**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)

###### **Specific Aims:** 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.

**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)

#### Specific Aims: SPECIFIC AIMS

Adipose tissue consists of adipocytes that are crucial in lipid synthesis and energy storage, and a smaller population of cells of the stromal vascular fraction (SVF). The SVF represents a heterogeneous mixture of endothelial, stem, and immune cells, including T cells, macrophages, B cells, and NK cells. It has become increasingly clear that the immune responses within adipose tissue, such as cytokine secretion and tissue remodeling, influence host health and metabolism. Much emphasis has been placed on the activation of T cells and macrophages and their role in the chronic low-grade inflammation seen in obesity. However, inflammation in response to infections has been less thoroughly investigated. Adipocytes infected by adenovirus display an increased size and density, and *Trypanosoma cruzi* directly infects adipocytes resulting in adipose tissue inflammation. Recently, adipose CD4 T cells were shown to provide a site of latent viral infection in Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV).

Another virus capable of persistence and latency is cytomegalovirus (CMV), and it has been long implicated in low-level, systemic inflammation. The primary site of persistence for CMV is believed to be the salivary gland, but primary sites of latency have been difficult to conclusively identify. CMV infection results in a strong T cell response; in an acute infection ~5% of mouse, and up to 40% in some human patients, peripheral T cells are specific for CMV antigen. In our hands, we find that ~10% of adipose CD8 T cells in a mouse CMV (mCMV) infection are specific to mCMV at early comparable acute infection time points. This suggests that adipose tissue is an underappreciated site of viral infection and immune activity during CMV infection. **The primary hypothesis of this proposal is that mCMV disseminates to adipose tissue, replicates, establishes latency, leading to an lifelong CD8 T cell response.** Our long-term research objectives are to identify the lifelong immunological consequences of CMV infection. *The objective of this proposal* is to determine if mCMV establishes a productive infection within adipose tissue, and the consequences, if any, of that infection. As CMV is highly specific for host species, we will employ the mCMV model of infection to achieve the primary objective of this proposal through the following specific aims:

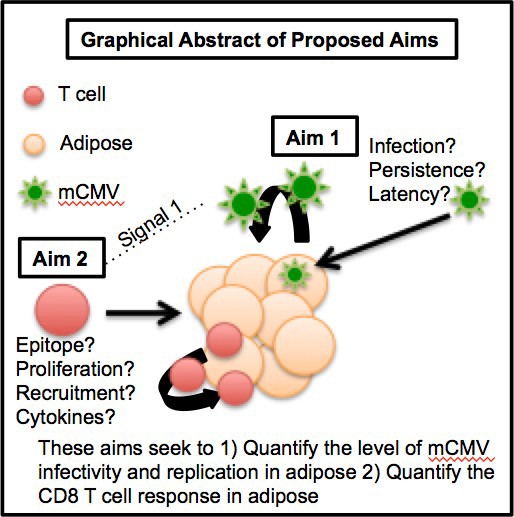
**AIM 1: Evaluate adipose tissue as a reservoir for replicative and persistent virus.** We hypothesize that adipose tissue is a location of active mCMV replication. To that end we will quantify viral load by plaque assay and qPCR in adipose compared to peripheral blood mononuclear cells (PBMCs) and salivary glands.

We will determine the extent to which mCMV persists in adipose in a chronically infected mouse using multiple modes of reactivation. We will also identify infected cells within adipose. These experiments will determine what cells within adipose tissue can harbor replicative and persistent infection.

**AIM 2: Determine the response of adipose tissue CD8 T cells during mCMV infection.** We hypothesize that mCMV specific T cells expand in adipose tissue. We have observed a significant expansion of mCMV specific CD8 T cells following infection. As the immune response to mCMV in adipose tissue has never been fully characterized we will determine the kinetics of mCMV specific CD8 T cell expansion and proliferation. We will determine if local T cells clonally expand or naïve cells are constantly recruited to adipose. These experiments will provide, for the first time, an understanding of adipose tissue mCMV immune response.

*See Graphical Abstract for Summary of Aims*

IMPACT: Upon the completion of these studies, we will have significantly advanced the understanding of mCMV cell tropism. We will have identified adipose tissue as a site of replicative and persistent virus that is capable of reactivation. The functional response of CD8 T cells and their mechanism of recruitment to adipose will have been identified. We will also have revealed the mechanism of viral spread into adipose tissue. These findings will have far reaching implications on the consideration of adipose tissue during vaccine design.

