Research Approach

Significance (about half a page)

- include background?
- innovation; model figure;
- how move field forward

Develop Aim 1; about 2.5 pages long

- re-state SA verbatim, then
- pilot data & rigor
- how & why you do the experiment
- who will help with new skills
- anticipated results. & alternatives

Look at samples F31: https://www.niaid.nih.gov/grants-contracts/three-new-f31-sample-applications

Reference manager

Endnote? Mendeley (free) Zotero (free)

Image design

Illustrator? BioRender (free)

Significance/ Background (0.5 page?)

- Explain the importance of the problem or critical barrier to progress in the field
- Explain how the proposed project will improve scientific knowledge, technical capability, and/or clinical practice
- Describe how the concepts, methods, technologies, treatments, that drive this field will be changed.
- Include only references directly related to the project's stated objectives
- Describe how current and previous work led to your proposed project.
- Fellowships aren't required to have Innovation, but include?

Examples of Significance

Contact PD/PI: Putnam, Nicole E

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 loc vasculature damage. Treatment requires drastic measures to eradicate the infection, and antibiotic resistar complicates this already difficult-to-treat invasive infection. The current standard of treatment for osteomye entails surgical debridement of the infected bone and prolonged courses of antibiotics (6). Unfortunat

What do you notice?

Contact PD/PI: Contreras, Nico

B. SIGNIFICANCE. Cytomegalovirus (CMV) is a ubiquitous betaherpesvirus that infects a large percentage of people worldwide[1,2–4]. Infection progresses from an acute replicative cycle leading to a latent and lifelong infection[5,6]. can be damaging in the immune compromised such as immune suppressed transplant patients, Human Immunodeficiency Virus (HIV) patients, Acquired Immunodeficiency Syndrome (AIDs) patients, and unborn fetuses[6–9]. The hallmarks of CMV disease progression are not seen in immune competent patients, and this is a result of the significant amount of resources that the adaptive immune system dedicates to control CMV infections. In fact, 5-10% of CD8 T cells during a primary CMV response can be specific for an antigen generated by CMV[12–15]. The magnitude of this response is largely unparalleled in any other infection, and due to this, CMV has been investigated for its role in age-related T cell memory inflation[3,5,16–18]. Studies of CMV T cell inflation and viral dissemination throughout the host have largely been focused upon spleen, lung, liver, blood, and salivary glands
into contact with mucosal
monocytes to the immune
been interrogated. Many c

<u>This proposal is of significant clinical and biological importance as CMV is a high vaccine priority[52] and there have been no studies demonstrating the immune response within adipose tissue specific to mCMV</u>. An understanding of the immune response to mCMV within adipose tissue and potential identification of a latent reservoir for mCMV will allow for more pointed future therapeutic and vaccine designs.

Research Approach

present a well-organized, visually appealing, and readable description of your proposed project.

good idea to restate the key points you've made about your project's significance, and your long-term goals.
How experiment tests hypothesis
Conceptualize what results will be, if right or wrong

Aims/ Experiments to test hypothesis (2.5 p per aim)

- preliminary data
- Design & measure with reproducible results
- interpretation/alternative approach

Research Approach-Tips

- Restate each Aim as on SA page
- Reiterate rationale for each specific aim
- Describe how pilot data or results in literature support your hypothesis
- State how your study will address the gap in knowledge

- Describe the overall strategy, methodology, and analyses
- Include how the data will be collected, analyzed, and interpreted. Sample size, power, significance of outcomes
- Describe expected results, potential problems and alternative approaches

Aim 1: Test XYZ

Hypothesis to test in this aim (sentence)
Relevant background to this aim (1/4 page)
Preliminary data to support this aim (up to 1/3 page)
Experimental Design to test this hypothesis (4-6 paragraphs of studies, perhaps with diagram)
Limits and Alternatives (paragraph)

Then do it again for Aim 2.

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.

