innate sensing of *S. aureus in vivo* by OBs and OCs will alter bone remodeling through signaling and induction of inflammation. The experiments outlined below will elucidate the role of downstream signaling through MyD88 on limiting *S. aureus* infection during osteomyelitis and in bone remodeling alterations.

Aim 2A. Determine how skeletal cell-specific MyD88 influences *S. aureus* clearance from bone and dissemination to other organs during osteomyelitis.

Experimental Design: Our *S. aureus* osteomyelitis model has previously been used to infect WT and MyD88-deficient mice **(Figure 4)**. To determine if MyD88 signal transduction is critical in skeletal cells during *S. aureus* osteomyelitis, we will infect skeletal cell-specific MyD88 knockout mice created with Cre-Lox breeding schemes. MyD88-floxed mice will be bred to Osteocalcin-*cre* and Cathepsin K-*cre* mice to create mice with MyD88-deficient mature OBs and OCs, respectively. To determine how immune defenses are affected when skeletal cells are unable to activate the downstream signaling through MyD88, *S. aureus* CFUs present at the primary infection site and distant organs will be enumerated following development of osteomyelitis.

Anticipated Results, Potential Problems, and Alternative Approaches: The creation of novel skeletal cell-specific knockout mice will allow us to determine the importance of MyD88 signal transduction in skeletal cells during bone infection. Specifically, we expect to observe changes in the ability of mice to limit *S. aureus* infection. MyD88 transduces many signals to mount an innate immune response, which likely led to the mortality phenotype in MyD88-deficient mice **(Figure 4B)**. However, we do not expect as severe of a mortality phenotype in cell-specific knockout mice. If mortality does remain prevalent, we will test a range of inocula between 10^3-10^6 CFUs. Additionally, the role of MyD88 in skeletal cell *precursor* cells may be of interest. Cell-specific deletion of MyD88 in OB and OC precursors can be accomplished by crossing MyD88 floxed mice with Osterix-*cre* or LysM-*cre* mice, respectively. However, these models are likely to have deletions in other cell lineages. Alternatively, to identify changes in MyD88 signaling through pre-OCs, BM transplants between WT and MyD88-deficient mice will be done, as OBs and mesenchymal cells are resistant to radioablation (59).

Aim 2B. Quantify changes in bone remodeling and osteoclastogenesis *in vivo* during experimental *S. aureus* osteomyelitis.

Experimental Design: Quantitative changes in bone architecture that occur during *S. aureus* osteomyelitis will be measured with the assistance of the Vanderbilt University Institute of Imaging Science (VUIIS) (see letter of support from Dr. Dan Perrien). Mock-infected bones will be used as an uninfected healing control relative to infected femurs from the same mouse strain to avoid direct comparisons between different mouse genotypes. To quantify bone remodeling, microCT will be used to measure cortical bone changes around the inoculation site and trabecular bone architecture, including bone volume/trabecular volume (BV/TV), trabecular thickness, trabecular spacing, and trabecular number.

Osteoclast changes during *S. aureus* osteomyelitis will be defined using histologic analyses of infected bone, with support from the VCBB facility (see letter of support from Josh Johnson). Following microCT scans, femurs will be decalcified and embedded for sectioning and staining with bone-specific hematoxylin and eosin and TRAP stains. OCs will be quantified from histological sections by counting TRAP-positive MNCs next to trabeculae in infected or mock-infected femurs. Bone resorption activity of OCs will also be monitored *in vivo* using a calvarial injection model, where concentrated *S. aureus* supernatants or vehicle control will be injected over the calvaria for 5 consecutive days. Resorption pits on calvaria will be quantified after 9 days using microCT analysis, and the mineral apposition rate (MAR) and bone formation rate (BFR) will be quantified using histologic measurement of calcein fluorescent double labels incorporated into the bone.

Anticipated Results, Potential Problems, and Alternative Approaches: We expect that changes in OC number and bone resorption will be discovered *in vivo* following stimulation with live *S. aureus* or concentrated supernatants. Specifically, we expect that *S. aureus* recognition or the induced proinflammatory cytokine response will promote OC differentiation. It is possible that defects in immunocompromised mice will lead to higher bacterial burdens at day 14 post-infection, which will complicate bone destruction data. To mitigate this issue we can surround mock-infected femurs from WT or cell-specific MyD88-deficient mice with scaffolds eluting heat-killed bacteria or concentrated supernatants, as previously described in a manuscript from our lab, to determine the effect of bacterial components on bone remodeling (60).

Collectively, these Aims will elucidate bacterial-induced mechanisms of altered bone remodeling and osteoclastogenesis, and will further define the ability of skeletal cells to respond to innate immune mediators, including pathogen associated molecular patterns and IL-1 family cytokines.

RESPECTIVE CONTRIBUTIONS

Concept

Dr. Jim Cassat (sponsor) and I share an interest in understanding host-pathogen interactions that trigger changes in human cell biology. We began discussing ideas for thesis projects when I joined the laboratory in May 2015. We are both excited to define how bacteria stimulate host skeletal cells to induce dramatic changes in bone remodeling processes, which are normally under tight regulation.

Project Design

In order to best study skeletal changes in bone remodeling, Dr. Cassat and I began to design my project with the support of Dr. Julie Sterling (co-sponsor), Assistant Professor in Medicine, Clinical Pharmacology, and Cancer Biology, and faculty member in the Vanderbilt Center for Bone Biology. Earlier work done in the Cassat laboratory demonstrates that bacterial strains deficient in toxins to induce skeletal cell death are still able to induce considerable bone destruction and aberrant bone formation during murine osteomyelitis. Relevant literature in the field describes inflammation-induced changes in bone homeostasis driven by alterations in bone-forming osteoblasts and bone-resorbing osteoclasts. Therefore, Dr. Cassat, Dr. Sterling and I designed preliminary experiments to test the impact of bacteria on osteoclast differentiation *in vitro* and the impact of innate immune recognition on bacterial clearance and bone homeostasis. From these promising results outlined in the Research Strategy, we developed a plan to discover the mechanism by which skeletal cells sense and respond to bacteria leading to alterations in bone remodeling.

Fellowship Application

I designed the studies, optimized the experiments, and generated all of the preliminary data included in this application. From these data, I have developed a series of hypotheses and formulated specific aims to test these hypotheses. I have documented these through writing of the Research Strategy of this F31 proposal, and then addressed feedback from Drs. Cassat and Sterling on this proposal.

Future Contributions

I will design and perform all of the experiments proposed in the Research Strategy, with some contributions from others. The advanced surgical techniques for the *in vivo* osteomyelitis model require Dr. Cassat, however, I will follow up with animal care and monitoring, as well as end point harvest and analysis. Additionally, Josh Johnson (see letter of support), who has years of experience in bone histology and is the manager of the Bone Histology core, will embed and section my samples. I will perform all staining of histological sections, all data interpretation from experiments, and statistical analyses with input from Drs. Cassat and Sterling.

