Descriptive Title: Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation

Submission Title: Watford R21 Plac8

Opportunity ID: PA-16-161

Opportunity Title: NIH Exploratory/Developmental Research Grant Program (Parent R21)

Agency Name: National Institutes of Health

Table of Contents

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

APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION

[ ] Pre-application [X] Application [ ] Changed/Corrected Application

2. DATE SUBMITTED | **Applicant Identifier**

4. a. Federal Identifier

b. Agency Routing Identifier

c. Previous Grants.gov Tracking ID

5. APPLICANT INFORMATION **Organizational DUNS:** 004315578

Legal Name: University of Georgia Research Foundation Inc.
Department: Division:
Street 1: 310 East Campus Rd Tucker Hall Room 409
Street 2:
City: Athens County/Parish:
State: GA: Georgia Province:
Country: USA: UNITED STATES ZIP / Postal Code: 30602-1589

Person to be contacted on matters involving this application
Prefix: First Name: Tammi Middle Name:
Last Name: Childs Suffix:
Position/Title: GRANTS OFFICER
Street 1: 0414 TUCKER HALL
Street 2:
City: ATHENS County/Parish:
State: GA: Georgia Province:
Country: USA: UNITED STATES ZIP / Postal Code: 30602
Phone Number: 706-542-5069 Fax Number: 706-542-5946
Email: tachilds@uga.edu

6. EMPLOYER IDENTIFICATION *(EIN) or (TIN)*: 581353149

7. TYPE OF APPLICANT: M: Nonprofit with 501C3 IRS Status (Other than Institution of Higher Education)
Other (Specify):
Small Business Organization Type [ ] Women Owned [ ] Socially and Economically Disadvantaged

8. TYPE OF APPLICATION:
[X] 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 [X] What other Agencies?

9. NAME OF FEDERAL AGENCY:
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation

12. PROPOSED PROJECT:

Start Date	Ending Date
5/1/2018 4:00:00 AM	4/30/2020 4:00:00 AM

13. CONGRESSIONAL DISTRICT OF APPLICANT
GA-010

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name: Wendy Middle Name:
Last Name: Watford Suffix:
Position/Title: ASSOCIATE PROFESSOR
Organization Name: University of Georgia
Department: INFECTIOUS DISEASES Division: College of Veterinary Medicine
Street 1: 0357 VET MED - 1
Street 2: 501 D. W. BROOKS DR.
City: ATHENS County/Parish:
State: GA: Georgia Province:
Country: USA: UNITED STATES ZIP / Postal Code: 30602
Phone Number: 706-542-4585 Fax Number:
Email: watfordw@uga.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	$412,500.00
b. Total Non-Federal Funds	$0.00
c. Total Federal & Non-Federal Funds	$412,500.00
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 [X] 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)

[X] 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

19. Authorized Representative

Prefix: First Name: Christian Middle Name:
Last Name: Heindel Suffix:
Position/Title: Grants Coordinator
Organization: University of Georgia
Department: Division:
Street 1: 501 D.W. Brooks Drive
Street 2:
City: ATHENS County/Parish:
State: GA: Georgia Province:
Country: USA: UNITED STATES ZIP / Postal Code: 30602
Phone Number: 706-542-1043 Fax Number:
Email: heindel@uga.edu

Signature of Authorized Representative — Completed on submission to Grants.gov

Date Signed — Completed on submission to Grants.gov

20. Pre-application	
21. Cover Letter Attachment	Plac8_Cover Letter_Final.pdf

The University of Georgia

College of Veterinary Medicine
Department of Infectious Diseases

Stacy E. Ferguson, Ph.D.
DAIT, NIAID, NIH, DHHS

June 15, 2017

Subject: R21 grant proposal in response to PA-16-161

Dear Dr. Ferguson,

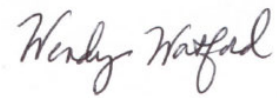
Please find enclosed a grant proposal entitled "**Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation**". This R21 application addresses the major public health issue of influenza virus infection and seeks to understand mechanisms of CD8 memory establishment by the novel cysteine-rich host protein, Plac8. The rationale for these studies is that knowledge gained about the mechanistic roles of Plac8 in T cell functions and memory establishment may inform the design of improved vaccines for viruses and cancers.

This proposal will generate genome-wide non-human expression data that will be made publicly available to the research community.

We request assignment to the following study section:
Cellular and Molecular Immunology – A study section – CMI-A

Should you need any further information, please contact me at 706-542-4585 or at watfordw@uga.edu. Thank you for your consideration.

Sincerely,



Wendy Watford
Associate Professor
Department of Infectious Diseases
University of Georgia, Athens

OMB Number: 4040-0010
Expiration Date: 10/31/2019

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: University of Georgia
DUNS Number: 619003127
Street 1: 501 D.W. Brooks Drive
Street 2:
City: Athens
State: GA: Georgia
Province:
Country: USA: UNITED STATES
ZIP / Postal Code: 30602-5023

County:

Project/Performance Site Congressional District: GA-010

Additional Location(s):

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 10/31/2019

1. * Are Human Subjects Involved? [X] Yes [ ] No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? [X] Yes [ ] No

If yes, check appropriate exemption number. [ ] 1 [ ] 2 [ ] 3 [X] 4 [ ] 5 [ ] 6

If no, is the IRB review Pending? [X] Yes [ ] No

IRB Approval Date:

Human Subject Assurance Number: 00003901

2. * Are Vertebrate Animals Used? [X] Yes [ ] No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? [ ] Yes [X] No

IACUC Approval Date: 7/20/2015 4:00:00 AM

Animal Welfare Assurance Number A3437-01

3. * Is proprietary/privileged information included in the application? [ ] Yes [X] No

4.a. * Does this Project Have an Actual or Potential Impact – positive or negative - on the environment? [ ] Yes [X] 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 designated, as a historic place? [ ] Yes [X] No

5.a. If yes, please explain:

6. * Does this project involve activities outside the United States or partnerships with international collaborators? [ ] Yes [X] No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract	Plac8_Abstract_Final.pdf
8. Project Narrative	Plac8_Project Narrative.pdf
9. Bibliography & References Cited	Plac8_References_Final.pdf
10. Facilities & Other Resources	Plac8_Facilities_Final.pdf
11. Equipment	Plac8_Equipment_Final.pdf
12. Other Attachments	

Abstract

Memory CD8 T cells are superior in protecting individuals from reinfection with highly mutagenic, evolving pathogens like influenza, HIV and malaria. This is primarily due to the fact that CD8 T cells, unlike antibodies, recognize conserved, internal proteins that are requisite for pathogen survival. Moreover, memory CD8 T cells correlate with improved overall survival in many cancers, emphasizing the broad benefits of memory CD8 T cell vaccines to human health. However, CD8 T cell responses to some live attenuated vaccines are not maintained long-term which results in waning immunoprotection. Therefore, methodologies to improve CD8 T cell based vaccines are warranted and dependent on a better understanding of the factors that regulate the development and maintenance of these long-lived memory cells. Our preliminary data demonstrate that the novel cysteine-rich protein, placenta-specific 8 (Plac8), is critical for the development and/or maintenance of CD8 T cell memory due to its functions within the T cells, themselves. However, how Plac8 regulates CD8 T cell responses is unknown. In this application, we will bring together expertise in the fields of molecular and cellular T cell immunology with expertise in proteomics and protein biochemistry to comprehensively dissect how Plac8 regulates memory CD8 T cell formation. In Aim 1, we will use a murine influenza infection model to test the hypothesis that Plac8 regulates autophagy, a process that can mitigate the cellular stress associated with effector to memory (ETM) cell transition. In Aim 2 we will examine the regulation and function of Plac8 in murine and human T cells and use an unbiased biochemical approach to gain insight into its functions through the identification of Plac8 interacting proteins. These data are essential to advance our understanding of Plac8 functions in T cells that underlie the generation of host protective CD8 T cell memory responses, a primary goal of vaccination.

Project Narrative

Annually administered influenza vaccines have variable efficacy rates due to inaccurate predictions of which strains of influenza will dominate the upcoming flu season and failure to evoke protective immune response. Our experiments will determine how a novel protein enhances the host's immune response to influenza infection. This knowledge will inform the design of vaccines that provide longer lasting protection healthy and high-risk populations.

References

1. Galaviz-Hernandez, C., et al., *Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis.* Gene, 2003. **309**: p. 81-89.
2. Ledford, J.G., M. Kovarova, and B.H. Koller, *Impaired host defense in mice lacking ONZIN.* Journal of immunology (Baltimore, Md. : 1950), 2007. **178**: p. 5132-5143.
3. Ledford, J.G., et al., *ONZIN deficiency attenuates contact hypersensitivity responses in mice.* Immunology and Cell Biology, 2012. **90**: p. 733-742.
4. Jimenez-Preitner, M., et al., *Plac8 is an inducer of C/EBP?? required for brown fat differentiation, thermoregulation, and control of body weight.* Cell Metabolism, 2011. **14**: p. 658-670.
5. Johnson, R.M., M.S. Kerr, and J.E. Slaven, *Plac8-dependent and inducible NO synthase-dependent mechanisms clear Chlamydia muridarum infections from the genital tract.* Journal of immunology (Baltimore, Md. : 1950), 2012. **188**: p. 1896-904.
6. Li, C., et al., *Excess PLAC8 promotes an unconventional ERK2-dependent EMT in colon cancer.* Journal of Clinical Investigation, 2014. **124**: p. 2172-2187.
7. Uehara, H., T. Takahashi, and K. Izumi, *Induction of retinol-binding protein 4 and placenta-specific 8 expression in human prostate cancer cells remaining in bone following osteolytic tumor growth inhibition by osteoprotegerin.* International Journal of Oncology, 2013. **43**: p. 365-374.
8. Kaistha, B.P., et al., *PLAC8 localizes to the inner plasma membrane of pancreatic cancer cells and regulates cell growth and disease progression through critical cell-cycle regulatory pathways.* Cancer Research, 2016. **76**: p. 96-107.
9. Kinsey, C., et al., *Plac8 links oncogenic mutations to regulation of autophagy and is critical to pancreatic cancer progression.* Cell Reports, 2014. **7**: p. 1143-1155.
10. Rogulski, K., et al., *Onzin, a c-Myc-repressed target, promotes survival and transformation by modulating the Akt-Mdm2-p53 pathway.* Oncogene, 2005. **24**: p. 7524-7541.
11. Tan, J.T., et al., *Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells.* The Journal of experimental medicine, 2002. **195**: p. 1523-32.
12. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nature immunology, 2003. **4**: p. 1191-8.
13. Xu, X.J., et al., *Autophagy is essential for effector CD8(+) T cell survival and memory formation.* Nature Immunology, 2014. **15**: p. 1152-1161.
14. Puleston, D.J., et al., *Autophagy is a critical regulator of memory CD8(+) T cell formation.* Elife, 2014. **3**.
15. Li, Y., et al., *The negative c-Myc target onzin affects proliferation and apoptosis via its obligate interaction with phospholipid scramblase 1.* Molecular and cellular biology, 2006. **26**: p. 3401-3413.
16. Schenkel, J.M. and D. Masopust, *Tissue-resident memory T cells.* Immunity, 2014. **41**(6): p. 886-97.
17. Teijaro, J.R., et al., *Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection.* J Immunol, 2011. **187**(11): p. 5510-4.
18. Anderson, K.G., et al., *Cutting edge: intravascular staining redefines lung CD8 T cell responses.* Journal of immunology (Baltimore, Md. : 1950), 2012. **189**: p. 2702-6.
19. Verbist, K.C., M.B. Field, and K.D. Klonowski, *Cutting edge: IL-15-independent maintenance of mucosally generated memory CD8 T cells.* Journal of immunology (Baltimore, Md. : 1950), 2011. **186**: p. 6667-71.
20. Wojciechowski, S., et al., *Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis.* J Exp Med, 2007. **204**(7): p. 1665-75.
21. Doi, K., et al., *ZFAT plays critical roles in peripheral T cell homeostasis and its T cell receptor-mediated response.* Biochem Biophys Res Commun, 2012. **425**(1): p. 107-12.
22. Zhang, X.a.L., Q., *Autophagy Assays (LC3B immunofluorescence, LC3B western blot, acridine orange assay).* Bio-protocol, 2013. **3**(24): p. e1012.
23. Wlodkowic, D., et al., *Apoptosis and beyond: cytometry in studies of programmed cell death.* Methods Cell Biol, 2011. **103**: p. 55-98.
24. Darzynkiewicz, Z., et al., *Fluorochrome-labeled inhibitors of caspases: convenient in vitro and in vivo markers of apoptotic cells for cytometric analysis.* Methods Mol Biol, 2011. **682**: p. 103-14.
25. Jeannet, G., et al., *Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9777-82.
26. Jenkins, M.R., et al., *Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting.* J Immunol, 2006. **177**(5): p. 2917-25.

27. Bakre, A., et al., *Identification of Host Kinase Genes Required for Influenza Virus Replication and the Regulatory Role of MicroRNAs.* PLoS One, 2013. **8**(6): p. e66796.
28. Huang, L., et al., *Induction and role of indoleamine 2,3 dioxygenase in mouse models of influenza a virus infection.* PLoS One, 2013. **8**(6): p. e66546.
29. Oksanen, A., et al., *Proprotein convertase FURIN constrains Th2 differentiation and is critical for host resistance against Toxoplasma gondii.* J Immunol, 2014. **193**(11): p. 5470-9.
30. Watford, W.T., et al., *Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii.* J Exp Med, 2008. **205**(12): p. 2803-12.
31. Li, X., et al., *Tumor Progression Locus 2 (Tpl2) Activates the Mammalian Target of Rapamycin (mTOR) Pathway, Inhibits Forkhead Box P3 (FoxP3) Expression, and Limits Regulatory T Cell (Treg) Immunosuppressive Functions.* J Biol Chem, 2016. **291**(32): p. 16802-15.
32. Acuff, N.V., et al., *Tumor progression locus 2 differentially regulates IFNgamma and IL-17 production by effector CD4+ T cells in a T cell transfer model of colitis.* PLoS One, 2015. **10**(3): p. e0119885.
33. Majumdar, S., et al., *Three CRISPR-Cas immune effector complexes coexist in Pyrococcus furiosus.* RNA, 2015. **21**(6): p. 1147-58.
34. Zhao, P., et al., *The human embryonic stem cell proteome revealed by multidimensional fractionation followed by tandem mass spectrometry.* Proteomics, 2015. **15**(2-3): p. 554-66.
35. Sun, T., et al., *One-Step Selective Exoenzymatic Labeling (SEEL) Strategy for the Biotinylation and Identification of Glycoproteins of Living Cells.* J Am Chem Soc, 2016. **138**(36): p. 11575-82.
36. Mastorci, K., et al., *Phospholipid scramblase 1 as a critical node at the crossroad between autophagy and apoptosis in mantle cell lymphoma.* Oncotarget, 2016. **7**(27): p. 41913-41928.

FACILITIES & OTHER RESOURCES

Laboratory:
Dr. Watford's (co-PI) laboratory space occupies approximately 1,000 sq ft. of laboratory space and is located in Room 330 of the College of Veterinary Medicine Building with a nearby office in Room 357. The space includes four double-sided work benches and dedicated desk/computer space along the rear wall. There is sufficient bench and desk space for 8 investigators.

Dr. Klonowski's (co-PI) laboratory occupies space in the Paul D. Coverdell Center for Biomedical Research at the University of Georgia. The PI's laboratory is centrally located on the 3rd floor of this building which was designed with an open lab set-up. The overall square footage of the laboratory is approximately 1,000 sq ft. and includes 2 double-sided work benches and dedicated desk/ computer space along the entire rear wall. An additional tissue culture room is dedicated to the PI.

Dr. Well's (co-I) laboratory occupies two 1,400 sq. ft. research laboratories in the Complex Carbohydrate Research Center, which are equipped for biochemistry, analytical chemistry, and molecular and cellular biology. In addition, the Wells group has a cold room and a fully equipped 250 sq. ft. mammalian cell culture laboratory with incubators and biosafety cabinets. A secondary office that is set up for computer-based data processing is also available. Specialized equipment that is in the laboratory includes 5 LC-MS/MS systems all equipped with nano-LC and sources (Thermofisher: Velos Pro with external ETD/HCD, LTQ-OrbitrapXL with ETD, Elite-Orbitrap with ETD, the Fusion Tribrid Orbitrap with ETD, and the Fusion II Lumos Tribrid Orbitrap with ETD), a Beckmann PF-2D, an Agilent 1100 HPLC, UV/Vis Spectrophotometer, UV/Vis 96-well Plate Reader, Mammalian/Bacterial Electroporator, BioRad VersaDoc 3000 Imaging Station, Nexcelom Cellometer, 2D-gel apparatus, Thermocycler, Inverted Microscope with digital camera, 2 biosafety cabinets, 3 CO_2 incubators, centrifuges, 2 sonicators, luminometer, polytron, and multiple DNA and protein gel rigs with power supplies. All shared equipment at the CCRC is also available for his use including multiple mass spectrometers (MALDI-TOF, Q-TOF, TOF-TOF, IT-TOF, Q-Exactive, GC-MS, etc.), fluorescent and confocal microscopes, preparative and ultra centrifuges, Biacore (SPR) instruments, scintillation counters and a microwave-assisted solid-phase peptide synthesizer. The Wells' research team consists of 1 Chemistry Graduate Student, 2 Biochemistry Graduate Students, 3 Post-Doctoral Fellows, a Research Assistant Professor, a Research Computing Professional, and a Laboratory Manager as well as 3 Undergraduate Students.