Schwartz SA Page <-

Aims I V

B. APPROACH

SPECIFIC AIM 1: Define dsRNA features and their context that lead to potent activation of OAS1.

Rationale: dsRNA binding to OAS1 requires a minimum of 18 bp and activation is potentiated by the presence of recently identified molecular signatures, including However, how these molecular signatures act in concert or competition to affect the level of OAS1 activity and thus an innate antiviral response is unknown. Our preliminary data (**Fig. 3**) illustrate that in each of the two consensus sequences leads to opposing affects: one no longer activates OAS1 ("non-activating") while the other dramatically increases OAS1 activation ("hyper-activating"). Determining which RNA features create a preferred OAS1 binding site activating or non-activating will give critical new insight into how viruses could mask otherwise activating motifs to evade detection by the innate immune system. *Hypothesis: Extent of OAS1 activation is determined by the context of potentially overlapping RNA features with the ability to activate or inhibit OAS1.*

Overview of Experimental Design: I will examine how specific dsRNA features control the ex activity *in vitro* by using our established chromogenic assay (28) to measure OAS1 enzyme wild-type and each variant dsRNA hairpin (see **Fig. 1B**). I will next compare the differences each dsRNA to activate OAS1 *in vitro* with their impact on the OAS1/RNase L pathway in liv experiments will provide important new insight into currently unknown features of dsRNA that OAS1 activation *in vitro* and in human cells.

Aim 1.1: Define the features of dsRNA that lead to potent activation of OAS1 *in vitro*.

Experimental Approach: I will *in vitro* transcribe the dsRNA hairpins from linearized plasmid DNA templates using T7 RNA polymerase and purify the RNAs by denaturing polyacrylamide gel electrophoresis using established protocols (30-33). Two "scrambled" versions of the dsRNA hairpin will also be transcribed as controls: Scramble 1 will maintain the wild-type activation consensus sequences, but will randomize all other nucleotides and in Scramble 2 all nucleotides will be randomized (while maintaining an 18 bp duplex in both RNAs). Human OAS1 will be expressed in *E. coli* BL21(DE3) and purified using sequential Ni^{2+}-affinity,

Be sure to lay out a plan for alternative experiments and approaches in case you get negative or surprising results.

Anticipated Results. It is expected that the viral load of adipose tissue to be comparable to that of spleen at day three and seven post-infection. At day fourteen post-infection we expect to see a reduction in viral load in adipose tissue and an increase in salivary gland as has been reported[58,69,80]. We anticipate that plaque assays at these time points will be sufficient to detect replicating virus. From these experiments we will have established adipose tissue and its cellular constituents as a site for productive mCMV replication.

Pitfalls and Alternatives. Our laboratory has experience with cell culture and it is not anticipated that these experiments will be technically challenging. The gold standard for mCMV plaque assays has been the use of MEFs but these cells are not immortalized and therefore must be repeatedly produced and stocks can vary from lot to lot. If these cells do not yield interpretable data or do not form plaques we will switch to a newly described protocol that allows for greater viral spreading during assay[70]. It is entirely possible that mCMV does not replicate within adipose tissue. This is not our expectation based upon the increased presence of mCMV specific CD8 T cells in comparison to uninfected adipose. If we find no virus, this would suggest that CD8 T cells are either searching for their cognate antigen or were trafficking through adipose at the time point we looked. Even if this null result occurs the presence of CD8 T cells within adipose tissue even without detectable