Committee meetings will be held biannually, chaired by Dr. Eric Skaar (see letter of reference), and attended by my co-sponsor Dr. Julie Sterling, as well as Drs. Jeff Rathmell, Dan Moore, and Isaac Thomsen (see letters of reference). These meetings will provide the opportunity for my committee members to give feedback on my proposed experiments and data analysis, which will further enhance the quality of this proposed Research Strategy.

SELECTION OF SPONSOR AND INSTITUTION

Selection of Institution

When applying for Ph.D. programs, I was in search of an institution with a history in exceptional in graduate education and extramural funding, with access to core facilities led by experts, and a highly collaborative environment. Vanderbilt University Medical Center (VUMC) has an incredible atmosphere for training in biomedical science and establishing lasting collaborations, due to its large incoming class in the Interdisciplinary Graduate Program (IGP) in Biomedical Sciences with comprehensive coursework covering topics from all eleven degree-granting departments. Additionally, between Vanderbilt University (VU) and VUMC, there are over 60 core facilities, providing expertise in biostatistics, histology, animal care, immunology, molecular biology, and advanced genetics techniques, as well as resources for access to DNA repository data, poster printing, and advanced imaging techniques, among many others. Furthermore, the Office of Biomedical Research, Education, and Training (BRET) provides several training programs and career development opportunities through the NIH BEST Grant-funded ASPIRE Program. The selection of Vanderbilt University as my Ph.D. institution was primarily driven by its record of excellence in graduate education, the highly collaborative environment between departments and physicians, the ease of access to sophisticated methods and experts, the comprehensive structure of the graduate program, and the unique training and career development resources available to students through the BRET office.

Selection of Sponsor

After receiving my Master's degree in Molecular Microbiology and Immunology, I hoped to continue my Ph.D. studying host-pathogen interactions. I was first excited about Dr. Jim Cassat's research during a series of presentations for student recruitment in the initial few weeks of IGP. His research was particularly intriguing because of the convergence of disciplines between microbial pathogenesis, host immune responses, and skeletal biology, and the use of the cutting-edge imaging resources at Vanderbilt University. My interest in studying infectious diseases of public health importance was matched with Dr. Cassat's first hand experience as a physician-scientist, who sees patients at the Monroe Carell Jr. Children's Hospital at Vanderbilt in Pediatric Infectious Diseases. I was enthusiastic about using my background in microbiology and immunology to ask and answer important questions on how staphylococci influence on bone remodeling. I chose to rotate in Dr. Cassat's laboratory first, and was thrilled that Dr. Cassat personalized a project to my incoming skillset. As a new faculty member, Dr. Cassat was able to invest the time and effort to train me directly. I was impressed by his investment to individually train rotation students and this was a very productive rotation. My decision to join Dr. Cassat's laboratory was ultimately guided by his training style, mutual interests to ask complex questions about the intersection between scientific disciplines to study *Staphylococcus aureus*-induced bone disease, and my enthusiasm to understand the interactions between bacteriology, immunology and bone biology in the context of this project.

Selection of Co-sponsor

Dr. Cassat's affiliation with the Vanderbilt Center for Bone Biology introduced me to several bone biology specialists, an area where I desired to expand my training outside the Department of PMI to fully develop my project. I met Dr. Julie Sterling during the weekly Bone Biology Seminars, and frequently found myself reviewing her published articles and meeting with Dr. Sterling and members of her laboratory to glean information, compare protocols, and develop experiments. Dr. Sterling primarily studies bone metastasis and tumor invasion of bone, among which several research parallels can be drawn between the impact of cancer and infection on bone remodeling. Shortly after joining Dr. Cassat's research team in 2015, I was granted funding through the Vanderbilt Center for Molecular Pathogenesis in the form of a Mini-Sabbatical Award to periodically train with Dr. Sterling's laboratory throughout 2015-2016 to be oriented on the proper equipment and facilities necessary to perform bone histology techniques. This was an extremely beneficial experience, and the Sterling laboratory has been a great resource to guide the development of my research proposal. Furthermore, Dr. Sterling serves as a member of my thesis committee, which meets biannually to offer research support and career guidance. Interactions with the Sterling laboratory have already contributed to the success of preliminary experiments. There is no doubt that the expertise Dr. Sterling offers will be a strong supplement to the success and completion of the proposed research.

TRAINING IN RESPONSIBLE CONDUCT OF RESEARCH

BRET RCR Training

Vanderbilt University provides introductory and ongoing training in Responsible Conduct of Research (RCR) to graduate students and postdoctoral fellows through the Biomedical Research, Education, and Training (BRET) Office to satisfy NIH RCR requirements.

Annual Introduction to RCR for Incoming Students

The BRET Office offers an introduction to laboratory ethics to incoming graduate students as a component of orientation. The focus of this course is to define and prevent research misconduct in the forms of plagiarism, fabrication, and falsification by proper methods of record keeping, data management, and sharing. The a 2 hour course led by Dr. Roger Chalkley, Senior Associate Dean for Biomedical Research, Education, and Training, includes lectures, discussions, and case studies. I completed this course in August 2014.

Annual RCR Symposium

Each May, the BRET Office organizes a full day (9 hour) symposium that addresses the RCR issues outlined by the Office of Research Integrity (ORI). I completed this training after choosing my thesis laboratory in May 2015, at the end of the first year in the graduate program. Faculty presented face-to-face lectures to expose students to the nine areas of focus for RCR, followed by case studies and small group discussions, with detailed ORI subject matter noted below each topic:

1. Overview of Institutional & NIH Policies regarding grants, research, animal use, and human subjects (Drs. Roger Chalkley, Alyssa Hasty)
    i. Policies regarding humans/live vertebrate animals, safe laboratory practices
    ii. Collaborative research

2. What biomedical scientists in training need to know about the NIH funding system: R01s, Training Grants, Program Project Grants, and Center Grants (Drs. Roland Stein, Tony Weil)
    i. The scientist as a responsible member of society, contemporary ethical issues in biomedical research, and the environmental and societal impacts of scientific research

3. Data Management, Record Keeping and Conflict of Interest (Drs. Danny Winder, Roger Colbran)
    i. Conflict of Interest
    ii. Data acquisition and laboratory tools, management, sharing and ownership

4. Authorship and Publication (Drs. Roger Colbran, Rebecca Ihrie)
    i. Peer review
    ii. Responsible authorship and publication

5. Reproducibility (Drs. Brian Welch, Melanie Ohi)
    i. Research misconduct and policies for handling misconduct

6. Mentorship (Drs. Rebecca Ihrie, Melanie Ohi)
    i. Mentor/mentee responsibilities and relationships

Ongoing Training in RCR

Training in Responsible Conduct of Research is constantly ongoing in the Cassat laboratory environment. On a weekly basis, Dr. Cassat and I meet individually to discuss the progress of my research Aims and informally discuss ORI issues as they develop, such as data acquisition, management, and sharing of data. These interactions extend into our weekly laboratory meetings, making RCR a common topic of conversation in the laboratory. For example, Dr. Cassat facilitates discussions in RCR whenever an opportunity arises, such as allowing graduate students in the laboratory to assist in the peer review of manuscripts. Finally, our ongoing training in RCR is formally supplemented with lab meeting presentations covering each of the 9 areas of RCR every 1-2 months.