All laboratory spaces are fully equipped with centrifuges, microscopes, scales, PCR and RT-PCR machines, molecular biology equipment, biosafety cabinets and CO_2 incubators and have access to additional shared space and core facililties located in the College of Veterinary of Medicine Building and Coverdell as detailed in the "Equipment" page.

Animals: Sufficient space for housing mice is available to the investigative staff in any of several vivariums on campus: the College of Veterinary Medicine's Central Animal Facility, the AHRC, and the Coverdell Research Vivarium located just across the street. Animal Resources staff provide daily monitoring, husbandry, and necessary veterinary care, while personnel from the Watford and Klonowski labs will perform all experimental manipulations and observations of test animals. All employees involved in the *in vivo* studies have hands-on rodent handling experience and all protocols are reviewed and approved by the Institutional Animal Care and Use Committee. Each of these facilities has a veterinarian on staff to ensure the well being of all animal subjects.

Dr. Watford's animals currently occupy two rooms (Rm 194 and Rm 111) in the Central Animal Facility. They are specific pathogen free, including routine testing as helicobacter-negative, and are housed in sterile microisolator cages with sterilized feed and water. Dr. Watford can house up to 320 cages in her combined rooms. Each of the two rooms is equipped with a BSL-2 certified biosafety cabinet. There are also two common procedure rooms that are available to the investigative staff within the Central Animal Facility. Dr. Watford also has a Mark I 68A Irradiator with a 2,200 Curie Cesium-137 source for irradiating large numbers of animals. Each of the three mouse holders is designed to hold up to 18 mice for a capacity of 54 mice per irradiation cycle. This irradiator is housed in the College of Veterinary Central Animal Facility where Dr. Watford's animals are housed.

Dr. Klonowski's mice are housed in the basement of the Coverdell building and cared for by personnel of the Animal Welfare and Veterinary Staff at The University of Georgia. Dr. Klonowski can house up to 1000 cages in her total rooms and one of Dr. Klonowski's spaces also has an ante room housing surgical equipment, a microscope, and other supplies used exclusively by the PI's lab for procedures including tissue harvesting. Inside each of the housing areas, there is also a BSL2 cabinet for infecting the animals with low pathogenic (mouse adapted) influenza.

The University of Georgia is registered as a research facility with the United States Department of Agriculture, and is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animal welfare assurances are filed with OPRR-NIH.

Clinical: Not applicable.

Computer: The research computing at the University of Georgia maintains an extensive data communication network. Dr. Watford uses a desktop iMac, and her laboratory is equipped with four desktop iMacs and 2 Dell PCs (1 laptop and 1 desktop). Dr. Klonowski has a Dell Latitude E6430 laptop, and the laboratory is equipped with 2 desktop PC and 2 desktop Macs. The most powerful Mac utilizes a quad core with two monitors that runs a virtual platform continuously on one of them for software compatibility purposes (also required for analysis of large volume of flow cytometry data). Back-up storage server systems exist for Dr. Watford's laboratory in the College of Veterinary Medicine and Dr. Klonowski's laboratory in the Coverdell Building, in the Department of Cellular Biology, and from periodic back-ups on external hard drives. Computers, printers, and other items are state-of-the-art and linked to the UGA intranet and have university-wide wireless internet access with all the needed programs. The Wells' laboratories and offices have multiple desktop and laptop computers and printers. There are also 3 dedicated multi-node server workstations with multiple software packages (including Proteome Discover, SequestHT, Mascot, Byonic, and ProteoIQ) for omics data analyses in the Wells' laboratory and a multi-terabyte mirrored backup server.

Office: Dr. Watford has an office ~168 sq ft. located in room 357 of the Veterinary Medicine Building just down the hall from her lab. Dr. Klonowski has an office ~168 sq ft. which is down a small corridor from her laboratory. Dr. Wells occupies a 180 ft^2 office adjacent to his respective lab. All labs and offices within the CCRC are interconnected by Fast Ethernet (1G switches).
Adequate office spaces are available for scientists, post-docs, technicians and students.

Scientific and intellectual environment: Dr. Klonowski's lab (co-PI) is embedded in the Center for Tropical & Emerging Global Diseases which studies the immunology, transmission, and genetic components of infectious diseases common to tropical climates. In addition, faculty with research interests similar to the PI's are located across the street in the CVM building (College of Veterinary Medicine), within the Department of Infectious Disease, where Dr. Watford's lab (co-PI) and office are located. In addition, the University of Georgia has made a significant investment over the last decade, and particularly the last 5 years, to position itself as a strong Southeastern hub of biomedical research. In 2012, the Center for Molecular Medicine (with strengths in stem cell biology) opened with a mission of understanding the cellular mechanisms of disease to develop novel therapies and diagnostics. Importantly, the Center for Vaccines and Immunology (CVI), headed by Dr. Ted Ross, opened in the summer of 2016. Virologists and immunologists with research programs involving respiratory pathogens/ respiratory biology certainly complementing this proposal are members of the CVI and both Drs. Watford and Klonowski are associate members. The Klonowski lab is well engaged within both the Departments of Cellular Biology and Infectious Diseases (PI holds dual appointment), attending weekly seminars and monthly research in progress talks. Monthly mixers within Departments and Centers are also valued for not only for their social aspect but also for facilitating scientific exchange between faculty, students and post-docs. In addition, there are also several open format immunology-related journal clubs that further promote scientific discussions and foster interaction among colleagues. Members of the Klonowski and Watford labs attend one of these jointly with Dr. Rick Tarleton's group.

ThermoFisher Center of Excellence in Glycoproteomics

In 2015, ThermoFisher appointed the Wells' and Tiemeyer's laboratories at the CCRC as a Center of Excellence in Glycoproteomics. Beyond the ThermoFisher-sponsored training this allows us to provide the community, it also provides us access to instrumentation and service. Further, it provides us an opportunity to have in-depth discussions with software developers with regards to needs related to identifying protein modifications and complexes.

Core Facilities and Other Support:

A number of core facilities are available to researchers on a for-fee basis. These include the Georgia Genomics Facility that conducts gene sequencing and expression analysis, the Flow Cytometry Facility which provides cell sorting capabilities and access to additional FACS analyzers, monoclonal antibody production and the Statistical Consulting Center that provides statistical support for research projects.

Additional support services include animal care, IT, library services, electronics technicians and machine shop facilities.

Equipment:

Wendy Watford, co-PI

The Watford lab is equipped with three BSL-2 certified biosafety cabinets, two air-jacketed CO_2 incubators that automatically switch to a back-up CO_2 tank, one non-CO_2 incubator, an AutoMacs cell separator, a heated shaking incubator, two tabletop refrigerated centrifuges, four microcentrifuges, a refrigerated microfuge, a standard balance, a four-point precision balance, an inverted Zeiss microscope, an Epoch monochromater-based microplate reader & spectrophotometer, one chromatography refrigerators, two -20°C freezers, one -80°C freezer, electrophoresis equipment (DNA, SDS-PAGE, Westerns), an iBlot transfer system, three thermocyclers, three thermomixers, a StepOnePlus RT-PCR machine, an automated Countess cell counter, and a heated water bath with two chambers.

Dr. Watford also has a Mark I 68A Irradiator with a 2,200 Curie Cesium-137 source for irradiating large numbers of animals. Each of the three mouse holders is designed to hold up to 18 mice for a capacity of 54 mice per irradiation cycle. This irradiator is housed in the College of Veterinary Central Animal Facility where Dr. Watford's animals are housed.

Dr. Watford's group also has access to equipment located in common space or in neighboring labs on the third floor of the Veterinary Medicine building: chemical fume hood, an additional StepOnePlus RT-PCR machine, liquid nitrogen storage, FluorChem Western blot developing system with UV, TissueLyser tissue processor, fluorescence and luminescence plate readers, Intravital Imaging System (IVIS), water purification systems, autoclaves, walk-in cold room, and glassware dishwasher.

All animal work is conducted in two BSL-2 biosafety cabinets that are located in our two designated animal rooms (one private and one shared with one other investigator).

Kimberly Klonowski, co-PI

The Klonowski lab contains a private tissue culture room equipped with a Zeiss inverted microscope, air-jacketed CO_2 incubators w/ decon cycle, incubator/ shaker, and a BSL-2 certified biosafety hood. We also have a refrigerated centrifuge, large, non-CO_2 incubator, TissueLyser tissue processor, Thermo Scientific Arktik thermal cycler, scales (analytical and gram), microfuges, electrophoresis apparatus (DNA, SDS-PAGE, Westerns) ,UV spectrophotometer, refrigerators, -20 and -80 freezers, and a Z2 Coulter particle counter. Additional space in our designated animal room contains all of our surgical equipment (instruments and sterilizers), a Zeiss dissecting microscope, and a secondary stock of general lab supplies appropriated for animal work.

Dr. Klonowski's group also has unrestricted access to the following equipment in laboratories flanking the Klonowski lab in the Coverdell building: Leica inverted fluorescent microscope, 2 Peltier thermal cyclers, Applied Biosystems ABI 7500 multicolor real-time PCR detection system, SpeedVac, Sorvall high speed centrifuge, ELISPOT reader, Cryostat, HPLC systems (3), SpectraMax 340 microtiter plate reader, Roche Light Cycler, Beckman CEQ 200XL DNA Sequencer, Hitachi Genetic System Spbio III microarray spotter, Genetic Micro Systems 418 array scanner, and a 30,000 sample Liquid Nitrogen Storage System (we rent space).

General facility equipment: autoclaves (large and small), cage washer, 72-box HEPA filtered mouse microisolator racks with watering system (4), walk-in cold room, liquid N2 cryostorage freezer and dewars Dishwasher, 4' and 6 BSC for animal handling.

Lance Wells, co-I

The labs of Dr. Wells are well equipped for research in biochemistry, analytical chemistry, molecular and cellular biology, tissue culture, and proteomics data analyses. Specialized equipment available for the research proposed in this application in the PI's laboratory include:

Mass Spectrometers and LC Systems: 1 Thermofisher Fusion II Lumos Tribrid Orbitrap with ETD, with nanospray ion source, autosampler, and Ultimate3000 nano-LC; 1 Thermofisher Fusion Tribrid Orbitrap with ETD, with nanospray ion source, autosampler, and Ultimate3000 nano-LC; 1 Thermofisher Elite-Orbitrap with ETD, with nanospray ion source, autosampler, and Ultimate3000 nano-LC; 1 Thermofisher LTQ-OrbitrapXL with ETD, with nanospray ion source, flow splitter, and surveyor LC; 1 Thermofisher Velos Pro LC-MS/MS system with external ETD/HCD, with nanospray ion source, autosampler, and Ultimate3000 nano-LC; 1 Agilent 1100 LC with fraction collector

Tissue Culture (in a dedicated cell culture room): 2 Biosafety Cabinets, 3 CO_2 Incubators, 1 Inverted microscope with digital camera, 1 Electroporator, 2 Centrifuges, 1 Nexcelom cellometer

Computer Room (in a dedicated secondary office): 3 Multi-node parallel processing workstations (all with licenses for ProteomeDiscover, SequestHT, Mascot, Byonic, and our customized versions of ProteoIQ and GRITs), 1 Mirrored,4 hard drives, backup server (32 TB)

Additional Resources in the CCRC and Connected CMM Building (available to the PI): 2 Confocal microscopes, 3 Fluorescence microscopes (1 inverted), 2 Biacore SPR Instruments, 1 Microwave-assisted solid-phase peptide synthesizer, 2 Flow cytometers, 1 Sterile flow sorter, Multiple NMR Instruments (600, 800, and 900 MHz), Multiple LC- and GC-mass spectrometers (including Q-TRAP, IT-TOF, MALDI-TOF, Q-Exactive)

Equipment in core facilities relevant to this grant:
A core facility is located in the Veterinary Medicine Building (where Dr. Watford's lab is located) that houses a 13-color BD LSRII, a 9-color BD LSRII, a FACs ARIA sorter and a Nikon Ti Inverted Confocal Microscope with A1R/Spectral Scanner and Environmental Chamber.

A second core flow cytometry facility is located in the Coverdell Center (where Dr. Klonowski's lab is located) and is managed by the Center for Tropical and Emerging Global Diseases. This site contains a Beckman Coulter-Cytomation 9-color CyAn and HyperCyAn analyzer and a MoFlo high-speed sorter, two 4-color Facscaliburs, and a BioRad Luminex bead array reader.

Also located in the Coverdell Center is the Biomedical Microscopy Core, which includes a Zeiss LSM 510 META and a Zeiss LSM 710 Confocal microscope, a DeltaVision I and DeltaVision II inverted microscope, a BD Pathway 435 Bioimager, a Zeiss Axio Examiner, and a Zeiss ELYRA S1 Super Resolution Microscope.

Other cores relevant to this application:
Histology Laboratory: Tissue Tek Embedding Center, Microm HM325 Microtome, Thermo Electrom Cryostat, Leica Autostainer XL, Tissue Tek VIP 5 Tissue Processor.

OMB Number: 4040-0001
Expiration Date: 10/31/2018

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE – Project Director/Principal Investigator					
Prefix:		First Name:	Wendy	Middle Name:	
Last Name:	Watford			Suffix:	
Position/Title:	ASSOCIATE PROFESSOR	Department:	INFECTIOUS DISEASES		
Organization Name:	University of Georgia		Division:	College of Veterinary Medicine	
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State:	GA: Georgia		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	30602	
Phone Number:	706-542-4585	Fax Number:			
E-Mail:	watfordw@uga.edu				
Credential, e.g., agency login	watfordw				
Project Role:	PD/PI	Other Project Role Category:			
Degree Type:	PhD				
Degree Year:					
Attach Biographical Sketch	Plac8_Watford_Biosketch.pdf				
Attach Current & Pending Support					

PROFILE - Senior/Key Person					
Prefix:		First Name:	Kimberly	Middle Name:	D
Last Name:	Klonowski			Suffix:	
Position/Title:	ASSOCIATE PROFESSOR	Department:	Cellular Biology		
Organization Name:	University of Georgia		Division:	Franklin College	
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City:	ATHENS	County/Parish:			
State:	GA: Georgia		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	30602	
Phone Number:	706-583-5576	Fax Number:			
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Project Role:	PD/PI	Other Project Role Category:			
Degree Type:	PhD				
Degree Year:					

OMB Number: 4040-0001
Expiration Date: 10/31/2018

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

Attach Biographical Sketch	Klonowski_Biosketch.pdf
Attach Current & Pending Support	

PROFILE - Senior/Key Person					
Prefix:		First Name:	Robert	Middle Name:	Lance
Last Name:	Wells			Suffix:	
Position/Title:	PROFESSOR	Department:	Complex Carbohydrate Res Ctr		
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Street 2:	315 RIVERBEND ROAD				
City:	ATHENS	County/Parish:			
State:	GA: Georgia		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	30602	
Phone Number:	706-542-7806	Fax Number:			
E-Mail:	lwells@ccrc.uga.edu				
Credential, e.g., agency login	lancewells				
Project Role:	Co-Investigator	Other Project Role Category:			
Degree Type:					
Degree Year:					
Attach Biographical Sketch	Wells_Biosketch.pdf				
Attach Current & Pending Support					

ADDITIONAL SENIOR/KEY PERSON PROFILE(S)

Additional Biographical Sketch(es)

Additional Current and Pending Support(s)

BIOGRAPHICAL SKETCH

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

NAME: Watford, Wendy Tharpe

eRA COMMONS USER NAME (credential, e.g., agency login): watfordw
POSITION TITLE: Associate 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
University of Georgia, Athens, GA	B.S.	05/1996	Genetics
Duke University, Durham, NC	Ph.D.	08/2001	Cell Biology
National Institutes of Health, Bethesda, MD		07/2009	Immunology

A. Personal Statement:

Dr. Watford trained with Dr. John O'Shea, a leader in the field of cytokine signaling and T helper cell differentiation at the National Institutes of Health, prior to establishing her own independent research group at the University of Georgia. Utilizing a combination of cellular and molecular immunological approaches, Dr. Watford has made significant scientific contributions regarding cytokine signaling and T helper cell differentiation. In particular, Dr. Watford identified the proprotein convertase, furin (*Nature*), and the serine-threonine kinase, Tpl2 (aka Map3K8 or Cot)(*J Exp Med*), as IL-12-induced Stat4 target genes. Dr. Watford has continued to study the role of Tpl2 in innate and adaptive inflammatory processes to better understand how Tpl2 activity could be modulated therapeutically for treating infectious and autoimmune diseases. Of particular importance to the current application, Dr. Watford's group has established the influenza virus infection model in the lab and has recently demonstrated that Tpl2 functions early during virus infection to induce IFN-lambda induction and impede influenza virus replication (*PLoS Pathogens*). The current proposal seeks to investigate how another Th1-candidate gene, Plac8, participates in antiviral immunity by promoting memory CD8 T cell formation. Dr. Watford's expertise in cell signaling, T helper cell differentiation, immunoprecipitation and Western blotting will facilitate the completion of many of the biochemical aspects of the application, and her experience generating bone marrow chimeric mice is also essential for *in vivo* studies. Information obtained from these studies will help inform the design of novel vaccines (potentially modulating Plac8 or its interacting proteins) that will enhance vaccine efficacy.