Contreras

As you develop an aim

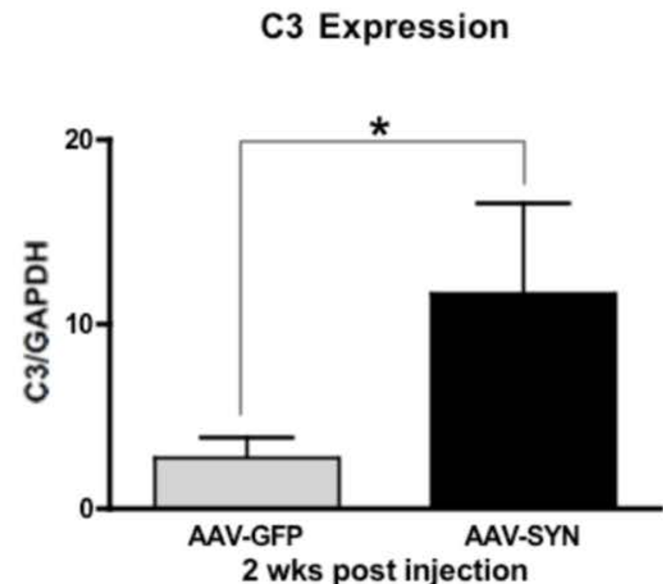
- Note expertise to do a specific task (or collaborators).
- Describe your/ expert's past accomplishments related to the project.
- Describe preliminary studies and new, relevant findings
- Outline research strategy for aim 1 and 2 including each experiment you will use for each aim.
- State how the experiment tests the hypothesis
- Explain how you plan to interpret data from the aim.
- Conceptualize what the results might be, if your hypothesis is right, or wrong
- Describe how to address potential pitfalls with contingency plans.

Approach

Aim 1: Using the AAV-SYN in vivo model of PD, determine whether a-syn expression leads to activation of classical pathway specific C1q and C4, common pathway C3 and C5, and terminal deposition of C9.

Rationale: In PD post-mortem brain tissue, there are increased amounts of complement components, C1q and C9, mRNA in the substantia nigra and caudate[27]. Also, different complement component isoforms are expressed in CSF from PD patients as compared to healthy controls and other neurodegenerative diseases[12,16].

Previous work in our laboratory on mice stereotactically injected with an AAV that overexpresses alpha-synuclein (AAV-SYN) shows a 30% loss of dopaminergic neurons in the substantia nigra 6 months post- injection[41]. The nigral pathology found in these animals contains a strong immune system component as evident by increased deposition of IgG, microglial activation, increased cytokine secretion, and increased



•Describe the overall strategy, methodology, and analyses to be used to accomplish the specific aims of the project.
•Include how the data will be collected, analyzed, and interpreted. sample size, power, significance of outcomes
•Describe expected results, potential problems and alternative approaches

What do you notice?

Experimental Design

Mouse Model: C57BL/6 mice will be injected stereotactically under isoflurane anesthesia with 2 μL of a recombinant adeno-associated virus 2 containing the gene for human alpha-synuclein (AAV-SYN) or green fluorescent protein (AAV-GFP) of the same viral titer. The stereotaxic coordinates target the right substantia nigra, and are: anterior-posterior (-3.2 mm from bregma), medio-lateral (-1.2 from midline) and dorso-ventral (-4.6 from the dura). After injection, mice will be sacrificed at prescribed time points as described below. Additionally, some mice will be injected with LPS acutely as a positive control in the same stereotaxic area. These mice will be sacrificed within 24 hours of injection. In previous studies, this injection leads to a consistent, progressive selective dopaminergic neuron loss of up to 30% by 6 months[41].

Time Points: 2 week and 4 week time points were chosen for this experiment. Initial inflammatory reactions are seen at 2 weeks post-injection of AAV-GFP, when viral proteins begin to express at high levels. At the 2 week time point, in the PD mouse model, we see an increase in IgG as assessed by immunofluorescence staining and an increase in pro-inflammatory cytokine expression as assessed by qPCR. At 4 weeks post-

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Project Design: give enough detail for reviewers to determine the appropriateness of the design in terms of its applicability to the project hypotheses.

Procedures: Describe data collection procedures. Include enough detail so that someone unfamiliar with the procedures could duplicate major aspects and be able to envision the environment

Methods of Analysis: Describe statistical approach and related data analyses in as much detail as possible. Associating the method of analysis with the hypothesis and specific aims is recommended

discuss the results you expect and how they will be interpreted.