SPONSOR AND CO-SPONSOR STATEMENTS

I. Sponsor Statement

A. Research Support Available

	Source	Identifying Number	Title of Research	Principal Investigator	Dates	Award Amount (annual)
Active	NIH/NIAID	6 K08 AI113107- 03	Host-pathogen interactions during osteomyelitis	James E. Cassat	6/1/2014-5/31/2019	$172,098
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

B. Sponsor's Previous Fellows and Trainees

After completing a residency in Pediatrics and a clinical fellowship in Pediatric Infectious Diseases in 2014, I joined faculty at Vanderbilt as an Assistant Professor on the physician-scientist tenure track in the Departments of Pediatrics and Pathology, Microbiology, and Immunology. My laboratory currently consists of two graduate students in the Microbiology and Immunology program, an MSTP student in the Biomedical Engineering program, and a co-sponsored graduate student in the Chemical and Biomolecular Engineering program. As a junior faculty member, I have no previously sponsored trainees. Therefore, to support Nicole Putnam during her graduate fellowship, two senior mentors will play key roles in the Training Plan. Dr. Eric Skaar serves as the mentor on my K08 award and will serve as the chair of Nicole's thesis committee. Dr. Skaar has trained 11 graduate students and 13 postdoctoral fellows, 6 of which now hold tenure track positions. Dr. Julie Sterling, a primary facultymember in the Vanderbilt Center for Bone Biology, will serve as Co-Sponsor (see below).

C. Training Plan, Environment, Research Facilities

Training Plan

Nicole joined the Vanderbilt Interdisciplinary Graduate Program in 2014 after completing undergraduate studies at the University of Wisconsin-La Crosse and a Master of Science Degree in Molecular Microbiology and Immunology from the Johns Hopkins Bloomberg School of Public Health. Her master's thesis work was performed in the laboratory of Dr. Diane Griffin, an international leader in the fields of viral pathogenesis and T-cell responses to viral pathogens. Nicole's project in the Griffin lab focused on characterization of Th17 responses to measles infection in non-human primates. This training experience provided strong foundational knowledge in innate and adaptive responses to human pathogens, and led to Nicole's desire to study microbial-host interactions during her doctoral training at Vanderbilt. I was thrilled when Nicole picked my laboratory for one of her rotations during the first year of graduate school. It was immediately clear that she was a talented and hard-working student, and that her prior research experiences positioned her to address fundamental questions in the pathogenesis of invasive infections. My laboratory studies host-pathogen interactions during osteomyelitis, a common and debilitating infection of bone most commonly caused by the human bacterial pathogen *Staphylococcus aureus*. Given her prior training in T-cell biology, Nicole immediately noted the field-changing observation from the early 2000's that the canonical differentiation factor for bone-resorbing cells, or osteoclasts, was a T-cell cytokine called RANK-ligand. She began to ask broad questions about how inflammation from invasive bacterial infections might perturb osteoclast biology, and the potential for osteo-immunologic crosstalk. She envisioned a project focused on understanding how *S. aureus* triggers

changes in osteoclast differentiation and behavior, and how innate and adaptive immune responses protect from, or contribute to, the pathogenesis of osteomyelitis. After completing her rotations, Nicole agreed to join my lab in 2015, where she has already had an enormous impact on my research program and on our understanding of how infectious and inflammatory stimuli impact bone homeostasis. We now propose a focused line of inquiry that will align her research and career goals as she completes doctoral training.

Although osteomyelitis is one of the most common manifestations of invasive bacterial infection, the pathogenesis remains poorly understood. Given the extreme morbidity and treatment recalcitrance associated with bone infections, there is an urgent need for new diagnostic approaches, antimicrobial treatments, and adjunctive therapies. The mechanisms by which bacterial pathogens invade, survive within, and ultimately trigger alterations in bone homeostasis are poorly understood. Moreover, very little is known about the immune responses to bone pathogens, largely do to the lack of genetically tractable animal models. Nicole therefore recognized the opportunity to accomplish two of her major career goals by training in my laboratory: to study an infectious disease with a drastic public health burden, and to conduct translational research on host-pathogen interactions. Shortly after joining my lab full time, Nicole made the exciting, and potentially groundbreaking, observation that *S. aureus* and other bacterial pathogens can trigger osteoclastogenesis independently of the canonical differentiation factor RANK-ligand. This finding suggests that bacterial factors can be sensed by skeletal progenitors, and that subsequent cellular responses can fundamentally alter bone cell behavior. In order to further elucidate the underlying mechanisms for bacterial-induced osteoclastogenesis, Nicole needed to incorporate a number of new assays into our lab. Of her many contributions to our research program, perhaps the most important was adopting all of the reagents and protocols necessary to study skeletal cell ontogeny and behavior in our lab. Since my training has been primarily in microbial pathogenesis, Nicole took the initiative to become the lab's expert in bone biology. In order to gain the necessary expertise, she reached out to Dr. Julie Sterling, a founding member of the Vanderbilt Center for Bone Biology and co-sponsor for Nicole's fellowship project (see co-sponsor statement below). Nicole will now unite her interests in host-pathogen interactions and skeletal cell biology to discover the microbial factors and host-pathways involved in altered osteoclastogenesis.

Nicole's project has the potential to significantly impact human health by uncovering pathways that lead to bone destruction, thereby contributing to the morbidity of osteomyelitis and facilitating treatment failure. Importantly, in research occurring parallel with Nicole's dissertation project, we are exploring new local drug delivery strategies to facilitate targeted therapy for bone diseases. This work will ensure that we are in a position to immediately capitalize on Nicole's basic science discoveries, thereby fulfilling her career goal to participate and direct translational research efforts. Additionally, by studying the host signaling pathways that govern skeletal homeostasis in response to infectious and inflammatory cues, Nicole's work is poised to reveal biologic responses that may impact other human diseases, such as rheumatologic, auto-inflammatory, and oncologic disease of bone.