B. Positions and Honors:

Employment

2015- Associate Professor, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia. Immunoregulation of host defense and tolerance.

2009-2015 Assistant Professor, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia. Immunoregulation of host defense and tolerance.

2001-2009 Post-doctoral training, Molecular Immunology and Inflammation Branch, Lymphocyte Cell Biology Section, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health. Regulation of T helper cell differentiation by Stat transcription factors (advisor Dr. John J. O'Shea)

Honors

2015	American Association of Immunology, Careers in Immunology Fellowship Award
2009	UGA Nominee for Pew Scholars Program in the Biomedical Sciences
2004	NIH Merit Award for Research on STAT5, NIH, USA
2003	Fellows Award for Research Excellence, NIH, USA
1996	Graduated Summa Cum Laude, The University of Georgia, Athens, GA, USA
1996	Phi Beta Kappa
1992-1996	The University of Georgia Honors Program
1992	High School Valedictorian, 1992 (class of 230)

C. Contributions to science.

1. Role of the serine-threonine kinase, Tpl2, in the regulation of T helper cell differentiation.
During my post-doctoral fellowship at the NIH, I discovered the serine-threonine kinase Tpl2 as an IL-12-induced STAT4 target gene. My analysis of Tpl2-deficient mice revealed that this kinase is important for ensuring optimal IL-12-induced IFN-γ production by CD4 T cells. Consequently, host resistance to the intracellular pathogen, *Toxoplasma gondii* was impaired in a T cell-intrinsic manner. I further demonstrated biochemically that Tpl2 promotes the expression of important Th1 transcription factors, STAT4 and T-bet, upon TCR stimulation. This finding that Tpl2 also participates in TCR-induced signal transduction led to the hypothesis that Tpl2 may also regulate the differentiation of other T helper cell lineages by modulating the perceived TCR signal strength. Indeed, ablation of Tpl2 also exacerbated Th2-mediated inflammation in an ovalbumin sensitization and challenge model of allergic asthma. Furthermore, we have also recently demonstrated that, despite promoting Th17 differentiation *in vitro* by suppressing Foxp3 expression, Tpl2 genetic ablation impaired Th1, but not Th17-driven inflammation *in vivo* in a T cell transfer model of colitis. In a separate study, we demonstrated that Tpl2 ablation resulted in reductions in iTreg differentiation, Foxp3 expression and immunosuppressive functions by impairing activation of the PI3K/Akt/mTOR pathway. Overall, these findings suggest that small molecule inhibitors of Tpl2 may be effective at treating chronic autoimmune diseases with a Th1 signature.
   - **Watford WT**, Hissong BD, Durant LR, Yamane H, Muul LM, Kanno Y, Tato CM, Ramos HL, Berger AE, Mielke L, Pesu M, Solomon B, Frucht DM, Paul WE, Sher A, Jankovic D, Tsichlis PN, O'Shea JJ. Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii. *J Exp Med*. 2008 Nov 24;205(12):2803-12.
   - **Watford WT**, Wang CC, Tsatsanis C, Mielke LA, Eliopoulos AG, Daskalakis C, Charles N, Odom S, Rivera J, O'Shea J, Tsichlis PN. Ablation of tumor progression locus 2 promotes a type 2 Th cell response in Ovalbumin-immunized mice. *J Immunol*. 2010 Jan 1;184(1):105-13.
   - Acuff NV, Li X, Kirkland R, Nagy T, **Watford WT**. Tumor progression locus 2 differentially regulates IFNγ and IL-17 production by effector CD4+ T cells in a T cell transfer model of colitis. *PLoS One*. 2015 Mar 17;10(3):e0119885.
   - Li X, Acuff NV, Peeks AR, Kirkland R, Wyatt KD, Nagy T, **Watford WT**. Tumor Progression Locus 2 (Tpl2) Activates the Mammalian Target of Rapamycin (mTOR) Pathway, Inhibits Forkhead Box P3 (FoxP3) Expression, and Limits Regulatory T Cell (Treg) Immunosuppressive Functions. *J Biol Chem.* 2016 Aug 5; 291(32):16802-15.

2. Role of the serine-threonine kinase, Tpl2, in host innate immune responses.
In addition to the regulation of adaptive immunity as discussed above, we have made significant advances in understanding how Tpl2 also regulates innate immune responses. First, we demonstrated that Tpl2 ablation in dendritic cells has less severe consequences on Toll-like receptor signaling compared to its ablation in macrophages. These findings illustrate important cell type specific effects of Tpl2 ablation. Despite this finding, IL-1β mRNA induction was severely impaired in both cell types and correlated with enhanced susceptibility of Tpl2-deficient mice to infection with *Listeria monocytogenes*. We also showed that Tpl2 is important for proper expression of chemokine receptors that direct macrophage recruitment during acute inflammation. In all of these different instances, we have shown that Tpl2 is an important innate pro-inflammatory molecule suggesting that its therapeutic targeting may be predicted to alleviate chronic inflammation in patients with autoimmune or autoinflammatory diseases. A systematic analysis of Tpl2 activation by diverse TLRs also revealed differences in how distinct TLRs engage Tpl2. TLRs 2, 4 and 7 rapidly triggered Tpl2 activation, ERK phosphorylation and TNF-alpha secretion. On the contrary, endosomal TLRs 3 and 9 failed to rapidly activated ERK and had delayed TNF secretion. Importantly, this

study revealed that delayed Tpl2 activation and ERK phosphorylation by TLRs 3 and 9 required autocrine ROS signaling. These data are the first to suggest that Tpl2 regulates ROS production and suggests that Tpl2 might play a role in ROS-mediated killing of bacteria by macrophages and neutrophils, a hypothesis that is currently being tested. Finally, and central to the current application, we demonstrated that Tpl2 ablation severely impairs host mucosal IFNλ responses to influenza virus infection and drives morbidity and mortality to an otherwise low pathogenicity virus strain. This indicates that Tpl2 is a central component in innate virus sensing and suggests that Tpl2 modulation could be exploited therapeutically for enhancing vaccine efficacy to a range of 'mucosal-tropic' viruses.

- Mielke LA, Elkins KL, Wei L, Starr R, Tsichlis PN, O'Shea JJ, **Watford WT**. Tumor progression locus 2 (Map3k8) is critical for host defense against Listeria monocytogenes and IL-1 beta production. *J Immunol*. 2009 Dec 15;183(12):7984-93.
- Rowley SM, Kuriakose T, Dockery LM, Tran-Ngyuen T, Gingerich AD, Wei L, **Watford WT**. Tumor progression locus 2 (Tpl2) kinase promotes chemokine receptor expression and macrophage migration during acute inflammation. *J Biol Chem*. 2014 May 30;289(22):15788-97.
- Kuriakose T, Rada B, **Watford WT**. Tumor progression locus 2-dependent oxidative burst drives phosphorylation of extracellular signal-regulated kinase during TLR3 and 9 signaling. *J Biol Chem.* 2014 Dec 26;289(52):36089-100.
- Kuriakose T, Tripp RA, **Watford WT**. Tumor progression locus 2 promotes induction of IFNλ, interferon stimulated genes and antigen-specific CD8+ T cell responses and protects against influenza virus. *PLoS Pathog*. 2015 Aug 4;11(8):e1005038.

3. Role of STAT transcription factors in T cell effector functions.

During my postdoctoral training, I clarified some significant controversies regarding how cytokine signaling via the Jak/Stat pathway regulates T cell differentiation and effector functions. Major findings include the demonstration that type I IFNs can activated Stat4 during a viral infection. It had previously been thought that type I IFNs were capable of activating Stat4 in human T cells, but not in murine T cells. We showed that type I IFNs can directly activated Stat4 in CD4 and CD8 T cells *in vitro*. Furthermore, using a murine model of LCMV viral infection, we demonstrated that phosphorylation of Stat1 versus Stat4 by type I IFNs is dynamically regulated, since Stat4 activation wanes as Stat1 protein levels are induced by virus-induced type I IFNs. These findings revealed that Stat4 activation by type I IFNs was, in fact, similar between mice and humans. I was co-first author on this study. Next, I demonstrated that Stat5 was absolutely essential for normal lymphoid development. Analysis of *Stat5a*$^{-/-}$*Stat5b*$^{-/-}$ mice allowed us to demonstrate that the somewhat modest immunological phenotype of the previous Stat5 KO mouse model was due to the presence of a hypomorphic allele. I was co-first author on this study. In additional studies that I co-authored, I performed colitis experiments that demonstrated the essential functions of Stat3 in promoting colitis in a T cell transfer model, as well as the essential function of the Stat4 target gene, proprotein convertase furin, in opposing colitis development in the same model.

- Nguyen KB*, **Watford WT***, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science.* 2002 Sep 20;297(5589):2063-6.
  *equal contribution
- Yao Z*, Cui Y*, **Watford WT***, Bream JH*, Yamaoka K, Hissong BD, Li D, Durum SK, Jiang Q, Bhandoola A, Hennighausen L, O'Shea JJ. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A.* 2006 Jan 24;103(4):1000-5.
  *equal contribution
- Durant L, **Watford WT**, Ramos HL, Laurence A, Vahedi G, Wei L, Takahashi H, Sun HW, Kanno Y, Powrie F, O'Shea JJ. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity.* 2010 May 28;32(5):605-15.
- Pesu M, **Watford WT**, Wei L, Xu L, Fuss I, Strober W, Andersson J, Shevach EM, Quezado M, Bouladoux N, Roebroek A, Belkaid Y, Creemers J, O'Shea JJ. T cell expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. *Nature*. 2008 Sep 11;455(7210):246-50.

4. Immunoregulatory functions of surfactant protein A within the lung.

During my doctoral training, I demonstrated the important immunoregulatory roles of surfactant protein A (SP-A) in the lung. I demonstrated that SP-A binds to and agglutinates group B streptococcus and was

important for its efficient uptake and clearance from the lung by alveolar macrophages. In addition, I demonstrated that SP-A interacts with complement component C1q to (1) antagonized complement activation and the induction of inflammation within the lung and (2) promote the phagocytosis of pathogens or particles opsonized by C1q. The result is the clearance of foreign bodies via a non-inflammatory mechanism to limit damage to the lung epithelium.

- LeVine AM, Kurak KE, Wright JR, **Watford WT**, Bruno MD, Ross GF, Whitsett JA, Korfhagen TR. Surfactant protein-A binds group B streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein-A-deficient mice. *Am J Respir Cell Mol Biol*. 1999 Feb;20(2):279-86.
- **Watford WT**, Wright JR, Hester CG, Jiang H, Frank MM. Surfactant protein A regulates complement activation. *J Immunol.* 2001 Dec 1;167(11):6593-600.
- **Watford WT**, Smithers MB, Frank MM, Wright JR. Surfactant protein A enhances the phagocytosis of C1q-coated particles by alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol.* 2002 Nov;283(5):L1011-22.
- **Watford WT**, Ghio AJ, Wright JR. Complement-mediated host defense in the lung. *Am J Physiol Lung Cell Mol Physiol.* 2000 Nov;279(5):L790-8.

URL to full list of publications in PubMed: http://www.ncbi.nlm.nih.gov/pubmed/?term=watford+w

D. Research Support:

Ongoing Research Support

- NIH Grant 1R01AI099058-01A1; NIAID; Principal Investigator, 11/22/2012-2017
Tpl2-dependent IFN-gamma production: contribution to host defense and autoimmunity.
The goal of this project is to define the role of the MAP kinase Tpl2 in TCR signaling, T helper cell development and contribution to autoimmune diseases.

- Institutional start-up funds, Department of Infectious Diseases, University of Georgia, 2009-2017.

Completed Research Support

- Institutional Faculty Research Grant #2234; UGA; Role: PI (07/1/12-06/30/13)
Title: Role of Tpl2 in host immunity to *Mycobacterium tuberculosis*

- NIH Grant R56AI099058-01; NIH/NIAID; Role: PI (09/25/12-11/30/12)
Title: MAP3K8-mediated regulation of adaptive immune responses and autoimmunity
The goal of this project is to define the role of the MAP kinase Tpl2 in TCR signaling, T helper cell development and contribution to autoimmune diseases.

- NIH Grant 1S10RR031791-01; NIH/NCRR; Role: PI (08/01/11-07/31/12)
Title: MarkI 68A Cesium-137 Gamma Irradiator
The goal of this project was to provide UGA researchers with an irradiator to support other NIH-funded projects.

- NIH Grant K22AR053953-03; NIH/NIAMS; Role: PI (08/01/09-7/31/12)
Title: Tpl2-dependent IFN-γ production: contribution to host defense and autoimmunity
The goal of this project was to define the role of the MAP kinase, Tpl2, as a regulator of IFN-γ and Type 1 immunity

BIOGRAPHICAL SKETCH

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

NAME: Klonowski, Kimberly D.

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

POSITION TITLE: Associate Professor of Cellular Biology

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
University of Delaware; Newark, DE	BA	1991-1995	Biology
Temple University School of Medicine; Philadelphia, PA	PhD	1995-2001	Microbiology and Immunology
University of Connecticut Health Center; Farmington, CT	Postdoctoral	2001-2006	Immunology

A. Personal Statement

My research interests stem from my fascination with CD8 T cells and the fact that culmination of environmental signals interpreted by these cells early during activation and within specific niches profoundly influences their differentiation and subsequent immune outcomes. My lab is perhaps best known for demonstrating that respiratory influenza infection generates distinct pools of CD8 memory T cells which develop, migrate and are maintained distinctly from the archetypical memory derived from non-physiological, yet well characterized, systemic infections. Understanding mechanistically what biases this unique respiratory CD8 signature is the primary focus of the lab and has significant implications on vaccine formulation. I would say our approach to this problem is multifaceted, considering how characteristics of the virus and novel cellular interactions imprint specific features on respiratory memory development. We have standing collaborations with virology labs on campus with whom we have worked with cooperatively to mentor students, submit research proposals and manuscripts. I also have a keen interest in teaching and training graduate and undergraduate students in immunology, both in the laboratory and the classroom. My lab participates in formal and informal programs which introduce elementary school children and underrepresented high school students to science. I also advise budding undergraduate scientists as the Undergraduate Coordinator in the Cellular Biology Department.

The goal of the current application is to dissect the Plac8 dependent molecular mechanisms responsible for programming CD8 T cell memory after respiratory infection. This has profound clinical relevance as the longevity of respiratory CD8 T cell memory is directly linked to the longevity of heterosubtypic immunity. We have extensive expertise in mapping anti-viral CD8 T cell responses, including those at low frequencies in non-lymphoid tissues. Using mouse models of influenza infection, the laboratory has made considerable progress defining how anti-viral CD8 T cells differ from their systemically derived counterparts. I have been studying memory CD8 T cell responses for 16 years beginning with my postdoctoral fellowship with Leo Lefrancois at the University of Connecticut. Since my appointment at UGA in 2006, I focused my research program on respiratory infections because of significant knowledge gaps of basic immunology in the lung and the need for improved influenza vaccines. Many projects have moved beyond CD8 T cell intrinsic factors and expanded to physically distinct environments and contributing cell extrinsic factors relevant to memory cell programming in vivo.

Publications particularly relevant to the current application:

Verbist, K.C., Field, M.B., and K.D. Klonowski. IL-15-independent maintenance of mucosally generated memory CD8 T cells. *Cutting Edge: J. Immunol.* 186(12):6667-71, 2011.

Shane, H.L. and K.D. Klonowski. A direct and nonredundant role for Thymic Stromal Lymphopoietin on anti-viral CD8 T cell responses in the respiratory mucosa. *J. Immunol.* 192(5): 2261-70, 2014.