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**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)

### Specific Aims: SPECIFIC AIMS

The innate immune system must accurately distinguish self from foreign molecules to provide a critical first line of defense against pathogens. Double-stranded RNAs (dsRNAs) are produced during the life cyclesof many viruses and their detection by cellular sensors signals that infection has occurred and antiviral defenses should be activated. 2’-5’-oligoadenylate synthetase 1 (OAS1) is an important innate immune sensor of cytosolic dsRNA. OAS1 activates the latent ribonuclease (RNase L) via dsRNA-dependent productionof 2’-5’- oligoadenylate second messengers. Activated RNase L then targets viral and cellular RNA for degradation and thereby halts viral replication. Attesting to the importance of the OAS1/RNase L pathway, viruses have evolved mechanisms to evade its effects by directly inhibiting OAS1, sequestering dsRNA produced as a result of viral infection, or producing 2’-5’-oligoadenylate analogs. Determining the molecular mechanism of dsRNA- mediated regulation of OAS1 is vital for understanding how the OAS1/RNase L pathway controls viral infection.

Previous structural studies revealed that dsRNA binding allosterically induces conformational changes in OAS1 to form its active site and thus drive polymerization of ATP into 2’-5’-oligoadenylates. These structures have given us some insight into how OAS1 is activated by dsRNA; however, we still understand very little about how *specific features of the dsRNA* contribute to the level of OAS1 activation. The dsRNA used in these structural studies contained two overlapping and antiparallel copies of a known OAS1 activation consensus sequence, one on each strand of the helix. Yet the dsRNA bound OAS1 in a single unique orientation. Our preliminary data indicate that this selectivity in binding orientation also exists in solution and plays a critical role in determining the potency of OAS1 activation. We designed three RNA hairpin constructs based on the model dsRNA used in the structural studies: the wild-type dsRNA hairpin with the two strands linked by a short stable loop, and two variants where a critical residue of one OAS1 consensus sequence was mutated. Remarkably, while mutation of one consensus sequence resulted in *complete loss* of OAS1 (“non-activating”),

*increased* OAS1 (“hyper-activating”). Despite these differences in ability to activate OAS1, preliminary data from our lab suggested that both variants bind OAS1 with similar affinity.

These findings suggest that the wild-type dsRNA hairpin contains competing OAS1 binding sites with remarkably different capacities to activate the protein. However, the molecular signatures defining these sites as activating and non-activating are unknown. The dsRNA hairpins we have generated are ideal tools for deciphering the structural requirements and molecular mechanisms of OAS1 activation. Thus, in the following two Specific Aims, I will use complementary assays of OAS1 activity *in vitro* and in human cells as well as biochemical, biophysical, and structural approaches to define the differences in protein-RNA interaction that lead to differential activation of OAS1 by these three dsRNA hairpin sequences.

**SPECIFIC AIM 1: Define dsRNA features and their context that lead to potent activation of OAS1.** *Hypothesis: Extent of OAS1 activation is determined by the context of potentially overlapping RNA features with the ability to activate or inhibit OAS1.* I will examine the role of specific RNA features *in vitro* by using an established assay to measure OAS1 enzyme kinetics in the presence of the wild-type and each variant dsRNA hairpin. I will correlate the differences in ability of each RNA to activate OAS1 *in vitro* with their impact on the OAS1/RNase L pathway by transfecting these dsRNAs into A549 cells and measuring cellular messenger RNA transcript level changes of known RNase L targets and cleavage of ribosomal RNA by RNase L. These experiments will provide important new insight into currently unknown features of dsRNA that result in potent OAS1 activation and the potential for competition between OAS1 binding sites.

**SPECIFIC AIM 2: Determine the molecular mechanism(s) by which the “non-activating” and “hyper- activating” dsRNAs differ in their effects upon OAS1 activity.** *Hypothesis: “Non-activating” and “hyper- activating” RNAs exert their effects through different impacts on binding kinetics and/or OAS1 structural changes despite both having similar binding affinity*. Biotinylated OAS1 will be immobilized on a streptavidin biosensor and dsRNA binding affinity and kinetics (on/off rates) measured using bio-layer interferometry. Next, I will use hydrogen-deuterium exchange coupled with mass spectrometry to determine the impact of each dsRNA hairpin on OAS1 conformational dynamics, including critical allosteric changes in the enzyme active site. Lastly, I will use x-ray crystallography to solve structures of dsRNA hairpins bound to OAS1. Collectively, these experiments will reveal the molecular mechanism(s) by which specific dsRNA signatures drive different levels of OAS1 activation.

**Summary**: The goal of this project is to determine how specific features in dsRNA, as well as their context, control regulation of OAS1. Such studies are critical to gain a broader understanding of host-pathogen interactions and the fundamental mechanisms of cellular translational control. For example, this work will give insight into how viruses could mask otherwise activating motifs in order to evade detection by the innate immune system and may provide a foundation for the development of novel antiviral therapeutics.