I believe that my laboratory, the Department of Pathology, Microbiology, and Immunology, the Center for Bone Biology, and the Vanderbilt academic community as a whole offer the ideal environment for Nicole to complete her doctoral training and achieve her career goal of becoming a translational scientist in the field of host-pathogen interactions. Of particular importance to Nicole's project are the outstanding core facilities at Vanderbilt, including a world-renowned imaging center – the Vanderbilt University Institute for Imaging Sciences (VUIIS). Our lab is uniquely positioned to study how bacterial pathogens impact bone remodeling in part because we have created a new genetically tractable animal model of osteomyelitis, and subsequently have adopted some of the outstanding imaging technologies available in the VUIIS. During her doctoral training, Nicole will learn these advanced imaging techniques for skeletal tissues, while also becoming proficient in a host of new skills that will empower her research on bone biology and ensure that she is well positioned for the next stage in her career. The Vanderbilt Center for Bone Biology provides exemplary resources for the study of bone pathology, and the inclusion of Dr. Sterling as a co-sponsor will allow Nicole to effectively bridge the fields of bone biology and infectious diseases to be positioned at the forefront of osteoimmunology research. Finally, as a new faculty member who has benefited enormously from outstanding mentoring, I recognize the paramount importance of establishing effective mentoring for Nicole. We have therefore carefully selected her thesis committee with experts in host-pathogen interactions, bone biology, immunology, and translational research. Nicole and I meet formally each week, and have daily discussions on her research and career development activities. The entire lab meets once weekly as a group, at which time we discuss research in progress, the responsible conduct of research, and essentials in public speaking and written science communication. To ensure that Nicole learns the skills necessary for critical review of

manuscripts and grant applications, she reviews 3-4 manuscripts per year under my guidance, and is expected to provide peer review of all funding applications submitted from the lab. Nicole also participates in a variety of seminars, interest groups, and career development programming across the institution. Collectively, these activities ensure that Nicole will have institutional support and mentoring to complete her research training.

Environment

Members of my laboratory participate in a number of meetings and seminars. Given Nicole's focus on host-pathogen interactions and bone biology, we have carefully selected institutional activities to ensure that she is surrounded by colleagues that will enrich her training experience and facilitate her career goals. Weekly seminars include the Pathology, Microbiology, and Immunology Departmental Seminar, in which local and invited scientists present topics across the spectrum of host-pathogen interactions, and the Microbiology and Immunology Research In Progress series, in which pre- and postdoctoral trainees present their own work and receive critical feedback on their project. Twice monthly, Nicole will participate in the Microbial-Host Interactions meeting, in which investigators across multiple departments and divisions at Vanderbilt come together to discuss microbial pathogenesis. Once monthly, Nicole will attend the new Frontiers in Infection, Inflammation, and Immunity seminar series, where field-leading scientists come to Vanderbilt to present cutting edge research. Since Nicole envisions a career in translational science, she applied, and was subsequently accepted to, the prestigious Vanderbilt Program in Molecular Medicine (VPMM). The VPMM was established through funding from the Howard Hughes Medical Institute in 2010, with the overarching goal of training the next generation of translational scientists. VPMM training seeks to align basic science trainees with a clinical mentor so that they can experience the clinical challenges associated with the pathologies that they study. This experience has allowed Nicole to see patients suffering from osteomyelitis and other invasive staphylococcal diseases, and also to gain an appreciation for the challenges in diagnosis and treatment of these infections. In addition to these transformative clinical experiences, Nicole attends a bi-monthly VPMM seminar series and an annual retreat. I therefore feel confident that Nicole is in an outstanding environment to support her growth as a translational scientist. Finally, Vanderbilt offers an exciting array of career development opportunities through the Office of Biomedical Research Education and Training. Notably, this includes a "Career Connections" seminar series and symposium aimed at exposing trainees to the diverse opportunities for careers in the biomedical sciences, rather than simply assuming that all students will run an independent, NIH-funded laboratory. Because of these outstanding research and career development activities, I feel strongly that Vanderbilt is the ideal environment for Nicole to complete her training and achieve her research and career objectives.

Research Facilities

Laboratory: The Cassat laboratory, located in 1035B Light Hall, consists of 850 square feet of space and is sufficient to accommodate a team of 8-10 people. The lab contains capital equipment, biologicals, two tissue culture hoods, and supplies required for standard molecular biology and biochemistry techniques (see Equipment). Adjacent facilities with immediate access include a cold room and equipment corridor. The Cassat laboratory is designated as a Biohazard Safety Level 2 facility meaning that all equipment and resources are in place to work with the biohazardous agent described in this application. Furthermore, all members of the Cassat laboratory undergo extensive training prior to working with biohazardous material. We have worked closely with the Institutional Biosafety Officer at Vanderbilt to ensure that BSL-2 practices are maintained during all facets of this proposal.

Computer: The Cassat lab is equipped with 4 PC and 3 iMac computers, each with ample video processing and memory capabilities to support advanced imaging analysis. Each computer is connected to the Vanderbilt network providing direct access to current versions of Genebank, EMBL, and other protein/nucleic acid sequence databases. Each computer includes software for imaging analysis, advanced image editing and graphics production (Adobe Professional Suite), word processing (Microsoft Office Suite), statistical software (Prism) and genomic analysis tools (CLC bio). All computers in the Cassat lab are linked to a shared server that can be accessed from each computer ensuring that all data are backed up on tape. A networked, color laser printer is housed in the Cassat laboratory space.

Equipment: The Cassat laboratory has all the requisite equipment to study the molecular biology of microbial pathogens and skeletal cell biology. In regards to this proposal, the equipment includes a Thermo Sorvall Lynx 6000 superspeed centrifuge with general purpose and ultraspeed rotors, Thermo Sorvall Legend XTR benchtop centrifuge; Thermo Micro21 and 21R benchtop microcentrifuges, Locator Jr. Plus Cryo Vessel for liquid nitrogen cell storage, Nuaire Class II Type A2 Biosafety Cabinet (2 ea), Olympus inverted microscope CKX53 with QImaging OQCLR5 digital camera, Eppendorf Nexus Mastercycler Gradient Thermocycler, Mettler

Toledo Excellence balance, Thermo Forma series CO_2 incubators (2 ea), New Brunswick Innova Model 44 stackable incubator shaker, Thermo MaxQ4450 tabletop shaking incubator, Fisher Isotemp General Microbiologic Incubators (3 ea), Isotemp bath incubators (3 ea), three variable speed rotating shakers, a BioTek Hybrid Synergy microplate reader, GeneSys 10S UV-VIS spectrophotometer, Next Advance Bullet Blender for bone homogenization, UVP GelDocIT Gel Documentation system, Thermo TSU series 600 -80°C freezer, Thermo IsoTemp -20°C freezer, Thermo MR49PA 4°C refrigerator, Mettler Toledo S220 pH meter, protein purification columns and reagents, BioRad polyacrylamide gel casting equipment and Western transfer apparatus, and a BioRad GenePulser Xcell electroporation apparatus. The Center for Small Animal Imaging (CSAI) in the VUIIS contains biomedical imaging instruments spanning a wide range of modalities, including MRI, CT, PET, SPECT, ultrasound, bioluminescence (BLI), fluorescence, and optical imaging. Equipment includes a 4.7T Varian MRI, 9.4T Varian MRI, 15.2T Bruker Biospec MRI, Xenogen IVIS 200 bioluminescent and fluorescent imaging system, Scanco μCT40 and μCT50 micro-CT scanners for *ex vivo* imaging, Siemens MicroCAT II X-ray micro-CT and Scanco VivaCT for *in vivo* imaging, 400Mhz vertical Bruker Avance III spectrometer for small molecule NMR, Siemens MicroPET Focus 220, Bioscan NanoSPECT SPECT/CT, CRI Maestro optical imaging systems for *in vivo* fluorescence, Visen FMT for quantitative optical tomography, and a VisualSonics high-resolution ultrasound system.

