**Nicole Putnam, Ph.D., of Vanderbilt University**   
[**“The impact of innate immune recognition of Staphylococcus aureus on bone homeostasis and skeletal immunity”**](https://www.niaid.nih.gov/sites/default/files/nicoleputnamapplicationF31.pdf)

**Vertebrate Animals:**

###### 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).

###### 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 2x108 progenitor cells to complete the proposed studies with replicates, including phenotypic staining (1x107 bone marrow cells) and functional analyses (6x107 bone marrow cells) for Aim 1A, transcriptional investigation following RNA isolation (8x107 bone marrow cells) for Aim 1B, and monitoring of protein expression changes (5x107 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 (2x108 bone marrow cells x 3 mouse genotypes x 2 stimulation conditions x 2 bone marrow cultures types), with an expectation of isolating approximately 2x107 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/MyD88fl/fl* mice, Cre-negative *MyD88fl/fl* littermates of *ocn-cre/MyD88fl/fl* mice, *ctsk-cre, ctsk-cre/MyD88fl/fl* mice, and Cre-negative *MyD88fl/fl* littermates of *ctsk-cre/MyD88fl/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.

###### 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.

###### 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.

###### Methods of Euthanasia

Mice will be euthanized by forced CO2 inhalation delivered in a sealed chamber from a cylinder with compressed CO2 gas, consistent with the recommendations from the American Veterinary Medical Association (AVMA). CO2 will be gradually displaced at a rate of 10 to 30% of the chamber volume per minute, followed by cervical dislocation to confirm death.

**Nico Contreras, University of Arizona**

[**“The Immunological Consequences of Mouse Cytomegalovirus on Adipose Tissue”**](https://www.niaid.nih.gov/sites/default/files/F31-sample-application_nico_contreras.pdf)

**Vertebrate Animals:**

## Vertebrate Animals

Use of animals

Male or female mice of varying ages (dominantly 3 months to 21 months) on the C57BL/6 genetic background (congenic, transgenic and targeted mutant derivatives) will be used for Specific Aims 1 and 2. As to date, we have found no sex-associated difference in immune responses between old male and female mice in response to cytomegalovirus (CMV), we will use males dominantly for this study.

Animals will be used for: (i) lymphoid organ harvest and adipose harvest for analysis of cytokine/chemokine and cellular (primarily T cell, macrophages, and NK cell) immune responses; (ii) blood and/or serum collection by retro-orbital bleeding or cardiac puncture (>200 ul); (iii) subcutaneous or intraperitoneal immunization for infection with live CMV (Smith strain and mutant derivaties). A number of animals could also be used for adoptive cell transfer followed by immunization and/or infection.

Justification for use

Mice are a logical and necessary choice for investigation of lifelong CMV infection and the effects on adipose tissue due to the similarity of CMV infection in both humans and mice, the existenece of congenic and recombinant strains of mice, defined genetics, availability of cell surface markers for flow cytometry, specific molecular probes, and monoclonal antibodies. Furthermore, a lifelong investigation on CMV infection and the consequences on human adipose tissue would not be feasible under the scope of this fellowship. The number of animals required provides statistical power needed for each experiment. For most experiments using 8 animals/groups allows us to observe

>30% difference between groups with 80% power at p<0.05. Power calculations were based on preliminary data.

Veterinary care

A full-time veterinarian is in charge of the animal care program at the University of Arizona.

The facility is accredited by AAALAC and in full compliance with NIH, as well as other federal, state, and local regulations and guidelines. All of the studies are covered by the IACUC UA protocol 08-102.

Procedures to minimize discomfort

Discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. Analgesia, anesthetic, and tranquilizing drugs will be used as determined by our veterinarian. Any discomfort or distress during bleeding will be minimized with the use of inhalation anesthetics such as isoflurane. If the mice experience distress (manifested by agitation), we will immediately halt the procedure. Mice will be observed until active after bleeding, injections, and surgery. All procedures have been approved under IACUC #08-102.

All injectables will be purchased as a pharmaceutical grade solution. All of the saline is purchased as sterile USP grade saline.

Euthanasia

Animals will be sacrificed by isoflurane overdose.

**Samantha Lynne Schwartz, Emory University**

[**“Regulation of 2'-5'-Oligoadenylate Synthetase 1 (OAS1) by dsRNA”**](http://www.niaid.nih.gov/sites/default/files/F31-Sample-Application_Samantha-Schwartz.pdf)

**Vertebrate Animals:**

Not included in this application