Shane, H.L., Verbist, K.C. and K.D. Klonowski. Respiratory infection alters archetypal anti-viral CD8 T cell differentiation programs resulting in diminished memory potential. *J Immunol* 192 (1 Supplement):134.4, 2014.

B. Positions and Honors

Positions and Employment

2001-2006 Postdoctoral Fellow, Department of Immunology, University of Connecticut Health Center, Farmington, CT
2006-2012 Assistant Professor, Department of Cellular Biology, University of Georgia, Athens, GA
2007- Course Director & Instructor: Immunology (CBIO 4100/6100), Adv. Immunology (CBIO 8100)
2007- Adjunct Assistant Professor, Department of Infectious Diseases, University of Georgia, Athens, GA
2008- Executive Council, Faculty of Infectious Diseases, University of Georgia, Athens, GA
2013- Associate Professor, Department of Cellular Biology, University of Georgia, Athens, GA
2015- Undergraduate Coordinator, Department of Cellular Biology

Other Experience and Professional Memberships

2006- *Ad hoc reviewer: Blood, Journal of Leukocyte Biology, European Journal of Immunology, Journal of Immunology, Frontiers in Immunological Memory, Nature Medicine, Nature Reviews Immunology, Mucosal Immunology, Plos Pathogens and PlosOne*
2007- Member, American Association of Immunologists
2007 Ad hoc reviewer: Swiss National Science Foundation
2008 Study Section, Molecular Immunology, Arthritis Foundation
2009- Member, Society of Mucosal Immunology
2010-2012 Editor, *Journal of Biomedicine and Biotechnology: "Cytotoxic T Lymphocytes and Vaccine Development"*
2011 Study Section, NIH, Special Emphasis Panel: Infant Immunity
2016 Study Section, NIH, Special Emphasis Panel: NIAID Investigator Initiated Program Project Applications

Awards and Honors

2000 NIH-sponsored Travel Award to attend Keystone Symposium, Steamboat Springs, CO
2000-2001 Temple University Dissertation Grant
2004 Federation of Clinical Immunology Societies (FOCIS) Travel Assistance Award to attend the 12th International Congress of Immunology in Montreal, Canada
2012 American Association of Immunologists Early Career Faculty Travel Grant
2014 American Association of Immunologists Laboratory Travel Grant
2015 American Association of Immunologists Careers in Immunology Fellowship
2015 American Association of Immunologists Travel Grant for the 4th European Congress of Immunology

C. Contribution to Science

1. Improved our understanding of the factors that regulate memory CD8 T cell development, migration and recall potential. My early publications on CD8 T cell memory contributed to our overall understanding of key

aspects of memory CD8 T cell biology. Co-authored papers delineated how naïve CD8 precursor frequency impacts memory cell potential (a) and defined how memory cells acquire growth signals in the steady-state (b). The former study (a) significantly impacted how the field studies memory T cell development in frequently used adoptive transfer models. My studies also demonstrated that CD8 memory T cell precursors are not selected by IL-7/IL-7Ralpha interactions (c), a pervasive theory at the time, demonstrating that memory formation is complex, with redundant mechanisms in place. Importantly, I discovered that memory cells could be maintained outside of lymphoid tissues (d). However, secondary lymphoid tissues were required for recall responses where the characteristics of the infectious agent and the migratory preferences of memory cells dictated the specific secondary lymphoid tissue required (d). Cumulatively, these studies not only advanced our basic understanding of memory CD8 T cell biology but also provided information about key checkpoints in CD8 memory T cell development and maintenance which have subsequently been targeted in vaccine development.

a) Marzo, A.L., Klonowski, K.D., Le Bon, A., Burrow, P., Tough, D.F. and L. Lefrancois. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat. Immunol*. 6(8): 793-799, 2005.
b) Schluns, K.S., Klonowski, K.D., and L. Lefrancois. Trans-regulation of memory CD8 T cell proliferation by IL-15Rα+ bone marrow-derived cells. *Blood*. 103:988-994, 2004.
c) Klonowski, K.D., Marzo, A.L., Puddington, L., and Lefrancois, L. Cutting Edge: IL-7 independent regulation of IL-7Rα expression and memory CD8 T cell development. *J. Immunol.* 177(7): 4247-4251, 2006.
d) Klonowski, K.D., Marzo, A.L., Williams, K.J., Lee, S., Pham, Q., and Lefrancois, L. CD8 T cell recall responses are regulated by the tissue tropism of the memory cell and pathogen. *J. Immunol.* 177(10): 6738-6746, 2006.

2. Discovered tissue resident memory (T_{RM}) CD8 T cells. Initially, memory CD8 T cells were broadly categorized into two populations based on homing preferences, circulating between secondary lymphoid organs as central memory T cells (T_{CM}) or less discretely throughout the periphery, including non-lymphoid tissues, defined as effector memory T cells (T_{EM}). We utilized parabiotic mice to ascertain the migratory potential of T_{EM}. While my studies determined that T_{EM} migrate through many non-lymphoid tissues, I also discovered a new pool of memory CD8 T cells which remained embedded in specific tissues and did not recirculate (a,b). Because T_{RM} provide a front-line defense, and are enriched at mucosal sites, understanding the biology and contribution of these cells to long-term protection has since become a hot topic for the development of CD8 T cell vaccines against viruses such as influenza (c), HIV and malaria.

a) Klonowski, K.D., Williams, K.J., Marzo, A.L., Blair, D.A., Lingenheld, E.G., and L. Lefrancois. Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity*. 20:551-562, 2004.
b) Klonowski, K.D. and L. Lefrancois. The CD8 memory T cell subsystem: integration of homeostatic signaling during migration. *Semin Immunol.*, 17(3):219-29, 2005.
c) Shane, H.L. and K.D. Klonowski. Every breath you take: the impact of environment on resident memory CD8 T cells in the lung. *Front. Immunol.*, 5:320, 2014.

3. Described a novel function for the cytokine IL-15. The cytokine IL-15 is associated with CD8 T cell activation, proliferation and the development and maintenance of memory CD8 T cells derived from systemic infection. However, we described a new function for IL-15 in promoting the migration of anti-viral effector CD8 T (a) and NK cells (b) to the site of infection, in our case the respiratory mucosa. Moreover, we demonstrated that addition of exogenous IL-15 complexes can be used therapeutically to augment CD8 T cell trafficking to the site of application (a). This novel chemotactic property of IL-15 has since been extended to vaccines (reviewed in (c)) and tumor models where IL-15 is used to simultaneously enhance CD8 T cell migration into tumors and enhance effector function.

a) Verbist, K.C., Cole, C.J., Field, M.B., and K.D. Klonowski. A role for IL-15 in the migration of effector CD8 T cells to the lung airways following influenza infection. *J. Immunol*. 186(1): 174-182, 2011.
b) Verbist, K.C., Cole, C.J., Field, M.B., Rose, D.L., and K.D. Klonowski. IL-15 participates in the respiratory innate immune response to influenza virus infection. *PlosOne,* 7(5):e37539, 2012.
c) Verbist, K.C. and K. D. Klonowski. Functions of IL-15 in anti-viral immunity: multiplicity and variety. *Cytokine*. 59 (3): 467-478, 2012.

4. Defined key properties of respiratory-derived memory CD8 T cells. My laboratory has published several important papers defining how memory CD8 T cells derived from respiratory infections are limited in terms of number and lifespan as a result of environmental influences. Early exposure of CD8 to the enzyme indoleamine 2,3-dioxygenase (IDO) elicited by influenza infection limits CD8 T cell expansion in the respiratory tract (a). Thymic Stromal Lymphopoietin (TSLP) also tempers CD8 T cell responses in the lung by limiting early proliferation in situ while maintaining a small population of memory CD8 T cells in the respiratory tract (b). Interestingly, those memory cells generated from an influenza infection are refractory to IL-15, which is essential for memory cells derived from systemic infections (c). We (c and unpublished data) and others have demonstrated that IL-15 independence is a key signature of respiratory T_{RM} and can be used to track T_{RM} development. Overall, our work has been very influential in understanding many key aspects of regulation of respiratory CD8 T cells in the lung (d), leading up to our current work attempting to delineate the inherent mechanisms in place within the respiratory tract which limit the development of long-lived T_{RM}.

a) Fox, J.M., Sage, L.K., Huang, L., Barber, J., Klonowski, K.D., Mellor, A.L, Tompkins, S.M., and R.A.Tripp. Inhibition of indoleamine 2,3-dioxygenase enhances the T-cell response to influenza virus infection. *J. Gen. Virol.* 94(Pt 7):1451-61, 2013.
b) Shane, H.L. and K.D. Klonowski. A direct and nonredundant role for Thymic Stromal Lymphopoietin on anti-viral CD8 T cell responses in the respiratory mucosa. *J. Immunol.* 192(5): 2261-70, 2014.
c) Verbist, K.C., Field, M.B., and K.D. Klonowski. IL-15-independent maintenance of mucosally generated memory CD8 T cells. *Cutting Edge: J. Immunol.* 186(12):6667-71, 2011.
d) Shane, H.L. and K.D. Klonowski. Every breath you take: the impact of environment on resident memory CD8 T cells in the lung. *Front. Immunol.*, 5:320, 2014.

PubMed/My Bibliography link to full publication list:
http://www.ncbi.nlm.nih.gov/sites/myncbi/kimberly.klonowski.1/bibliography/40832956/public/?sort=date&direction=ascending

D. Research Support

Ongoing

R21 AI131093 Klonowski (PI) 1/2017-1/2018
NIH/ NIAID
Monocyte regulation of tissue resident memory CD8 T cells
The goal of this proposal is to determine how monocytes with a suppressive phenotype regulate CD8 T cell programming after respiratory infection

Bridge funding 7/2015-present
University of Georgia, Office of the Vice President of Research & Department of Cellular Biology
The purpose of these funds is to bridge the PI between NIH awards.
Role: PI

Completed (last 3 years)

R01 AI081800 Klonowski (PI) 2009-2015 (no cost extension)
NIH/NIAID
Cytokine regulation of memory CD8 T cell responses
The goal of this proposal is to understand how the cytokine Thymic Stromal Lymphopoietin regulates immune responses in the lung following influenza virus infection.

U01 AI83005 Klonowski (Co-I) 4/2009- 4/2014
Manipulating natural host immunoregulation via IDO during viral infection
The goal of this proposal is to understand how the enzyme IDO regulates anti-influenza immunity and viral burden.

BIOGRAPHICAL SKETCH

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

NAME: Wells, Lance

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

POSITION TITLE: Professor of Biochemistry and Molecular Biology and Endowed Investigator

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
Georgia Institute of Technology, Atlanta	B.S.	1991	Chemistry
Emory University School of Medicine, Atlanta, GA	Ph.D.	1998	Biochem & Mol Biol
Johns Hopkins University School of Medicine, Baltimore MD	Postdoc	1998-2003	Biological Chemistry

A. Personal Statement

My laboratory is focused on understanding the role that post-translational modifications and protein complexes play in increasing functional diversity of proteins. For the last 14 years, my lab has primarily focused on the biomedical significance of O-glycosylation, specifically the nutrient-sensing O-GlcNAc modification of nuclear and cytosolic proteins and the O-mannoslyation pathway that is defective in many cases of congenital muscular dystrophy. The laboratory also has extensive experience in analytical mass spectrometry and the development of glycomic and glycoproteomic quantitative platforms. We also have experience in enzymology, molecular biology, mammalian cell culture manipulation, model organisms, protein expression and purification, and peptide/protein chemistry. Our lab has also taken an active role in building consortiums to standardize and improve reliability and reproducibility in the field of glycoproteomics highlighted by the following 2 manuscripts:
1. **Wells L**, Hart GW (2013) Glycomics: building upon proteomics to advance glycosciences. *Mol Cell Proteomics* **12:** 833-5. PMID: 23378519.
2. York WS, Agravat S, Aoki-Kinoshita KF, McBride R, Campbell MP, Costello CE, Dell A, Feizi T, Haslam SM, Karlsson KH, Khoo KH, Kolarich D, Liu Y, Novotny M, Packer NH, Paulson JC, Rappe E, Ranzinger R, Rudd PM, Smith DF, Struwe WB, Tiemeyer M, **Wells L**, Zaia J, Kettner C (2014) MIRAGE: the minimum information required for a glycomics experiment. *Glycobiology* **24:** 402-6. PMID: 24653214.

B. Positions and Honors

Professional Experience

1991-1993	Research Specialist for Dr. Jan Pohl, Microchemical Facility, Winship Cancer Center, Emory University School of Medicine, Atlanta, GA
1998-2003	Postdoctoral Fellow, Department of Biological Chemistry (Laboratory of Dr. Gerald W. Hart), Johns Hopkins University School of Medicine, Baltimore, MD
2003-2009	Assistant Professor, Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center, and Adjunct in Chemistry, University of Georgia, Athens, GA
2004-Present	Georgia Cancer Coalition Distinguished Scholar
2008-Present	Director of Graduate Studies, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA
2010-2014	Associate Professor Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center and Adjunct in Chemistry

2011-Present	Georgia Research Alliance Lars G. Ljungdahl Distinguished Investigator, University of Georgia
2015-Present	Professor, Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center and Adjunct in Chemistry

Honors and Awards

1999-2002	National Research Award (NRSA) (F32 CA83261), National Cancer Institute at NIH
2007, 09, 13, 17	Invited Speaker and Session Chair (2013), Glycobiology Gordon Conference
2008, 12, 14	Invited Speaker and Co-organizer (2012), Warren Workshop on Glycoconjugate Analysis
2008-2011	Reviewer for Wellcome Trust Senior Fellowships
2009	Reviewer for NIH/NHLBI Proteomic Program Project Study Section
2010	Organizer and lecturer at ASMS Fall Workshop on Glycoproteomics
2010, 11	Reviewer for NIH Special Emphasis Panel ZRG1 Study Section
2010, 16	Invited Speaker, Rare Disease Day Symposium, San Diego, CA
2010, 12, 15-17	Invited Speaker and Session Chair (2012, 17), Society for Glycobiology Annual Meeting
2011-Present	Endowed Position, Georgia Research Alliance Lars G. Ljungdahl Distinguished Investigator
2011-Present	Editorial Board Member for *J. Biol. Chem.*
2011-Present	Editorial Board Member for *Mol. and Cell. Proteomics*
2012	Invited Speaker , ASBMB/Experimental Biology Annual Meeting
2012	Co-organizer*, Mol. and Cell. Proteomics/*ASBMB Glycomics Standards Checklist Meeting
2012	Invitee and Rapporteur , National Academy of Science Workshop on Glycoscience,
2012	Site Visit Reviewer for NCRR/NIGMS P41 Program
2012	Invited Speaker, ASBMB Workshop on Post-translational Modifications
2012	Invited Speaker, International HUPO Workshop, ThermoFisher Workshop
2012, 13	Ad-Hoc Reviewer for NIH Intracellular Interactions (ICI) Study Section
2013	Guest Editor for *Mol. and Cell. Proteomics*, Glycomic/Glycoproteomic Special Issue
2013	Invited Speaker, US-HUPO
2013	Keynote Lecturer at Short Course in Glycoanalytics, University of Copenhagen, Denmark
2013	Visiting Professor, Short Course in Cancer Biology, Eppley Institute, University of Nebraska
2013, 16	Theme Organizer, Session Chair, and Speaker, ASBMB/Experimental Biology
2013-2016	Member of Board of Directors, Society for Glycobiology
2013-Present	Editorial Board Member, *Glycobiology*
2014	Co-organizer, CFG Workshop at NIH, Exploring Glycoscience
2014	Selected Speaker, ThermoFisher Speaker at ASMS Annual Meeting
2015	Member, Organizing Committee for the Society for Glycobiology Annual Meeting
2015	Invited Plenary Speaker, Johns Hopkins Training Course in Glycobiology
2015	Invited Speaker and Panelist for Bill &Melinda Gates CAVD Annual Meeting
2015	Reviewer for NHLBI/NIH P01 Program
2015-Present	Co-Director, ThermoFisher Appointed Center of Excellence in Glycomics/Glycoproteomics
2015-Present	Co-Director of Bill and Melinda Gates Foundation Global Health Vaccine Accelerator Platform (GH-VAP) in Glycomics/Glycoproteomics.
2016	Co-Organizer and Speaker, Harnessing Glycoscience to Understand and Optimize HIV Env Immunogencity, Bill and Melinda Gates Foundation
2016	Co-Organizer, Session Chair, and Speaker, Biochemistry Society Hot Topic: O-GlcNAc London UK
2016	Student Invited Speaker, UCLA Muscle Cell Biology Seminar Series, Los Angeles, CA
2016	Award Winner and Invited Speaker, *Molecular and Cellular Proteomics ASBMB Lectureship* at Society for Glycobiology Annual Meeting
2017	Invited Speaker, GLYCO 24, Jeju, S. Korea
2017	Invited Speaker and External Examiner for Ph.D. Defense, Copenhagen Center of Glycomics, University of Copenhagen, Denmark

C. Contributions to Science

1. Quantitative Glycomics/Glycoproteomics and site mapping methodologies. Our laboratory has invested significant efforts to develop better glycomic, site-mapping, and proteomic platforms so that we can begin to assign functions to individual post-translational modifications and test glycans as potential biomarkers of disease. Towards this we have built high-thoughput glycomic platforms, developed quantitative glycomic strategies at the in vitro and in cell culture level, developed triggered collision approaches with an instrument manufacturer for site-mapping of labile post-translational modifications, developed and/or applied tagging and enrichment strategies, and applied our glycomic and proteomic technologies to modifications beyond glycosylation as well as to defining individual components in protein complexes.