Animal Facility. Animals for this study will be procured through and housed in an ABSL-2 Animal Facility located adjacent to Light Hall in Medical Center North. The animal facility includes an animal suite with anesthesia machines and biohazard cabinets equipped for surgical procedures. The DAC provides procurement, husbandry and veterinary care services in support of research and teaching at VUMC. The DAC's comprehensive preventative medicine and veterinary care program includes daily observation of animals (including weekends and holidays) by animal care, veterinary, and research staff.

D. Number of Fellows/Trainees to be Supervised During the Fellowship

My laboratory currently consists of 5 individuals (two graduate students, one MSTP student, a co-sponsored student in Biomolecular and Chemical Engineering, and a lab manager). A second MSTP student will join the lab full time in August of 2017. The laboratory manager oversees day-to-day operations of the lab such as animal husbandry, autoclaving, media preparation, dishwashing, and ordering, ensuring that Nicole can focus on her research project without undue administrative tasks. The presence of two other graduate students with complementary projects will allow Nicole to have ongoing intellectual stimulation and camaraderie in the lab as she completes her doctoral research. Nicole will also benefit from a number of colleagues at different stages in training within the labs comprising the Center for Bone Biology (see co-sponsor statement below).

E. Applicant's Qualifications and Potential for a Research Career

I have had the pleasure of working closely with Nicole for over two years now, and I can confidently say that she possesses all of the characteristics that portend success for a career in biomedical sciences. She is incredibly hard working, bright, curious, and resilient. She has a technological courage that many trainees at her stage lack, and this has greatly facilitated her growth in the fields of skeletal cell biology and osteoimmunology. She is an extremely effective teacher, having trained four rotation students in our laboratory, one of which ultimately decided to join our group full time. Nicole's greatest attribute is her tenacity in acquiring new skills, including many experiences and techniques that are outside of my area of expertise. This has greatly benefited our research program, and Nicole has played a key role in bridging our strengths in microbial pathogenesis with established and emerging technologies in the field of bone biology. She is the perfect applicant for a Ruth L. Kirschstein Individual National Research Service Award Fellowship.

Looking back on Nicole's accomplishments prior to joining my lab, it was already evident that she had a bright future as a young scientist. In her Master's thesis work, Nicole made fundamental observations regarding how Th17 and Tc17 cells respond to wildtype measles virus in nonhuman primates, and how measles vaccine impacts adaptive immune responses. This work is detailed in a first-author manuscript that is currently under revision, and a middle author paper in the *Journal of Virology*. Because of her outstanding undergraduate and Master's thesis scholarship, Nicole was accepted into the Interdisciplinary Graduate Program at Vanderbilt in 2014. During her four research rotations, her incredible work ethic and productivity were readily apparent, and her work in one of these two-month rotations resulted in a manuscript in the *American Journal of Transplantation*. After joining my lab in 2015, Nicole quickly established several *in vitro* models of skeletal cell differentiation and function. Her preliminary data allowed for the successful publication of two additional manuscripts in the journals *PLoS Pathogens* and *Antimicrobial Agents and Chemotherapy*. In total, Nicole is

now included as an author on five publications, a remarkable accomplishment given that we have yet to publish the exciting preliminary data forming the foundation of this fellowship application.

At present, Nicole has completed all graduate coursework and successfully passed her dissertation candidacy exam. She has assembled an outstanding thesis committee, with experts from all facets of her project: microbiology, immunology, and skeletal cell biology. Her induction into the highly prestigious Vanderbilt Program in Molecular Medicine will ensure that her research project remains grounded in the most important clinically relevant questions, and that she is receiving targeted support towards her goal of a career in translational science. In the coming years Dr. Sterling and I will work closely together, and in concert with her thesis committee, to ensure that we continue to develop Nicole's mentoring skills, to fortify her written and oral scientific communication, and to nurture the leadership skills that she is already displaying. I anticipate that Nicole will write a first-authored paper describing her exciting preliminary data on bacterial-induced osteoclastogenesis in the next year. I envision that this work will lead to fundamental shifts in how we view bone homeostasis in the presence of both pathogenic and commensal organisms. As you will see from her enclosed letters of recommendation, Nicole is truly a special student. I am very fortunate to have her in my laboratory, and I support her NRSA fellowship application with the highest possible enthusiasm.

II. Co-sponsor Statement

A. Research Support Available

	Source	Identifying Number	Title of Research	Principal Investigator	Dates	Award Amount (annual)
Active	Department of Veterans Affairs	1I01BX001957-01	Strategies for early treatment of bone metastases	Julie A. Sterling	7/1/2013-6/30/2017	$237,399
Active	NIH/NCI	1R01CA163499-01A1	The Role of Mechanotransduction in Progression of Tumor-induced Bone Disease	Julie A. Sterling, Scott A. Guelcher	9/1/2012-6/30/2017	$205,424
Active	NIH/NIAMS	1R01 AR064772-01A1	Biofilm Dispersive Bone Grafts to Improve Healing of Contaminated Fractures	Scott A. Guelcher	4/1/2014-3/31/2019	$315,539
Active	Department of Defense / CDMRP	BC141789	Targeted Drug Nanocarriers for Inhibiting Bone Metastatic Breast Cancer	Julie A. Sterling	9/30/2015-9/16/2018	$119,468
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

B. Co-Sponsor's Previous Fellows and Trainees

During my 8 years at Vanderbilt, I have had the opportunity to train 9 graduate students and one post-doctoral fellow. Many of these trainees are still in my laboratory, however, those that have completed training have secured advanced positions in the biomedical sciences. Five representative prior trainees are listed below:

Trainee	Position and tenure in Sterling lab	Current institution and position
Nazanin Ruppender	Graduate Student (2007-2011)	Teaching faculty, North Seattle College
Rachelle Johnson	Graduate Student (2008-2011)	Assistant Professor, Vanderbilt University
Sabrina Danilin	Postdoctoral Fellow (2009-2011)	Research Scientist, University of Strasbourg
Shellese Cannonier	Graduate Student (2012-2016)	Analyst at Proactive Worldwide
Ushashi Dadwal	Graduate Student (2012-2016)	Postdoctoral Fellow, IUPUI (Shankar Lab)

C. Training Plan, Environment, Research Facilities

Training Plan

I am thrilled to serve as a Co-Sponsor for Nicole Putnam in her application to the Ruth L. Kirschstein Individual National Research Service Award Fellowship Program. I first met Nicole about two years ago when she and Dr. Cassat approached my lab to learn new techniques in the study of skeletal cells. Our lab focuses on the skeletal and inflammatory events surrounding tumor-induced bone disease, with a specific interest in cancer metastases. Since the Cassat lab is interested in studying how inflammation during osteomyelitis triggers changes in bone biology, I felt that our labs could have considerable synergy moving forward. As someone who has trained in bone cell biology, I can say without hesitation that Nicole's project is very unique and exciting, and is poised to move the emerging field of osteoimmunology forward. Nicole's observation that bacterial factors may induce osteoclastogenesis independently of canonical differentiation factors is potentially groundbreaking, and could have enormous ramifications not only for bone infections, but also more generally for our understanding of how bone cells respond to inflammation and microbes.