Results, interpretations & alternatives: If we find that the number of mutations in the VH sequences of BRMs remains the same on days 15, 30 and 70, but that the number of mutations in GC B cells in the LN continue to accumulate over these times, then we will conclude that BRMs are established in the lung early after infection and, once established, are maintained without significant immigration of GC-derived memory B cells. This result would imply that the BRMs in the lung have relatively low affinity BCRs. Alternatively, we may find that VH sequences of BRMs in the lung continue to accumulate mutations through day 70. This result would contradict our parabiosis data, which shows that only about 5% of the BRMs in the lung are imported between 30 and 45 days after infection and may suggest the surprising possibility that BRMs accumulate mutations in situ in the lung, possibly in locations like bronchus associated lymphoid tissue (BALT).

Interestingly, we may see differences in the number of mutations between the BRM subsets. For example, since CD73 is a marker of GC-emigrants[8] we may find more mutations in subsets A and B compared to subsets C and D, which are likely derived from the EFA. Since the extrafollicular reaction is rapidly resolved after infection, we may find that subsets C and D fail to accumulate more mutations over tim
A and B continue to accumulate mutations, suggesting that BRMs in these subsets are
GC-derived precursors. To test this possibility, we will ablate GCs starting on day 15 by
a blocking antibody to CD40L[24] and assay the number of mutations in BRMs in the lu
number of mutations increases in control mice, but fails to increase in CD40L-blocked
conclude that at least some BRMs are continually seeded by GC-derived precursors.

What do you notice?

In addition to seeding of BRMs from cells outside the lung, the number and quality of BRMs in the lung is likely to be affected by local homeostatic proliferation and cell death. To better understand the dynamics of BRM turnover in the lung, we will quantify apoptotic cells by staining with Annexin V and proliferating cells by BrdU (or EdU) incorporation. We will also determine the expression of survival markers like BcL-2. If we find that some populations have low Annexin V, high Bcl-2 and incorporate BrdU, whereas others have high Annexin V, low Bcl-2 and fail to incorporate BrdU, then we would conclude that the former are likely to outcompete the latter and to dominate the BRM populations in the lung over time.

Pitfalls and alternative approaches: I have spent the last year developing the parabiosis model following influenza infection and characterizing influenza-specific BRMs in the lung using tetramer reagents and flow cytometry. Moreover, I have extensive experience with preparative and analytic flow cytometry, including analysis of Annexin V, BrdU and intracellular staining. Moreover, I will have help with the single cell sequencing from Dr. Rodney King here at UAB, who routinely performs similar experiments to amplify mouse

Example, statistics

Statistical analysis: All *in vivo* experiments comparing the course of EAE will be performed with a minimum of 15 mice per genotype or treatment group with replication, and all *in vitro* experiments will be performed at least three times to ensure reproducibility of the findings. These experimental group sizes are based on power calculations and feasibility. Statistical analyses will be done in coordination with biostatistics resources and experts available at our institution. Pooled data will be expressed as mean±SEM for *in vivo* and mean±SD for *in vitro* experiments, and longitudinal measures assessed by repeated-measures ANOVA. When significant differences are observed ($p<0.05$) post-hoc pair-wise least-squared difference tests will be performed with Bonferroni corrections. When data are not normally distributed analysis will be performed after transformation.

F31 NINDS Wrights

Consult with biostatistician!

CTSI-CN SPARC request portal

Check

1. Background and data to give reviewers context
2. Each Aim has a set of experiments
    1. tests the hypothesis
    2. alternative approaches are shown
3. Experiments yield meaningful data to test hypothesis
4. Enough detail to convince reviewers I understand a method.
5. It is clear what I do well and what unique skills I bring to the research. I explicitly state my team's resources and expertise.
6. If team has experience with a method, cite it; otherwise include enough detail to convince reviewers feasible
7. The results I anticipate are described and their implications.
8. explain who will do what, what they will do, when and where they will do it, how long it will take,
9. My timeline shows when I expect to complete my aims.