1.1 Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, **Wells L**, Terns RM, Terns MP (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139:** 945-56. PMID: 19945378

1.2 Zhao P, Viner R, Teo CF, Boons GJ, Horn D, **Wells L** (2011) Combining high-energy C trap dissociation and electron transfer dissociation for protein O-GlcNAc modification site assignment. *J Proteome Res* **10:**4088-104. PMID: 21740066

1.3 Teo CF, **Wells L** (2014) Monitoring protein O-GlcNAc status via metabolic labeling and copper-free click chemistry. *Anal Biochem* **464:** 70-2. PMID: 24995865

1.4 Yu SH, Zhao P, Sun T, Gao Z, Moremen KW, Boons GJ, **Wells L**, Steet R (2016) Selective exo-enzymatic labeling detects increased cell surface sialoglycoprotein expression upon megakaryocytic differentiation. *J Biol Chem* **291**: 3982-9. PMID: 26733198

2. O-GlcNAc cycling, function, and site mapping. O-GlcNAc is a dynamic and inducible single sugar modification of nuclear and cytosolic proteins discovered by my post-doctoral mentor Dr. Jerry Hart in 1984. Since that time more than a thousand proteins have been identified that carry this modification and multiple functions have been assigned. There is only one mammalian gene for the transferase (OGT) and one for the hydrolase (OGA) that add and remove O-GlcNAc, respectively. Thus, characterizing the regulation of the O-GlcNAc cycling enzymes is a major endeavor of my laboratory. Furthermore, assigning function to specific sites of modification has been a major challenge and focus. Towards this end we have developed site-mapping methodologies and have proposed a working model along with Dr. Hart and others that O-GlcNAc is a nutrient sensor that modulates signaling and transcription. We have also recently assigned causality to mutations in OGT associated with X-linked intellectual disability (XLID).

2.1 **Wells L**, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ, Hart GW (2002) Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. *Mol Cell Proteomics* **1:** 791-804. PMID: 12438562

2.2 Durning SP, Flanagan-Steet H, Prasad N, **Wells L** (2016) O-linked beta-N-acetylglucosamine (O-GlcNAc) acts as a glucose sensor to epigentically regulate the insulin gene in pancreatic beta cells. *J Biol Chem* **291**: 2107-18. PMID: 26598517

2.3 Teo CF, El-Karim EG, **Wells L** (2016) Dissecting PUGNAc-mediated inhibition of the pro-survival action of insulin. *Glycobiology* **26:** 1198-1208. PMID: 27072814

2.4 Vaidyanathan K, Niranjan T, Selvan N, Teo CF, May M, Patel S, Weatherly B, Skinner C, Opitz J, Carey J, Viskochil D, Gecz J, Shaw M, Peng Y, Alexov E, Wang T, Schwartz C, **Wells L** (2017) Identification and characterization of a missense mutation in the O-GlcNAc transferase gene that segregates with X-linked intellectual Disiability. *J Biol Chem* **ePub** March 16. PMID: 28302723

3. The O-mannosylation pathway and Congenital Muscular Dystrophy (CMD). Defects in the dystrophin-glycoprotein (alpha-dystroglycan) complex that bridges the cytoskeleton to the extracellular matrix lead to multiple forms of muscular dystrophy. Of interest, defects in enzymes involved in the complex O-Mannosylation pathway that modifies alpha-dystroglycan are causal for congential muscular dystrophy, specifically secondary dystroglycanopathies including Walker-Warburg syndrome, Muscle-Eye-Brain-Disease, Fukuyama syndrome, and Limb-Girdle disease. Our laboratory is extensively investigating the O-Mannosylation pathway in terms of defining the glycan structures made, assigning and characterizing the enzymes responsible (including naturally occurring mutants in patients), and examining the function of the O-mannose glycans in binding extracellular matrix proteins and certain classes of arenaviruses.

2.1 Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, **Wells L**, Campbell KP (2010) O-Mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science* **327:**88-92. PMID: 20044576

2.2 Praissman JL, Live DH, Wang S, Ramiah A, Chinoy ZS, Boons GJ, Moremen KW, **Wells L** (2014) B4GAT1 is the priming enzyme for the LARGE-dependent functional glycosylation of alpha-dystroglycan. *Elife* **3**.doi: 10.7554/eLife.03943. PMID: 25279697

2.3 Praissman JL, Willer T, Sheikh MO, Toi A, Chitayat D, Lin YY, Lee H, Stalnaker SH, Wang S, Prabhakar PK, Nelson SF, Stemple DI, Moore SA, Moremen KW, Campbell KP, **Wells L** (2016) The functional O-mannose glycan on alpha-dystroglycan contains a phospho-ribitol primed for matriglycan addition. *Elife* **April 29**.doi: 10.7554/eLife.14473. PMID: 27130732

2.4 Halmo SM, Singh D, Patel S, Wang S, Edlin M, Boons GJ, Moremen KW, Live D, **Wells L** (2017) Protein O-linked mannose beta-1,4-N-acetylglucosaminyl-transferase 2 (POMGNT2) is a gatekeeper enzyme for functional glycosylation of alpha-dystroglycan. *J Biol Chem* **292:** 2101-09. PMID: 27932460

Complete list of Published Work (in NCBI My Bibliography):
https://www.ncbi.nlm.nih.gov/myncbi/browse/collection/40460426/?sort=date&direction=descending

D. Current Research Support

P41GM103490 — Pierce (PI) — 09/01/03 – 06/30/18
NIH/NIGMS — Role: Senior Investigator, TR&D2 Leader
National Center for Biomedical Glycomics
Goal: Develop MS-based qualitative and quantitative strategies for the analysis of glycans and glycoproteins

U01CA128454-01 — Pierce (PI) — 07/25/07 – 06/30/17
NIH/NCI — Role: Co-I — **Non-renewable**
Tumor Glycomics Laboratory for Discovery of Pancreatic Cancer Markers
Goal: Determine glycans and glycoproteins that may serve as markers of disease

P01GM107012 — Boons (PI) — 07/01/13 – 06/30/18
NIH/NIGMS — Role: Project #3 Leader — **Non-renewable**
Mammalian Glycosyltransferases for Use in Chemistry & Biology
Goal: Determine the in vivo specificity of glycosytransferases involved in terminal modifications

R01GM111939 — Wells (PI) — 08/01/14 – 04/30/18
NIH/NIGMS — Role: PI (with David Live)
Structure and Function in Alpha-Dystroglycan Glycosylation
Goal: Determine the enzymes and structures required for functional glycosylation of alpha-dystroglycan

R21AI123161 — Wells (PI) — 07/01/15 – 06/30/17
NIH/NIAID — Role: PI (with Christ West and Rick Tarleton) — **Non-renewable**
Enabling tools for protist pathogen glycobiology
Goal: Genetic Tools for studying glycosylation in Toxoplasma and T. cruzi biology/virulence/persistence

CAVD Program — Alter (PI, Ragon Institute) — 10/01/13 – 3/31/18
Bill and Melinda Gates Foundation — Role: Co-PI
Glyco-adjuvanting HIV vaccines
Goal: Investigate the glycosignatures and glycosylation differences that predict or improve HIV vaccines.

Medical Research Grant Program — Tiemeyer (PI) — 01/01/15 – 12/31/17
The W.M. Keck Foundation — Role: co-PI — **Non-renewable**
The Glycomics of Human Neurodegenerative, Developmental, and Cognitive Disorders
Goal: Uncover the roles of glycans as drivers and markers of neurological disease.

PHS 398 Cover Page Supplement

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

1. Human Subjects Section

Clinical Trial?	[ ] Yes	[X] No
*Agency-Defined Phase III Clinical Trial?	[ ] Yes	[ ] No

2. Vertebrate Animals Section

Are vertebrate animals euthanized?	[X] Yes	[ ] No
If "**Yes**" to euthanasia		
Is method consistent with American Veterinary Medical Association (AVMA) guidelines?	[X] Yes	[ ] No

If "**No**" to AVMA guidelines, describe method and provide scientific justification

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

[ ] Yes [X] No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

*Does the proposed project involve human embryonic stem cells? [X] No [ ] Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. 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) (Example: 0004):

5. Inventions and Patents (RENEWAL)

*Inventions and Patents: Yes [ ] No [ ]

If the answer is "Yes" then please answer the following:

*Previously Reported: Yes [ ] No [ ]

6. Change of Investigator / Change of Institution Section

[ ] Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

[ ] Change of Grantee Institution

*Name of former institution:

OMB Number: 0925-0001

PHS Modular Budget

Budget Period: 1

Start Date: 5/1/2018 4:00:00 AM End Date: 4/30/2019 4:00:00 AM

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A	$150,000.00
Consortium F&A	
Total Direct Costs	$150,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Research/Fed/OnCampus (MTDC)	50	$150,000.00	$75,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): US DHHS, Steven Zuraf, 301-492-4855

Indirect Cost Rate Agreement Date: 8/23/2016 4:00:00 AM

Total Indirect Costs: $75,000.00

C. Total Direct and Indirect Costs (A+B)

Funds Requested ($): $225,000.00

Budget Period: 2

Start Date: 5/1/2019 4:00:00 AM End Date: 4/30/2020 4:00:00 AM

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A	$125,000.00
Consortium F&A	
Total Direct Costs	$125,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Research/Fed/OnCampus (MTDC)	50	$125,000.00	$62,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): US DHHS, Steven Zuraf, 301-492-4855

Indirect Cost Rate Agreement Date: 8/23/2016 4:00:00 AM

Total Indirect Costs: $62,500.00

PHS Modular Budget

C. Total Direct and Indirect Costs (A+B)	Funds Requested ($)	$187,500.00

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$275,000.00
Section A, Total Consortium F&A for Entire Project Period	$0.00
Section A, Total Direct Costs for Entire Project Period	$275,000.00
Section B, Total Indirect Costs for Entire Project Period	$137,500.00
Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$412,500.00

2. Budget Justifications

Personnel Justification	Plac8_personnel budget justification_Final.pdf
Consortium Justification	
Additional Narrative Justification	Plac8 Additional narrative justification.pdf

PERSONNEL BUDGET JUSTIFICATION

Senior Key Personnel

Wendy Watford, PhD (co-PI) - (1 summer month effort) will be responsible for providing oversight for the entire project, be responsible for supervising and training personnel and will serve as the contact PI for NIH, including submitting annual reports. Dr. Watford will manage fiscal and administrative aspects of this project. Dr. Watford will assume joint oversight of Aim 1 with Dr. Klonowski and primary oversight of Aim 2, which includes the collaboration with Dr. Wells and interfacing with the UGA Genomics Core Facility.

Kimberly Klonowski, PhD (co-PI) - (0.5 summer month effort) will be responsible for supervising and training personnel and overseeing Aim 1 jointly with Dr. Watford. Both Drs. Watford and Klonowski will be involved in both Aims and will interact regularly with each other, trainees and other personnel, continuing joint lab meetings and student oversight. They will share responsibility for experimental design and generation and analysis of data, utilizing the unique strengths each brings to the table. Both Drs. Watford and Klonowski will jointly oversee the preparation of manuscripts for publication.

Lance Wells, PhD (co-I) - (0.2 academic month effort) will oversee personnel and experiments for the identification of Plac8-interacting proteins by mass spectrometry in Aim 2.3 and will oversee the preparation of these results for publication.

Other Personnel

[redacted] (PhD candidate; 12 Calendar months, 50% effort) will serve as a Graduate Student co-mantored by Drs. Watford and Klonowski. [redacted] is proficient in analysis of antigen-specific CD8 T cell responses of mice at acute and memory time points during influenza infection and has generated the majority of preliminary data provided in this application. [redacted] will perform experiments to characterize the size and quality of the effector and memory pool *in vivo* (Aim 3).

[redacted] (Undergraduate student worker, 12 Calendar months, 50% effort). [redacted] is a rising Junior and dual major in Biological Sciences and Animal Sciences at the University of Georgia. [redacted] has assisted in animal husbandry and genotyping of all WT, *Plac8*$^{+/-}$, *Plac8*$^{-/-}$ and congenic mice used for these studies. Because of [redacted] exceptional diligence and reliability, [redacted] will continue to assist with animal colony maintenance for this proposal [redacted] will be responsible for intercrossing *Plac8*$^{-/-}$ mice with OT-1 *Rag*$^{-/-}$ mice for studies in Aim 1.4.

Graduate student, TBD (PhD candidate; 6 Calendar months, 50% effort) will serve as a Graduate Student in the Watford lab and/or the Klonowski lab. This student will delineate the mechanisms regulating Plac8 expression in human and murine CD4 T cells as well as the functional consequences of Plac8 ablation in murine and human CD4 T cells (Aim 3). In addition, this student will evaluate Plac8-dependent generation of antigen-specific CD4 T cells during influenza infections by characterizing recall responses to MHC class II-restricted influenza peptides.

ADDITIONAL NARRATIVE JUSTIFICATION

One extra module is requested in year 1, because more of the animal studies will occur in year 1.

PHS 398 Research Plan

OMB Number: 0925-0001

Introduction

1. Introduction to Application (Resubmission and Revision)

Research Plan Section

2. Specific Aims	Plac8_Specific Aims_Final.pdf
3. *Research Strategy	Plac8_Research Strategy_Final.pdf
4. Progress Report Publication List	

Human Subjects Sections

5. Protection of Human Subjects	Plac8_Protection of Human Subjects_Final.pdf
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	

Other Research Plan Sections

9. Vertebrate Animals	Plac8_VertebrateAnimals.pdf
10. Select Agent Research	
11. Multiple PD/PI Leadership Plan	Plac8_multi-PI.pdf
12. Consortium/Contractual Arrangements	
13. Letters of Support	Plac8_Combined_LOS.pdf
14. Resource Sharing Plan(s)	Plac8_ResourceSharing.pdf
15. Authentication of Key Biological and/or Chemical Resources	Plac8_Authentication_Final.pdf

Appendix

16. Appendix

Specific Aims

Memory CD8 T cells are superior in protecting individuals from reinfection with highly mutagenic, evolving pathogens like influenza, HIV and malaria. This is primarily due to the fact that CD8 T cells, unlike antibodies, recognize conserved, internal proteins that are requisite for pathogen survival. Moreover, high numbers of memory CD8 T cells in tumor sites are correlated with improved overall survival in many cancers, suggesting that the potential benefit of memory CD8 T cell vaccines to human health is broad. While the contribution of memory CD8 T cells to vaccine efficacy has not been tested comprehensively or longitudinally, limited studies have shown that CD8 T cell responses to some live attenuated vaccines are not maintained long-term. Therefore, methodologies to improve CD8 T cell based vaccines are warranted and dependent on a better understanding of the factors that regulate these long-lived memory cells.

Antigen-specific CD8 T cells clonally expand and acquire potent cytotoxic effector functions to kill infected cells. Pathogen clearance removes antigenic survival cues, leading to massive contraction of the antigen-specific T cell pool by apoptotic programmed cell death. However, a small pool of antigen-specific CD8 T cells persists as memory cells that acquire a stem cell-like capacity for slow self-renewal dependent upon the cytokines IL-7 and IL-15, in the absence of antigen. Memory CD8 T cells are poised numerically and epigenetically to provide rapid protective immunity upon re-exposure to antigen. Therefore, promoting the development and maintenance of memory CD8 T cells after pathogen encounter is a hallmark of adaptive immune responses and the central goal of vaccination. Identifying the mechanisms that regulate the development and/or persistence of memory CD8 T cells is critical for improving vaccine efficacy and is currently an area of intense investigation. We recently demonstrated that a novel cysteine-rich protein, placenta-specific 8 (Plac8), is critical for the development and/or maintenance of memory CD8 T cells (See *Preliminary Studies*). How Plac8 regulates this process, however, is still unclear. Although the molecular sequence of Plac8 is evolutionarily conserved, its function remains enigmatic. This is an important problem, because lack of this knowledge is a barrier to developing better vaccine strategies against intracellular microorganisms and tumors.