Our lab has considerable expertise in the isolation, differentiation, and characterization of primary skeletal cells, and we are happy to share all relevant reagents and protocols with Nicole and the rest of the Cassat lab. Dr. Cassat and I will coordinate our mentorship of Nicole, meeting with her formally as a group at least once monthly, and individually at least once weekly. Nicole will present her data at our lab meetings and at research in progress meetings in the Vanderbilt Center for Bone Biology (VCBB). She will have ample interactions not only with members of my laboratory, but also with the numerous other trainees of primary investigators in the VCBB. It is my expectation that these experiences, coupled with the Training Plan that Dr. Cassat has outlined above, will allow Nicole to emerge from her dissertation training as an expert not only in host-pathogen interactions, but also in bone biology. Collectively, this skillset will position Nicole to achieve her career goal of becoming a translational scientist that bridges infectious diseases and cell biology.

Environment

My laboratory is part of the Vanderbilt Center for Bone Biology (VCBB), located two floors above the Cassat laboratory. The VCBB was created to investigate diseases of bone and mineral metabolism, and primary investigators associated with the Center study embryonic bone development, neuroskeletal biology, biomechanics, fracture repair, osteoporosis, bone infections, and cancers such as breast cancer and prostate cancer, which frequently affect the skeleton. Dr. Cassat and I are primary faculty members of the Center and expect that Nicole will benefit greatly from the collegial interactions with experts in skeletal cell biology. Additionally, the Center has a number of specialized resources for the study of skeletal biology, including the recent addition a full-time bone histotechnologist.

Equipment

In addition to standard equipment for molecular biology, the VCBB shared laboratory includes the following specialized equipment related to Nicole's project – mammalian surgical area, ultraturax homogenizer, Miltenyi MidiMACS manual separator, cell culture hoods and incubators, automated Bio-Rad cell counter, sonicators, cryotanks, two real-time qPCR instruments (ABI), Bio-Rad CF x 96 qRT-PCR thermal cyclers, DNA and protein gel migration apparatus, equipment for soft and calcified tissue histology, a Wehmer plastic embedding grinder, 3 Leica RM2255 microtomes, an Olympus microscope with fluorescence and high-resolution camera, a computer with the histomorphometry analysis software inclucing Metamorph, Bioquant, and Osteomeasure.

D. Number of Fellows/Trainees to be Supervised During the Fellowship

My laboratory currently consists of three graduate students and a lab coordinator. The graduate students are working on projects in cancer biology, focusing on the molecular events and inflammatory pathways that promote bony metastasis. The presence of three graduate students at different stages of training and all working on bone cell biology will offer Nicole a unique perspective as she continues her work on osteomyelitis.

E. Applicant's Qualifications and Potential for a Research Career

To echo Dr. Cassat's statements above, Nicole is an outstanding student and has a very bright future as a translational scientist. I have been very impressed with her work ethic, her creative approaches to defining host responses to bone infection, and her ability to articulate both her research findings and her career goals. She has a very unique, interdisciplinary project, and I commend her for her efforts to bridge two diverse fields to answer fundamental questions about osteo-immunologic crosstalk. I am confident that the training plan we have outlined, the resources from my lab and the VCBB, and her thesis committee will serve as strong foundation for her continued growth as a scientist. I am absolutely thrilled to support her NRSA application.

DESCRIPTION OF INSTITUTIONAL ENVIRONMENT AND COMMITMENT TO TRAINING

Institutional Environment

Vanderbilt University has outstanding institutional resources and shared core facilities, providing an ideal environment for the completion of the proposed Research Strategy. Through the coordinated actions of the Biomedical Research, Education, and Training (BRET) office, Vanderbilt University displays a strong commitment to graduate students and post-doctoral fellows by providing resources such as career development, the ASPIRE program, Responsible Conduct of Research training, poster-printing services, and additional training programs.

Departmental Environment

Commitment to the training of students is also robust at the departmental level. The Department of Pathology, Microbiology, and Immunology (PMI) has faculty members that are heavily involved in the training of students in the Graduate Program in Microbiology and Immunology (M&I), through training at the classroom level and through sustained interactions with graduate students at research seminars and journal clubs. This graduate program benefits immensely by being housed within the larger department of PMI, with close access to resources, seminars, and expertise available from the Vanderbilt Center for Microbial Pathogenesis (VCMP) and the Vanderbilt Center for Immunobiology (VCI).

Proposed Development Plan

My research proposal is at the crossroads of microbial pathogenesis, immunology, and bone biology. These areas are well-represented on my Ph.D. Committee through PMI faculty members and physicians. This research is supported by Directors of VCMP and VCI, and investigators affiliated with the Vanderbilt Center for Bone Biology (VCBB) and the Vanderbilt University Institute for Imaging Sciences (VUIIS). Furthermore, there are a number of investigators at Vanderbilt University conducting research complementary to the work detailed in this proposal, including:

- Dr. Eric Skaar, Director of the Center for Microbial Pathogenesis, PMI faculty: Heme iron nutrient utilization in staphylococcal pathogenesis (complementary to Aims 1 and 2)
- Dr. Jeff Rathmell, Director of the Center for Immunobiology, PMI faculty: Mechanisms by which metabolism modulates inflammatory diseases (complementary to Aims 1 and 2)
- Dr. Dan Moore, Vanderbilt Children's Hospital, PMI faculty: Mechanisms of immune tolerance and immune-mediated tissue injury in autoimmunity (complementary to Aims 1 and 2)
- Dr. Isaac Thomsen, Vanderbilt Children's Hospital: Serologic responses to toxins during osteomyelitis and other invasive *S. aureus* infections (complementary to Aim 2)
- Dr. Dan Perrien, VUIIS, VCBB: Fracture repair, distraction osteogenesis, and bone remodeling (complementary to Aim 2)
- Dr. Rachelle Johnson, VCBB: Hypoxic regulation of cancer metastases in bone (complementary to Aim 2)
- Dr. Scott Guelcher, VCBB: Local drug delivery in bone fractures complicated by invasive *S. aureus* infection (complementary to Aim 2)