The *objective* of this application is to understand how Plac8 promotes memory CD8 T cell formation. Influenza provides an excellent model for addressing this problem due to the well-characterized expansion and contraction of antigen-specific CD8 T cells and the wealth of available reagents. Our *central hypothesis* is that Plac8 facilitates the cellular catabolic process of autophagy, which enables CD8 T cells to endure cellular stresses associated with antigen withdrawal at the effector to memory (ETM) cell transition. This hypothesis was formulated on the basis of our preliminary data that *Plac8*$^{-/-}$ CD8 T cells are less capable of developing into long-lived memory cells due to a T cell-intrinsic defect that occurs *after* effector expansion (see *Preliminary Studies*). The *rationale* for the proposed research is that a better understanding of the mechanisms regulating memory CD8 T cell differentiation and maintenance are necessary to improve vaccine strategies for both intracellular microorganisms and cancers. To address our central hypothesis, we will test the following Specific Aims:

Specific Aim 1: Determine the mechanism(s) by which Plac8 promotes CD8 memory establishment.

Specific Aim 2: Explore Plac8 biology and translational potential in human and murine T cells.

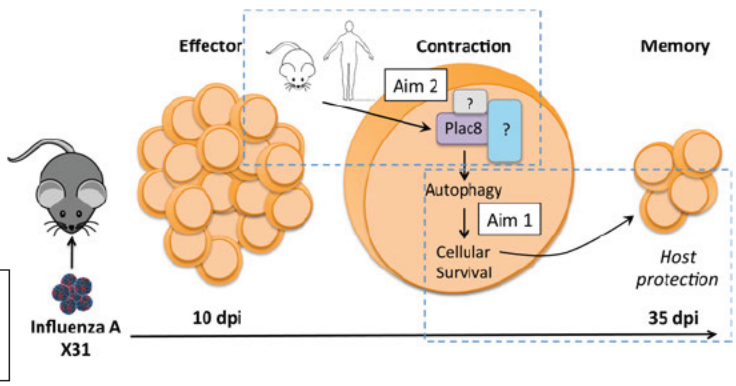

Figure 1: Aims. The overall goal of the study is to determine how Plac8 is expressed and functions in murine and human T cells to promote establishment of CD8 T cell memory.

The *expected outcomes* of the proposed studies are (1) an increased understanding of how memory CD8 T cells develop and are maintained *in vivo* and (2) the elucidation of Plac8 biochemical functions in T cells. This proposal is appropriate for the R21 funding mechanism as it is highly novel, exploratory and expands our laboratory research in a new direction. These studies are expected to have an important *positive impact* on human global health and well-being, because they will provide new information that can be exploited to enhance memory CD8 T cell development, a central goal of anti-viral and anti-cancer vaccines.

Research Strategy

(A) Significance

Identifying the mechanisms that regulate the development and/or persistence of memory CD8 T cells is critical for improving vaccine efficacy and is currently an area of intense investigation. The goal of this application is to determine the mechanism(s) by which Plac8 enhances memory CD8 T cell formation and to gain insights into Plac8's biochemical functions in T cells. Plac8 (aka onzin or C15) is a small (~16 kDa), cysteine-rich protein, originally identified in the spongiotrophoblast layer of mouse placental tissue [1]. Plac8 mRNA has been detected in both lymphoid and myeloid immune cells, and *Plac8*$^{-/-}$ mice have defects in innate immunity, attenuated contact hypersensitivity, and impaired brown and white fat differentiation [2-4]. In activated neutrophils Plac8 is associated with granules, and *Plac8*$^{-/-}$ neutrophils have impaired anti-microbial functions [2]. *Chlamydia muridarum*-specific CD4^{+} T cells express high levels of Plac8, which was associated with bacterial clearance via an iNOS-independent but degranulation-dependent mechanism [5]. Despite mounting evidence of its immunological importance, Plac8 regulation of T cell functions has been largely overlooked to date. Furthermore, its biochemical functions are also unclear. Many Plac8 studies are concentrated in the field of cancer research, as Plac8 is upregulated in colon, prostate, and pancreatic cancers [6-9]. Of note, a majority of non-Hodgkins lymphomas showed high Plac8 cytoplasmic staining (Human Protein Atlas), suggesting a possible role for Plac8 in development of T cell cancers. Plac8 inhibition reduced the spread of pancreatic cancers, although there are conflicting explanations for this effect, including Plac8-dependent regulation of apoptosis [10], autophagy [9] and the cell cycle [8]. These conflicting reports demonstrate that the role of Plac8 is likely context-dependent but emphasize the high therapeutic potential in learning how this protein and its binding partners can be modulated for treating cancers and enhancing vaccine efficacy.

The *scientific premise* for this application is our recent identification of Plac8 as a gene that is highly expressed in previously activated mouse and human CD8 T cells and is essential for the maintenance of influenza-specific memory CD8 T cells (See *Preliminary Studies* subsection). In particular, strengths of our preliminary data are that Plac8 is preferentially expressed in memory CD4 and CD8 T cell populations in mice. This expression pattern is recapitulated in human cells, suggesting the potential translational benefit of these studies to humans. Global Plac8 ablation rendered CD8 T cells unable to efficiently and/or durably generate memory CD8 T cells. Furthermore, multiple experiments conducted in mixed bone marrow chimeras clearly demonstrated that the requirement for Plac8 expression was intrinsic to the T cell compartment. These preliminary studies, coupled with the previously attributed role for Plac8 in T cell-mediated resistance to *Chlamydia* [5] and the high incidence of strong Plac8 expression in non-Hodgkins lymphomas justify additional studies into Plac8 functions in T cells. The proposed studies are anticipated to provide a better understanding of the mechanisms regulating memory CD8 T cell differentiation and maintenance, which is essential for improving vaccine strategies for both intracellular microorganisms and cancers.

(B) Innovation

This proposal is *conceptually innovative* in that we have identified *a protein that has not been attributed to memory cell development* that we hypothesize regulates the requisite metabolic transition of CD8 T cells from effector to memory. We propose a comprehensive and multifaceted approach to unravel the mechanistic pathway in which Plac8 regulates T cell functions, including CD8 memory cell programming, using the limited known functions of Plac8 coupled with an unbiased approach. These include chimeric mouse models which allow us to ascribe a specific T cell intrinsic role for Plac8 in memory cell development coupled with RNA-Seq and proteomics to identify novel Plac8 signaling pathways and interacting partners for future targeted interventions to regulate memory CD8 T cell immunity.

(C) Approach: Preliminary studies

We identified placenta-specific 8 (Plac8) as a gene highly expressed within multiple effector cell types involved in cell-mediated immunity, including Th1 cells, CD8 T cells and natural killer (NK) cells (data not shown). Further analysis revealed that Plac8 expression is more highly expressed in naïve CD8 T cells compared to CD4 T cells, and its expression is enhanced in antigen-experienced memory T cells **(Figure 2)**. *Plac8*$^{-/-}$ mice were generated by Beverly Koller [2] and show normal immune development of peripheral CD4 and CD8 T cell subsets (data not shown). To determine how Plac8 affects CD8 T cell development and function *in vivo*, a well-characterized murine Influenza A virus (IAV) model was chosen due to the wealth of available reagents. Mice were intranasally infected with 10^4 plaque forming units of influenza A virus (X31, H3N2). Mice were either weighed daily or euthanized 3, 5 or 7 days post infection (dpi) for quantitation of lung viral titers. *Plac8*$^{-/-}$ mice

displayed similar weight loss kinetics and viral titers as wild type (WT) mice **(Figure 3)**. To determine whether Plac8 regulates the T cell response to influenza infection, mice were euthanized at either the peak CD8 T cell effector (10 dpi) or memory (35 dpi) time point. Bronchoalveolar lavage fluid (BAL), spleen, mediastinal lymph node (mdLN), and lung cells were harvested. Lymphocytes were gated for $CD8^+$ $CD44^{high}$ nucleoprotein (NP)-tetramer$^+$ to distinguish influenza-specific CD8 T cells. When comparing the antigen-specific CD8 T cells between WT and *Plac8*$^{-/-}$ mice at 10 dpi, the frequencies and total cell numbers were equivalent **(Figure 4A-B)**. However, when comparing the frequency and total cell number of the antigen-specific CD8 T cells between WT and *Plac8*$^{-/-}$ mice at 35 dpi, the frequencies and total cell numbers were significantly reduced in all tissues of the *Plac8*$^{-/-}$ mice, with the exception of lung total cell numbers (which trended towards reduction) at 35 dpi **(Figure 4C-D)**. While these data provide evidence that Plac8 contributes to CD8 T cell memory formation, the mechanism remains unknown. To begin to resolve the mechanism, we generated mixed bone marrow chimeras (BMC). Heterozygous CD45.1/2 mice were lethally irradiated, and their hematopoietic compartments were reconstituted with a 1:1 mixture of bone marrow from CD45.2 *Plac8*$^{-/-}$ and CD45.1 WT mice. After two months, chimeras were infected with X31, sacrificed at either 10 or 36 dpi and lymphocytes recovered from the BAL, spleen, mdLN. X31-specific CD8 T cells were identified as $CD8^+$ cells and further distinguished as WT (CD45.1) versus *Plac8*$^{-/-}$ (CD45.2) in each recipient by flow cytometry. Subsequent gating within the two donor populations identified $CD44^{hi}$NP-tetramer$^+$ cells. When comparing effector CD8 T cells at 10 dpi, both WT and *Plac8*$^{-/-}$ X31-specific CD8 T cells were present at similar frequencies **(Figure 5A)**. However, there was a striking, statistically significant reduction in the frequency of *Plac8*$^{-/-}$ compared to WT cells within each tissue at 36 dpi **(Figure 5B)**. The specific reduction of *Plac8*$^{-/-}$ influenza-specific CD8 memory T cells in the mixed BMC demonstrates that Plac8 is acting via a T cell-intrinsic mechanism.

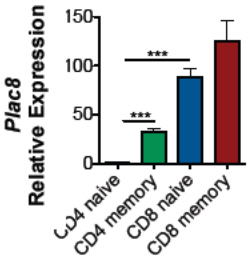

Figure 2 – Plac8 expression increases from naïve to memory transition. Plac8 expression in sorted naïve and memory $CD4^+$ and $CD8^+$ T cells from murine spleens and lymph nodes relative to naïve $CD4^+$ T cells and an endogenous actin control. Pooled from N=3 expts.

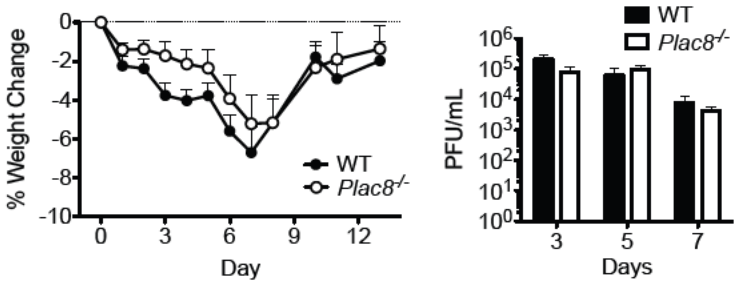

Figure 3 – Plac8 is dispensable for control of viral titers during primary influenza infection. Infection-induced weight loss (A) and viral titers (B) determined by plaque assay after X31 infection.

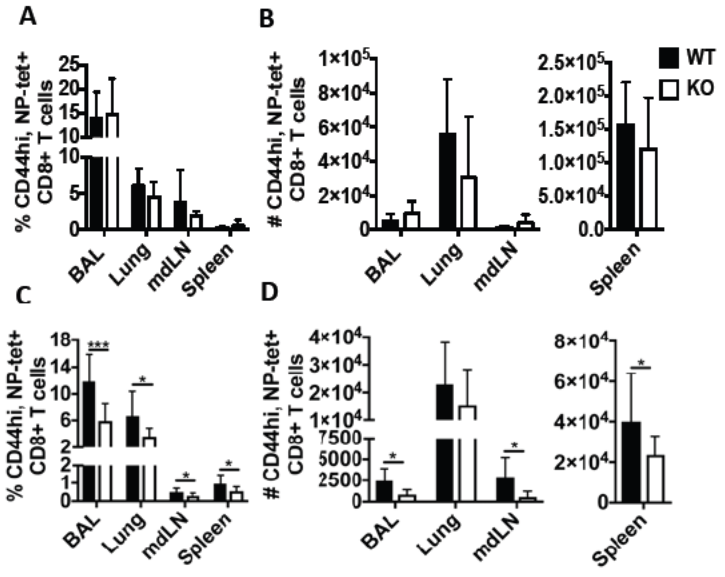

Figure 4 - Plac8*$^{-/-}$ **mice have a significant reduction of CD8+ memory T cells. Frequency (A) and number (B) of influenza-specific effector $CD8^+$ T cells in WT and *Plac8*$^{-/-}$ mice 10 dpi (effector). Frequency (C) and number (D) at 35 dpi (memory).

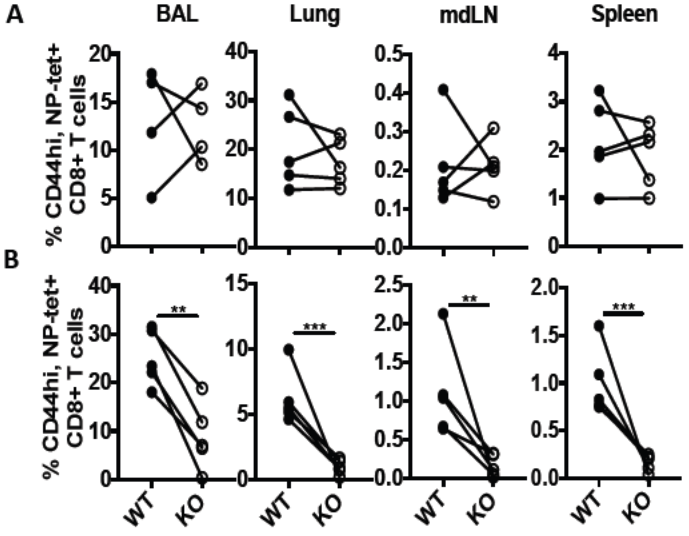

Figure 5 – Plac8 promotes CD8 memory via a T cell intrinsic mechanism. Frequencies of antigen-specific WT and *Plac8*$^{-/-}$ CD8 cells within mixed BMC at the effector time point 10 dpi (A) and the memory time point 36 dpi (B).

Research Strategy

Scientific Rigor and Reproducibility. To ensure rigor and reproducibility in our research, we will strictly adhere to the scientific method, include appropriate controls to ensure proper interpretation of results, validate that all studies are reproducible, and apply appropriate statistical tests when drawing conclusions about findings. Statistical analyses will be performed in consultation with University of Georgia statisticians. Experimental procedures will be reported in sufficient detail to enable laboratories to independently reproduce the findings. Both male and female age-matched mice will be used in experimental cohorts to address the gender effect.

Biohazards. Influenza strains will be handled as BSL2 and ABSL2 by properly trained personnel.

Specific Aim 1: Determine the mechanism(s) by which Plac8 promotes CD8 memory establishment.

Introduction. Antigen-specific memory CD8 T cells are an essential part of protective cell-mediated immunity (CMI) against viruses, and CMI is critical in protecting individuals prior to the generation of neutralizing antibodies. We observed that Plac8 expression is increased in murine memory T cell populations **(Figure 2)**, suggesting that Plac8 may regulate memory cell development, persistence or functions. Indeed, Plac8 ablation impairs memory CD8 T cell establishment via a T cell-intrinsic mechanism **(Figures 4-5)**. The mechanism by which Plac8 regulates this process is unknown. Memory CD8 T cell development requires a dynamic transition from the rapidly proliferating effector stage to a slowly proliferating quiescent memory phenotype. Clonal expansion of antigen-specific CD8 T cells results in antigen elimination and loss of T cell survival signals. As a result, the vast majority of antigen-specific CD8 T cells die via apoptotic programed cell death. Those that survive critically depend upon IL-7 and IL-15 for their continued survival [11, 12]. Autophagy, an evolutionarily conserved process in which cytoplasmic components and organelles are catabolized and recycled as an adaptation to cellular stress, increases dramatically at the initiation of contraction during lymphocytic choriomeningitis virus infection [13]. Furthermore, genetic ablation of autophagy-related proteins critically limits memory CD8 T cell formation without altering effector responses during viral infection models, including influenza, lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) [13,14]. Notably, Plac8 has been implicated in the regulation of autophagy [9]. Plac8 silencing in cell lines inhibited tumor growth by blocking autophagy [9], whereas Plac8 overexpression leads to resistance to apoptosis [15]. The *objective* of this aim is to determine the cellular mechanism(s) by which Plac8 facilitates memory CD8 T cell formation and/or maintenance. We will test the *working hypothesis* that *Plac8 initiates autophagy in late antigen-specific effector CD8 T cells to mitigate cellular stresses associated with the transition into memory.* Based on the precipitous decline in *Plac8*$^{-/-}$ memory CD8 T cells we observe at contraction, we predict that *Plac8*$^{-/-}$ memory CD8 T cells will exhibit reduced autophagy and correspondingly increased apoptosis.

Experimental Design

1.1. Define the requirement for Plac8 during CD8 differentiation and effector to memory transition. WT and *Plac8*$^{-/-}$ mice will be infected with 10^4 pfu of influenza A (X31), and the generation of effector versus memory cells will be measured longitudinally in blood at days 0, 8, 10, 14, 17, 24 and 35 to determine the timing of the defect. In each experiment, cohorts of 10 WT mice (5 male and 5 female) and 10 *Plac8*$^{-/-}$ mice (5 male and 5 female) will be used for infection with influenza. Five mice/group gives 90% power to detect T_{mem} differences (1 way ANOVA, α set at 0.05). Three such experiments will be performed. Cell surface staining for KLRG-1 and CD127 is useful for distinguishing short-lived effector cells (SLECs, CD127^{lo} KLRG1^{hi}) from long-lived memory precursor effector cells (MPECs, CD127^{hi} KLRG1^{lo}). Peripheral blood will be stained with influenza NP MHC class I tetramers plus antibodies against CD4, CD8, CD44, CD127 and KLRG1 to distinguish SLECs and MPECs. CD8 memory T cells are further categorized according to their migratory behavior into central memory, effector memory and resident memory, with tissue-resident memory T cells (Trm) residing permanently in non-lymphoid tissues where they can respond rapidly to control infection [16,17]. On day 35, mice will be euthanized, and both the magnitude and localization of the CD4 and CD8 T cell memory pools will be examined by flow cytometry. Three minutes prior to euthanasia, mice will be injected intravenously with FITC-labeled anti-CD45.2 to stain recirculating cells [18]. In contrast, cells resident within tissues (i.e. Trm) will be protected from the antibody. Cells from BAL, lung parenchyma, draining mdLN and spleens will be stained with MHC-I tetramers and antibodies against CD4, CD8, CD44, and the absolute numbers of Trm CD8 T cells will be quantitated. These experiments should delineate the time point at which impaired antigen-specific T cell responses are first observed in *Plac8*$^{-/-}$ mice, which will provide insights into the mechanism(s) responsible. Whether the memory response is also qualitatively changed to produce Trm versus recirculating memory CD8 T cells will also be determined.

1.2. Determine if Plac8 promotes self-renewal of memory CD8 T cells.

1.2.a. Self-renewal/Proliferation: WT and *Plac8*$^{-/-}$ mixed BMC will be generated, infected with X31 and rested for 35 days to allow memory establishment as in Figure 5. CD8 T cell proliferation will be determined using 5-Bromo-2'-deoxyuridine (BrdU) which is a synthetic analog of thymidine that is incorporated into the DNA of proliferating cells. For up to 10 days prior to sacrifice, mice will be administered BrdU (0.8 mg/mL) in their drinking water to gradually label the slowly proliferating memory cells as we have previously described. Mice will be euthanized at 10, 17, 24 and 35 days which correspond to the peak CD8 effector response, CD8 T cells presently undergoing contraction and memory CD8 T cells as determined by serial blood draws (data not shown). BrdU-labeled DNA will be measured using a FITC-labeled anti-BrdU antibody (BD Parmingen) by flow cytometry. The proportion of BrdU^{+} (proliferating) antigen-specific CD8 T cells (CD8^{+}CD44^{hi}NP-Tet^{+}) will be determined by flow cytometric analyses as we have demonstrated previously [19].

1.2.b. Responsiveness to IL-7 and IL-15: WT and *Plac8*$^{-/-}$ mice will be infected with X31 and euthanized at 10, 17, 24 and 35 dpi. The expression of the IL-7R and IL-15R on CD8 T cells in BAL, lungs, mediastinal lymph nodes and spleens will be assessed by flow cytometry. CD8 T cells (CD8^{+}CD44^{hi}NP-Tet^{+}) will be analyzed for surface expression of each receptor chain (IL-7Rα, IL-2/-15Rβ and CD132) using fluorescent antibodies (BD Biosciences). Responsiveness of the antigen-specific CD8 T cells to IL-7 and IL-15 will also be examined via induction of anti-apoptotic Bcl-2 [20] upon *in vitro* cytokine stimulation [21].

1.3. Determine if Plac8 initiates autophagy or apoptosis to mitigate cellular stress during memory transition. *1.3.a. Autophagy measurements:* WT and *Plac8*$^{-/-}$ mixed BMC will be generated and infected with X31 as in Figure 5. Mice will be euthanized at 10, 17, 24 and 35 dpi, and autophagy levels will be quantitated within CD8^{+}CD44^{hi}NP-Tet^{+} cells from BAL, lungs, mediastinal lymph nodes and spleens. Autophagy will be measured in two ways. First, we will use the commercially available CytoID Autophagy Detection Kit (Enzo Life Science), which quantitates autophagic vesicles via flow cytometry or microscopy. Second, antigen-specific CD8 T cells will be sorted from WT and *Plac8*$^{-/-}$ mice at 10, 17, 24 and 35 dpi, and autophagy will be measured by immunoblotting for classic markers, including LC3b-I conversion to LC3b-II and the degradation of ubiquitin-binding scaffold protein, p62 [13,22]. For the latter approach, we will avail ourselves of the OT-I congenically marked system described in sub aim 2.3 below for obtaining sorted antigen-specific cells for LC3b analysis.

1.3.b. Apoptosis measurements: WT and *Plac8*$^{-/-}$ mice will be infected with X31 and rested for 35 days. At that time, leukocytes harvested from BAL, lungs, mdLN and spleens will be analyzed for apoptosis by two complementary flow cytometric approaches that measure either caspase activation or phosphatidylserine externalization [23]. Fluorochrome-labeled inhibitors of caspases (FLICA) bind to active centers of caspases with high affinity and allow measurement of individual caspases [24]. Activation of central executioner caspases, caspases 3 and 7, will be measured directly *ex vivo* in CD8^{+}CD44^{hi}NP-Tet^{+} T cells using the Vybrant FAM Caspase-3 and -7 Assay Kit (Invitrogen) [25]. The second approach relies upon loss of phosphatidylserine (PS) asymmetry within the plasma membranes of apoptotic cells and the fact that PS becomes accessible to Annexin V binding during apoptosis. WT and *Plac8*$^{-/-}$ cells will be stained with FITC-labeled Annexin V and a vital dye (PI or 7-AAD) to distinguish viable, apoptotic and necrotic cells.

1.4. Determine how Plac8 ablation within CD8 T cells globally alters the transcriptome of developing CD8 memory cells. To gain a global and unbiased view of which T cell-intrinsic pathways are regulated by Plac8, we will perform RNA sequencing (RNA-Seq) analysis on WT and *Plac8*$^{-/-}$ antigen-specific CD8 T cells infected with influenza A/X31. CD45.2 WT OT-I and *Plac8*$^{-/-}$ OT-I cells will be independently adoptively transferred into CD45.1 congenic recipient mice, which will subsequently be infected with a recombinant influenza virus expressing the OT-I OVA peptide [26]. Antigen-specific CD45.2 donor CD8 T cells will be purified by cell sorting on days 10, 17, 24 and 35 which correspond to the peak CD8 effector response, CD8 T cells presently undergoing contraction and memory CD8 T cells, respectively. We have defined these time points based on blood tetramer levels measured serially in WT mice infected with the same dose of influenza A/X31 (data not shown). Total cellular RNA will be extracted using an E.Z.N.A.^{TM} kit (Omega). RNA-Seq will be performed in house at UGA's Georgia Genomics Facility in collaboration with Dr. Magdy Alabady (See Letter of Support). The RNA sequencing library will be prepared from isolated RNA isolated and sequenced on an Illumina NextSeq 500 instrument as described previously [27]. For quantification of gene expression, transcript alignment and analysis will be performed by Dr. Walter Lorenz, lead consultant of UGA's Quantitative Biology Consulting Group (See Letter of Support). Ingenuity pathway analysis (IPA) will also be performed to identify specific biological pathways that are regulated in a Plac8-dependent manner within memory CD8 T cells.

1.5. Determine the requirement for Plac8 in the generation of protective memory responses to influenza. Infection with influenza virus induces CD8 T cell responses against the internal viral proteins that not only mediate acute lung viral clearance, but also mediate protection against reinfection with a heterosubtypic virus. Plac8 expression is increased in memory T cell populations **(Figure 2)**, suggesting that

Plac8 may regulate memory cell development, persistence or functions. Functional memory responses to influenza A/X31 (H3N2) in WT and *Plac8*$^{-/-}$ mice will be assessed upon challenge 45 dpi with a lethal dose ($5xLD_{50}$) of the heterosubtypic virus, influenza A/PR8 (H1N1) [28]. Mice will be weighed prior to infection and daily thereafter until the X31-immunized WT mice recover their pre-infection body weights. Survival data will be analyzed by Kaplan-Meier plots. We hypothesize that *Plac8*$^{-/-}$ mice will fail to establish protective memory against a challenge with a heterosubtypic influenza virus.

Aim 1 Anticipated results, potential pitfalls & alternative approaches:

Collectively, these experiments should delineate the mechanism(s) by which Plac8 promotes antigen-specific T cell responses during respiratory virus infection as well as whether the memory response is qualitatively altered between tissue resident versus recirculating memory CD8 T cells. It is anticipated that *Plac8*$^{-/-}$ mice will be susceptible heterosubtypic influenza challenge due to impaired generation of CD8 T cell memory. RNA-Seq and ingenuity pathway analysis on antigen-specific CD8 T cells before, during and after contraction will delineate alterations in cellular responses that contribute to impaired memory establishment in *Plac8*$^{-/-}$ CD8 T cells. Because of the limited biochemical information available about Plac8 functions, this study is necessary for advancing the Plac8 field. It is anticipated that proteins implicated in autophagy will be differentially regulated by Plac8, but it is also likely that other significant pathways (for example, apoptosis and proliferation) will exhibit Plac8-dependent regulation. Plac8 dependent pathways will be validated and pursued. Most of the assays in this Aim are standard immunological protocols, therefore we do not foresee any technical barriers. One possible exception is that, depending upon the cell cycling rates, the the BrdU pulse duration may need adjusting, particularly for the 10 dpi time point, which will require a shorter pulse than memory time points.

Specific Aim 2: Explore Plac8 biology and translational potential in human T cells.

Introduction. In mice, we have observed that naïve CD4 T cells express low levels of Plac8 and that Plac8 expression is significantly induced in memory CD4 T cells **(Figure 2)**. Alternatively, Plac8 expression is already basally high in naïve CD8 T cells and remains high in memory CD8 T cells. Furthermore, loss of Plac8 significantly reduces the number of memory CD8 T cells generated after influenza virus infection **(Figures 4-5)**. Very little is known regarding the biochemical functions of Plac8, which limits our understanding of how Plac8 mechanistically regulates T cell development and functions. This Aim will further characterize Plac8 expression and functions within both naïve and memory CD4 and CD8 T cells from *mice and humans*. This *translational and hypothesis-generating Aim* should provide important clues about Plac8 function based on the regulation of its expression and the identification of Plac8-interacting proteins via an unbiased proteomics approach. *We hypothesize that Plac8 promotes Th1 immunity via regulation of both CD4 and CD8 T cell functions and further predict that Plac8 physically associates with proteins implicated in autophagy within CD8 T cells*.

Experimental Design

2.1. Characterize the regulation of Plac8 expression in murine and human T cells.

Plac8 mRNA and protein expression will be monitored in naïve and memory T cells sorted from mouse and human samples by RT-PCR or Western blotting with anti-Plac8 antibodies (mouse, Bioss; human, Cell Signaling Technologies). In a pilot experiment, we examined Plac8 mRNA expression in naïve and memory T cell populations isolated from a healthy human donor. We observed that Plac8 expression increases in antigen-experienced CD4 memory cells compared to naïve cells but is basally high in naïve CD8 T cells **(Figure 6)**, a trend reminiscent of its expression in murine cells **(Figure 2).** In this particular donor, Plac8 was already maximally expressed in naïve CD8 T cells and was not further up-regulated in memory CD8 T cells. Plac8 expression in these sorted human T cell populations will first be replicated in at least four more independent healthy human donors. Healthy human PBMCs are provided by Dr. Balazs Rada and contain no identifying information. Next, naïve mouse and human T cells will subsequently be activated with plate-bound anti-CD3 +/- anti-CD28 antibodies for 6, 24, 48, and 72 hours, and Plac8 mRNA and protein expression will be determined. These experiments will determine whether TCR signaling and co-stimulation induce Plac8 expression in T cells. The regulation of Plac8 expression in murine and human T cells by cytokines associated with Type I Immune responses, including, IL-12, IFN-γ and type I IFNs, will also be examined. Naïve CD4 and CD8 T cells will be activated for three days with anti-CD3/28 harvested and restimulated with 10 ng/mL of each cytokine for 6 or 24 hours prior to Plac8 quantitation.

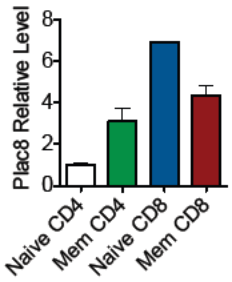

Figure 6 – Plac8 expression in human T cells. Naïve and memory CD4 and CD8 T cells were sorted from PBMC of a healthy human donor. Plac8 expression was determined by RT-PCR relative to 18s rRNA endogenous control and naïve CD4 T cells.

2.2. Characterize the function of Plac8 in murine and human T cells. T cells from WT and *Plac8*$^{-/-}$ mice will be activated *in vitro* with immobilized α-CD3/28 antibodies and assessed for their ability to proliferate,

differentiate into distinct T helper lineages (i.e. Th1, Th2, Th17 and iTreg), and undergo activation-induced cell death (AICD) as we have described previously for *Furin* [29] and *Map3k8* [30]. WT or *Plac8*$^{-/-}$ naïve CD4 and CD8 T cells will be isolated from spleens and lymph nodes of mice and labeled with CFSE prior to stimulation with different doses of anti-CD3 in the presence or absence of anti-CD28 co-stimulation. Cell culture supernatants will be harvested at 48 and 72 hours for quantitation of cytokine secretion using a Th1/2/17 cytokine bead array (Becton Dickenson), and proliferation and apoptosis of cells will be measured by flow cytometry based upon CFSE dilution and Annexin V/propidium iodide staining [31]. For some experiments, T cells will be activated for three days followed by re-stimulation with anti-CD3 to induce AICD, which will be quantitated via Annexin/PI staining. Finally, the ability of *Plac8*$^{-/-}$ CD4 T cells to differentiate into distinct T helper cell lineages will be examined by *in vitro* culture of naïve CD4 T cells under Th1, Th2, Th17 or iTreg conditions as we have done previously [29-32]. Polarization will be confirmed by appropriate cytokine secretion, intracellular staining and expression. We have observed that Plac8 is highly expressed in polarized Th1 cells **(Figure 7)**, reinforcing a possible function in Th1 responses. If there is a phenotype in murine cells, standard TCR and cytokine signaling pathways will be investigated as underlying mechanisms. The phenotype will also be validated and investigated in human cells using Plac8 si-RNA silencing as for *Map3k8* [30].

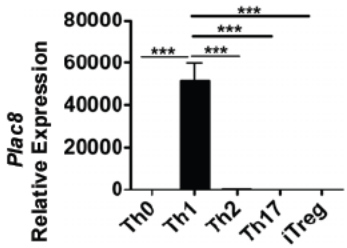

Figure 7 – Plac8 is highly expressed in Th1 cells. Naïve CD4 T cells were polarized to various T helper lineages, and Plac8 expression was determined by RT-PCR relative to actin endogenous control and Th0 cells.

2.3. *Determine the identity of Plac8-interacting proteins in human T cells.*

Plac8-interacting proteins in T cells will be determined by performing mass spectrometry on Plac8 co-immunoprecipitating proteins in naïve and memory CD4$^+$ and CD8$^+$ T cells populations isolated from peripheral blood of healthy human donors. Human CD4$^+$ and CD8$^+$ T cells will be sorted into naïve (CD3$^+$CD45RA^{hi}CCR7^{hi}) and memory (CD3$^+$CD45RA^{lo}) subsets and lysed in protein lysis buffer. Protein concentration will be determined by BCA assay (Pierce) and an aliquot of whole cell lysate will be reserved for Western blot (input). The remainder will be subjected to overnight IP with anti-Plac8 antibody or normal rabbit control IgG (Caltag) at 4°C. We have validated this antibody for both Western blotting an immunoprecipitation using human peripheral blood mononuclear cells (PBMCs) **(Figure 8)**. Immunoprecipitated proteins will be washed, and co-immunoprecipitating proteins will be reduced, alkylated, and digested with trypsin using standard protocols [33]. Resulting peptides will be desalted, and shotgun proteomics (LC-MS/MS) will be performed on a ThermoFisher Orbitrap Lumos Fusion Tribrid mass spectrometer that will use a combination of HCD/CID/ETD fragmentation [34]. All data will be analyzed using a combination of SequestHT, MASCOT, and Byonic algorithms running through Proteome Discover. Final resulting data will be collated, filtered to 1% false-discovery rate, and quantified using normalized spectral counts using ProteoIQ software [35]. We will validate interactions by immunoprecipitating with an antibody reactive to the identified protein and blotting for Plac8.