Intellectual Environment

After reaching candidacy, the student's remaining time in the Ph.D. program is dedicated primarily to research and participation in seminars and journal clubs. Students are required to give two lectures on their research in the M&I Research in Progress weekly seminar series attended by students, postdoctoral fellows, and faculty, which I will be presenting in the spring of 2017 and spring of 2019. Additionally, the Microbial-Host Interaction bi-monthly meeting supports presentation of research to graduate students before their third year and on a more regular basis; I was able to share my research in the spring of 2016 and will again present in January 2017. M&I also supports a bi-monthly journal club run by students, focusing on microbial pathogen-host interactions. In this journal club, I presented a manuscript in 2015 from the laboratory of Gabriel Nunez, to introduce his work prior to his visit to Vanderbilt later that month. Furthermore, VCBB holds weekly seminars for Vanderbilt students, faculty, and physicians across departments and disciplines with the common theme of studying bone-related research or disease. These seminars and journal clubs create venues for the productive interaction between students, postdoctoral fellows, faculty members, and physicians on a scholarly level to discuss student research, published research articles, clinical presentation of disease, or to introduce previous

work from visiting faculty members. In addition to these internal events, there is also a PMI weekly seminar series that boasts both internal and external speakers, a monthly Inflammation, Infection, and Immunity Frontiers seminar series that invites distinguished research faculty to Vanderbilt, and an annual Vanderbilt Symposium on Infection and Immunity. Vanderbilt facilitates broad discussions to enhance the intellectual environment through discussions between trainees and faculty through seminars and journal clubs and opportunities to meet with outstanding internal and external speakers.

Resources for Proposed Research

Vanderbilt University has an exceptional system of core facilities that I will utilize throughout the completion of my proposed Research Strategy. All proposed research is dependent on primary cell utilization and animal infection models, which is supported by the Division of Animal Care and veterinary care services. Resources for the completion of bone histological techniques and analyses, as well as the ability to render 3-dimensional images and perform quantitative analyses of bone remodeling changes are dependent on the unique and state-of-the-art resources available through the VCBB and VUIIS. Additionally, the VCMP and the Vanderbilt Institute for Clinical and Translational Research (VICTR) have contributed to my training and development of this research proposal. These resources are detailed in the Facilities and Other Resources document.

Resources for Career Development

The BRET office facilitates career development opportunities at Vanderbilt University, with Career Connection seminars and an annual BRET Career Symposium, both of which I attended in 2016. My interest in establishing a career as an independent scientific investigator with a focus on clinically-important infectious disease and translational research has been supported by Vanderbilt University on several fronts already. BRET offers training programs, such as the Vanderbilt Program in Molecular Medicine (VPMM), which I was accepted into in 2015 to shadow and receive clinical experience in Pediatric Infectious Diseases with my clinical mentor, Dr. Isaac Thomsen. This program includes tailored coursework, including Introduction to Clinical and Translational Research and VPMM Rounds with physicians, basic researchers, and patients. Moreover, the VPMM offers weekly seminars presented by clinicians, researchers, students, and postdoctoral fellows, Bench-to-Bedside symposia, and opportunities to attend clinical conferences, clinical boards, and clinical research seminars.

Vanderbilt has a remarkable career development program for its students, the ASPIRE Program, developed and funded through the 2013 Broadening Experiences in Scientific Training (BEST) NIH Award. M&I graduate students are encouraged to take advantage of the many offerings of this program during their graduate studies to explore career options through workshops and symposiums and expand skill sets through ASPIRE modules, externships, and internships. Due to my future career interests, I applied and was accepted into the ASPIRE Module on Clinical Laboratory Medicine (Clinical Microbiology) in 2016, which will start in January of 2017.

VERTEBRATE ANIMALS

1. Experimental procedures involving vertebrate animals

All animal procedures will be performed in the Vanderbilt University Medical Center ABSL-2 animal facility. Osteomyelitis will be induced in 7-8 week old female C57BL/6J *Mus musculus* (mice) according to published protocols. A subset of the animal procedures will also be performed in age-matched males of the same strain as noted below. Anesthesia will be induced with 3-5% inhaled isoflurane and maintained with 1-3% isoflurane delivered via a nosecone for the duration of the surgical procedure. Analgesia will be provided pre-operatively with 0.1 mg/kg buprenorphine injected subcutaneously. To induce osteomyelitis, a small incision will be made overlying the left mid-femur. Soft tissues will be carefully retracted to expose the mid femur. An approximately 1 mm defect will be created in one side of the bone cortex by trephination with a 21 -gauge needle. Osteomyelitis will be induced by injecting *S. aureus* into the bone defect with a micropipettor and sterile pipette tip. Muscle fasciae and overlying skin will subsequently be closed with Vicryl and Ethilon suture, respectively, and mice will be recovered from anesthesia on a 37°C warming pad. Mice will be monitored every 12 hours for the first 48 hours post-operatively, and then every 24 hours thereafter until the completion of the experiment. Analgesia (0.1 mg/kg buprenorphine injected subcutaneously) will be provided every 12 hours for the initial 48-hour post-operative period and then as needed for the duration of the experiment. To isolate primary murine bone marrow, mice will be euthanized, and the femurs removed for subsequent cell culture processing. We calculate the need for 540 mice to complete the Specific Aims (see below), which will be bred from purchased breeding pairs (immunodeficient mice, *cre*, and floxed mouse lines).

2. Justification for species selection and number of animals

In vivo investigations of the pathogenesis of bone infections by definition require the use of vertebrate animals. Tissue culture systems have been used successfully by many groups to recapitulate individual steps involved in the differentiation of osteoclasts from primary bone marrow cultures, and will be used in Specific Aim 1. However, no suitable *in vitro* system using tissue culture or mathematical models has been developed which assesses the complex interaction between skeletal and immune cells with *S. aureus* in bone. To accurately assess the impact of *S. aureus* on bone homeostasis, musculoskeletal tissues and immune responses are required. We have chosen the mouse as a model species for studying osteomyelitis, as it is the simplest organism available to complete the proposed surgical procedures, and because of the wealth of genetically modified strains available to study host responses.

Specific Aim 1 will require 120 mice.

Experiments in Aim 1 will require the isolation of primary bone marrow from long bones of mice deficient in TLR2, TLR9, or IL-1R. Osteoclastogenesis assays will test two stimulation conditions (with and without canonical osteoclastogenic stimulation) in whole bone marrow and bone marrow macrophage cultures. Analyses of osteoclastogenesis assays will require $2x10^8$ progenitor cells to complete the proposed studies with replicates, including phenotypic staining ($1x10^7$ bone marrow cells) and functional analyses ($6x10^7$ bone marrow cells) for Aim 1A, transcriptional investigation following RNA isolation ($8x10^7$ bone marrow cells) for Aim 1B, and monitoring of protein expression changes ($5x10^7$ bone marrow cells) for Aim 1C. To investigate the role of *S. aureus* supernatants on osteoclast differentiation with and without canonical stimulation, 120 mice will be required ($2x10^8$ bone marrow cells x 3 mouse genotypes x 2 stimulation conditions x 2 bone marrow cultures types), with an expectation of isolating approximately $2x10^7$ viable bone marrow cells per mouse. Preliminary data show no differences between male and female bone marrow in the ability of canonical stimulation and bacterial stimulation to induce osteoclastogenesis.

Specific Aim 2 will require 420 mice.

Aim 2A will use genetic approaches to test the role of MyD88 signaling in pathogen clearance during osteomyelitis. The genetic approach will utilize a Cre-Lox breeding strategy to ablate MyD88 signaling in mature osteoblasts (*ocn-cre*) or mature osteoclasts (*ctsk-cre*) and requires 120 mice (10 mice per group for bacterial enumeration x 1 *S. aureus* strain x 2 endpoints: 7 and 14 days x 6 mouse strains: *ocn-cre, ocn-cre/MyD88$^{fl/fl}$* mice, Cre-negative *MyD88$^{fl/fl}$* littermates of *ocn-cre/MyD88$^{fl/fl}$* mice, *ctsk-cre, ctsk-cre/MyD88$^{fl/fl}$* mice, and Cre-negative *MyD88$^{fl/fl}$* littermates of *ctsk-cre/MyD88$^{fl/fl}$* mice). Additionally, because sex differences in pathogen clearance may exist in mice with skeletal cell deletion of MyD88, this investigation requires 60 age-matched male mice (10 mice per group for bacterial enumeration x 1 *S. aureus* strain x 1 endpoint: 14 days x 6 mouse strains: see above). If sex differences are observed, these will be investigated further. Sample sizes are calculated based on a meaningful difference of 50%, standard deviation of 35%, and 80% power to

detect the meaningful difference with a type I error probability equal to 0.05. Dr. Cassat's considerable experience with the osteomyelitis model (>3,000 mice infected) allows for accurate predictions of standard deviation. To address reproducibility, the requirement of 10 mice per group will be spread over two independent trials using separate groups of 5 mice.

A standard two-step Cre-Lox breeding strategy will be used to generate mice with targeted deletions of MyD88 in the osteoblast or osteoclast lineage. Specifically, we will first mate homozygous *fl/fl* mice to the respective hemizygous *cre* mice to produce *cre*$^{+}$/MyD88$^{fl/+}$ mice (50% of the expected litters). These mice will then be mated back to the homozygous *fl/fl* mouse to produce homozygous knockouts (25% of the mice from this second mating). Another 25% of the mice from this second mating will be homozygous for the floxed allele but have no *cre* transgene. These will be used as experimental controls.

Aim 2B will require separate groups of mice for imaging/histopathologic analysis, comparing mock-infected and *S. aureus*-infected conditions. As skeletal remodeling may differ between male and female mice, we will infect both sexes in the experiments outlined in Aim 2B. Therefore, Aim 2B will require 240 mice (5 mice per group for imaging/histopathologic analysis x 6 mouse strains (see above) x 2 infection conditions (*S. aureus* and mock) x 2 endpoints (7 or 14 days) x 2 sexes). The requirement for 5 mice per group for imaging analysis is based on a meaningful difference of 25%, standard deviation of 12.5%, and 80% power to detect the meaningful difference with a type I error probability equal to 0.05.

3. Veterinary care

Vanderbilt University Medical Center is accredited by the American Association of Laboratory Animal Care and The Department of Health and Human Services (DHHS). Animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS "Guide for the Care and Use of Laboratory Animals". Veterinary care is under the direction of full-time resident veterinarians who are boarded by the American College of Laboratory Animal Medicine. Additional veterinary staff and veterinary technicians provide a comprehensive program of diagnostics, preventive, and clinical medicine at our facilities.

4. Procedures to limit discomfort, distress, pain, and injury

Animals subjected to experimental osteomyelitis will be administered preemptive analgesia and remain anesthetized throughout the duration of surgical procedures. Upon recovery from anesthesia, these animals may experience discomfort at the infection site. Mice will be administered post-operative analgesia (0.1 mg/kg buprenorphine) every 12 hours via subcutaneous injection for 48 hours following surgery. Mice who experience signs of pain (decreased mobility, altered gait, hunched posture, excessive grooming of surgical site) after this initial 48-hour period will be given additional analgesia on an as needed basis. Animals that are infected with *S. aureus* may suffer from acute disease. The signs for judging acute disease in mice are: ruffled fur, hunched posture, impaired mobility and apparent weight loss. In preliminary studies, MyD88-deficient mice were more susceptible to disseminated infection leading to acute disease. Animal infections in immunocompromised mice (Aim 2) may necessitate a decrease in inoculum to prevent the withdrawal of mice from experiments before the end point. Use of non-steroidal anti-inflammatory agents and anti-bacterials that are indicated for bacterial infection would compromise the purpose of the model. Mice will be weighed daily and any animals experiencing weight loss greater than or equal to 20% of their starting weight will be withdrawn from the study and euthanized. Mice who experience signs of secondary wound infection will also be withdrawn from the study and euthanized. All animals infected with *S. aureus*, irrespective of whether the animals develop symptoms or not, will be euthanized at the completion of the experiments.

5. Methods of Euthanasia

Mice will be euthanized by forced CO_2 inhalation delivered in a sealed chamber from a cylinder with compressed CO_2 gas, consistent with the recommendations from the American Veterinary Medical Association (AVMA). CO_2 will be gradually displaced at a rate of 10 to 30% of the chamber volume per minute, followed by cervical dislocation to confirm death.

RESOURCE SHARING PLAN

Sharing Data

All investigators are committed to the timely distribution of the results and methods obtained in this research proposal. All data generated will be published in peer-reviewed scientific journals, and following acceptance, will be deposited in the NIH National Library of Medicine's (NLM) *PubMed Central.* Additionally, these results will also be presented at national and international scientific meetings.

Sharing Model Organisms

This proposal plans to generate two new mouse models. These will be made available in a timely manner to the biomedical research community according to the NIH Model Organism Sharing Policy and the 1998 NIH, Jackson Laboratory, and DuPont Pharmaceuticals Cre-lox™ Technology Use Agreement. We will provide protocols and published phenotypic data relevant to this proposal upon request in accordance with the Guidelines for Recipients of NIH Grants and Contracts. Material transfers will be made with no more restrictive terms than in the Simple Letter Agreement (SLA) or the Uniform Biological Materials Transfer Agreement (UBMTA) and without reach-through requirements. Should any intellectual property arise which requires a patent, we will ensure that the technology remains widely available to the research community in accordance with the NIH Principles and Guidelines document.