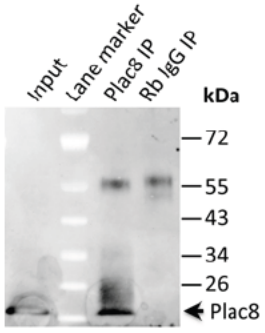

Figure 8 – Plac8 Immunoprecipitation. Human Plac8 was immunoprecipitated from peripheral blood mononuclear cells overnight with anti-Plac8 or an isotoype control antibody. Whole cell lysate (input) and immunoprecipitates were immunoblotted with anti-Plac8 antibody followed by protein A-HRP.

Aim 2 Anticipated results, potential pitfalls and alternative approaches:

We expect that Plac8 will be shown to have important functional roles in both CD4 (Th1) and CD8 T cell responses, and that insights about Plac8's biochemical functions will be revealed. We anticipate that phospholipid scramblase 1 (Plscr1) will be identified among the Plac8 interacting partners [15], although we expect that distinct Plac8 binding partners likely exist in T cells. Importantly, Plscr1 has been identified as a negative regulator of Plac8 [15] and, consistent with our hypothesis, Plscr1 has also been identified as a negative regulator of autophagy in mantel cell lymphomas [36]. We have validated the human Plac8 antibody in IP **(Figure 8)**, and therefore do not anticipate barriers to completing this aim. However, as an alternative approach we will purchase a commercially available Plac8 Myc-tagged protein expression vector (Origene) that can be transfected into Jurkat T cells followed by immunoprecipitations with anti-Myc antibody. Dr. Lance Wells has significant expertise in identification of protein-interacting partners and will oversee the proteomics.

Proposal summary: Our preliminary data implicate Plac8 as an important regulator of transitioning memory CD8 T cells. The experiments proposed herein will provide important new insights into how Plac8 mechanistically regulates the development, function and survival of memory CD8 T cells after infection which can be translated into methods for broadly improving CD8 T cell based immunity in cancer and infectious diseases.

Protection of Human Subjects

Human peripheral blood mononuclear cells used in this study will be provided by Dr. Rada (See Letter of Support). The PBMC are an unused by-product from his blood collections. The identity of the blood-donating volunteers is unknown, and therefore this research is exempt from Federal regulations under exemption 4.

VERTEBRATE ANIMALS

Introduction

The purpose of the proposed research with vertebrate animals is to determine how the novel protein, Plac8, affects CD8 memory establishment during influenza A virus (IAV) infection and how Plac8 might be exploited therapeutically to enhance CD8 memory responses. We have recently demonstrated that Plac8 ablation leads to significantly reduced CD8 memory T cells in IAV infected mice and it occurs through a CD8 T cell-intrinsic mechanism. By utilizing murine models of IAV infection, we will determine the mechanism by which this defect is occurring and how Plac8 contributes to host protection. Furthermore, the use of murine T cells in experiments *in vitro* will also reveal mechanistic information about how Plac8 contributes to both CD4 and CD8 T cell activation, proliferation, and survival. Results from these studies may provide a basis for improving vaccines against viruses like IAV as well as tumors. Wild type (WT) and genetically mutated mice must be used for these experiments.

1. Description of Procedures

Tissue harvesting. For harvesting spleen and lymph node T cells for *in vitro* studies, animals will be euthanized by compressed CO_2 gas hypoxia as this is the SOP for our institution, and it does not interfere with data interpretation. To verify death, cervical dislocation will be performed prior to tissue removal. For harvesting tissues for *in vivo* studies, mice will be anesthetized by i.p. administration of Tribromoethanol (TBE, aka Avertin 180-250mg/kg). Carcasses will be disposed of as medical pathological waste (MPW) in double lined plastic bags. *Numbers of animals*. Both male and female mice will be used between 6-12 weeks of age. Approximately 130 WT mice (C57BL/6J) and 130 *Plac8*$^{-/-}$ mice will be needed to perform *in vitro* experiments in Aims 2.1 and 2.2 and maintain breeding colonies over the two year project period. Therefore, 260 mice will be required for in vitro experiments and breeders over the project period for *in vitro* studies.

Influenza infections. Mice will be anesthetized by i.p. administration of 180-250mg/kg TBE, then intranasally instilled with 0.05 ml of PBS containing 10^4 PFU X31/mouse using a pipette and disposable tips. IAV infection induces some weight loss due to the induction of inflammatory cytokines. X31 is typically non-lethal to mice. WT and *Plac8*$^{-/-}$ mice will experience flu-like symptoms but will recover fully. For heterosubtypic viral challenge, mice that have previously infected with X31 or uninfected control mice will be challenged at 45 dpi with 5xLD50 of influenza PR8. Failure to mount a sufficient memory response to primary infection may result in morbidity in some of these animals. In order to minimize their pain or distress, they will be closely monitored and euthanized according to humane endpoints described below. *Numbers of animals*. Both male and female mice will be used between 6-12 weeks of age. Approximately 100 WT mice (C57BL/6J), 100 *Plac8*$^{-/-}$ mice, 375 C57BL/6 CD45.1 (Ptprc) mice, 260 WT heterozygous mice (C57BL/6 CD45.1 x CD45.2), 50 CD45.2 OT-I control mice, and 50 CD45.2 OT-I *Plac8*$^{-/-}$ mice will be needed to perform *in vivo* experiments and maintain breeding colonies. A total of 935 mice are needed for *in vivo* IAV infection studies and breeders.

Total numbers of animals required over the project period: 1195

2. Justification

For short-term studies, it is important to use primary cells in which signaling and cell behavior is unaltered by transformation. Experiments proposed to address the mechanism of Plac8 on CD8 T cell activity are most accurate using non-transformed cells from living animals. For long-term experiments, CD8 T cells will be isolated directly from IAV-infected WT and *Plac8*$^{-/-}$ mice to understand how these cells utilize Plac8 for memory development. These studies are only possible in genetically engineered animals because the process of memory formation cannot be replicated *in vitro*. Mice are the accepted small animal model for studies of immunity and disease pathogenesis caused by respiratory virus infections, including IAV. By using mice, we will be able to determine the effect of Plac8 *in vivo* under conditions that mimic human respiratory virus infection. In this regard, *Plac8*$^{-/-}$ mice are already available in the lab, and the mouse immune system is well characterized and similar in many respects to the human immune system. These studies will provide important information about host immune responses to viral infection and may contribute to novel disease intervention strategies.

3. Minimization of Pain and Distress

Influenza infections. Mice will be monitored daily (or more frequently if more pronounced disease is expected) for level of activity and general appearance. These will include piloerection, hunched posture, rapid breathing, and lethargy, for example. Body weights will be measured at the time of infection and daily thereafter. Mice will be euthanized when weight loss exceeds 30% initial body weight or if mice show above-mentioned signs of illness as outlined in the following section describing objective determination of humane endpoints. Animals will be euthanized whenever they reach a total score of 5 points as follows: 20% loss of body weight - 1 point; 25% loss of body weight - 2 points; 30% loss of body weight – 5 points; piloerection - 1 point; lethargy - 2 points; hunched posture - 2 points; labored breathing - 3 points. In the event that mice show more significant signs of illness before the endpoint of the study, they will be euthanized. Affected animals will be monitored daily by investigators and animal care staff and euthanized according to the guidelines in the Humane Endpoints Section of our IACUC protocol to minimize suffering, as death is not the intended endpoint of this study.

4. Euthanasia

The methods of euthanasia proposed for these studies is consistent with the recommendations of the American Veterinary Medical Association's Guidelines for the euthanasia of animals.

Project Leadership Plan for Multiple PI Grant Applications

Both PIs (Drs. Watford and Klonowski) will jointly provide oversight for the entire project, be responsible for supervising and training personnel, postdocs, and PhD students and ensuring progress on the overall research project. The PIs already share a lab meeting and co-mentor a PhD student working on the Plac8 project. Dr. Watford will serve as the contact PI for NIH and will submit the annual reports. She will also assume fiscal and administrative management of this project. Drs. Watford and Klonowski will have primary oversight of the work proposed in Aim 1 while Dr. Watford will have primary oversight of the work proposed in Aim 2 which includes the collaboration with Dr. Wells (Co-I). Dr. Watford will also maintain communication with the core facility personnel in Aim 2. However, both Drs. Watford and Klonowski will be involved in all Aims and will interact regularly with each other, trainees and other personnel, continuing joint lab meetings and student oversight. They will share responsibility for experimental design and generation and analysis of data, utilizing the unique strengths each brings to the table. Drs. Watford and Klonowski will share responsibility for manuscript generation, review and publication, determining senior authorship by source of data (i.e. Aim 1 versus Aim 2) and/or the primary mentorship of the 1st author trainee.

Drs. Watford and Klonowski will share responsibility for all aspects of compliance.

1785
The University of Georgia

Department of Infectious Diseases

June 14th, 2017

Wendy Watford Ph.D.
Associate Professor
University of Georgia
Department of Infectious Diseases

Wendy,

I write in strong support of your grant application entitled "Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation".

To complement your mouse studies, my laboratory is glad to provide you with human peripheral mononuclear blood cells (PBMC) needed to perform the experiments on primary human leukocytes as detailed in your application. My group mainly studies primary human white blood cells for different projects. We have recruited more than 700 blood donors in the past five years to donate venous blood and we plan to keep this rate of donor recruitment in the coming years, as well. Providing PBMCs three times a week for your Plac8-focused project is a minor effort for us and can be done easily. Our study is anonymous, the identity of the blood-donating volunteers is unknown but their gender, age and ethnicity is recorded. Therefore, PBMCs of a donor pool with a composition of your choice can be provided. Since your laboratory is only a few feet away from mine in the same building on the same floor, PBMCs can be used for experimentation in your laboratory immediately after their purification in my laboratory is completed.

I have known you since I arrived at UGA and our groups have had tight and productive collaborations in the past. This is proven by two manuscripts published together in The Journal of Leukocyte Biology and Journal of Biological Chemistry. I have greatly appreciated our collaborations to date and look forward to further such interactions.

I wish you good luck with the current grant proposal.

Best regards,

Balázs Rada, Ph.D.
Assistant Professor
University of Georgia
College of Veterinary Medicine
Department of Infectious Diseases

R verbend South, Room 161
110 R verbend Road
Athens, GA 30602

The University of Georgia
Georgia Genomics Facility

Te ephone (706) 542-6409
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http://dna.uga.edu

Tuesday, June 13, 2017

Wendy Watford, PhD
Associate Professor,
Department of Infectious Diseases
College of Veterinary Medicine
University of Georgia, Athens
Phone: 706.542.4585

Dear Wendy,

I am very pleased to offer the assistance and support of the Georgia Genomics Facility (GGF) at the University of Georgia to your NIH R21 grant application "Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation."

GGF is fully equipped and prepared to provide the sequencing needs for your study. As you know GGF supports a wide range of sequencing projects ranging from single-tube Sanger sequencing to high throughput next- generation sequencing using Illumina platforms (Miseq, Nextseq500, and Hiseq) and PacBio platform (Sequel). GGF offers a variety of sequencing applications, including RNA-Seq, DNA-Seq, small RNA-Seq, Exome capture, RAD-Seq, methyl-Seq, and ChIP-Seq. Also, GGF provides whole genome optical mapping service using the BioNano Irys Optical System.

Your RNA-Seq project will include making stranded RNA-Seq libraries from total RNA from WT and Plac8-/- antigen-specific CD8 T cells infected with influenza A/X31. The sequencing will be performed on the Illumina NextSeq500 platform. For sequencing, we will use the paired-end 75-nucleotide protocol, as it is the most appropriate to your questions. At GGF, we have developed and tested hundreds of barcodes to enable high levels of multiplexing in large-scale projects. You can find more details about our library preparation methods at GGF website. Using our large-multiplexing system, we will be able to pool together any number of libraries for sequencing in the same run. When we receive your total RNA samples, our workflow is as following: total RNA quality and quantity assessment, sequencing library prep, library quality and quantity assessment, and multiplexing libraries at equi-molar quantities. We will multiplex samples according to the coverage you need. We perform rigorous quality control checks on each prepared library before and after sequencing.

Overall, the GGF has the expertise and capacity to provide high quality, state of the art services for your research. We fully support your application and we look forward to assisting you on this project. Please feel free to contact me if I can provide any additional information that will assist your program officer or the review team. Good luck with your application.

Magdy S. Alabady, PhD
GGF Director of Science and technology

UNIVERSITY OF
GEORGIA

Quantitative Biology Consulting Group
Institute of Bioinformatics

June 13, 2017

Dear Dr. Watford,

I'm writing to describe the extensive resources and comprehensive bioinformatics environment at the University of Georgia that will support your NIH proposal entitled "**Defining the mechanisms by which Placenta-specific 8 (Plac8) facilitates CD8 memory formation.**" You will have access to the latest bioinformatics approaches and computational infrastructure needed to be successful with your NGS data analysis.

The University of Georgia operates a centralized and fully-staffed computational resource center, the GACRC (http://gacrc.uga.edu), that consists of two Linux clusters (zcluster and sapelo) with > 7,500 CPU cores for massive parallel computation in addition to over 1 petabyte of storage capability (>350 TB of fast-access storage and >700 TB of slower storage). All resources are available free of charge (except archival storage) including backup. Multiple jobs can be submitted to the cluster, and analyses can be run in parallel with a job distributed across as many as 128 cores simultaneously. Additionally, the GACRC has high memory resources including one 48-core 1TB node and multiple 32 and 48- core 512GB, 256GB and 192GB compute nodes for jobs requiring more memory. Resources and software required for analyses are maintained by GACRC staff.

The Quantitative Biology Consulting Group (QBCG) is a university core offering bioinformatics support and operated by the Institute of Bioinformatics (IOB). With our close working relationships with the GACRC, the Georgia Genomics Facility (GGF) and the Departments of Statistics and Biostatistics, experts from these other cores and disciplines are leveraged to help researchers negotiate experimental design, data processing and storage and data analysis using a comprehensive approach.

We are glad that you have chosen the QBCG to assist you in analyzing your RNA-Seq data. For this project we will be performing a workflow that includes quality assessment and filtering of your short read Illumina sequence data followed by read alignment to the *M. musculus* mm10 genome (Ensemble GRCm38.p4, GCA_000001635.6). We will utilize STAR aligner to map reads and employ multiple statistical approaches, i.e. DESeq2 and EdgeR, to identify and quantify gene expression from normalized read counts. Ultimately, we will provide you with cohorts of statistically validated and annotated differentially expressed transcripts to data mine across the control and experimental groups you are proposing to investigate. We wish you much success in securing funding for this proposal, and we look forward to helping you decipher the transcriptional mechanisms by which Plac8 ablation within CD8 T cells affects developing CD8 memory cells in response to influenza challenge.

Sincerely,

W. Walter Lorenz, Ph.D.
Lead Bioinformatics Consultant

Davidson Life Sciences Bldg., B320A| 120 E. Green St. | Athens, GA 30602
Telephone 706-542-7974 | Fax 706-542-1738
An Equal Opportunity, Affirmative Action, Veteran, Disability Institution

Resource Sharing

Any resources generated from this project will be made available to the scientific community upon publication as required by the NIH. Any large data sets, including RNA-Seq and proteomics analyses, will be deposited into an appropriate data repository at the time of publication.

Authentication

Key Biological Resources that will be utilized in this proposal include:

Mice:
Most mice (C57BL/6 and relevant CD45 congenics) will be purchased from reputable commercial vendors (Charles River, The Jackson Laboratory) and bred and maintained in house. The CD45 status of animals is verified before use using the appropriate antibodies by flow cytometry. *Plac8*$^{-/-}$ mice were originally generated by Beverly Koller and were kindly provided by Raymond Johnson. Genotypes of experimental mice and breeders are confirmed by PCR. Every effort has been made to ensure that mice used in these studies remain specific pathogen-free and are well-controlled to investigate the specified gene's functions. To ensure that experimental results are reflective of Plac8 ablation and not differences in the underlying background, confirmatory experiments are also conducted in WT and *Plac8*$^{-/-}$ littermates generated from intercrossing *Plac8*$^{+/-}$ mice. All mice are maintained in autoclaved caging and provided sterilized food and autoclaved water. Mice undergo routine sentinel testing in which used bedding from experimental mice is transferred to cages of sentinel mice that are subsequently euthanized for specific pathogen testing by UGA's Diagnostic Laboratory. Wild type C57BL/6 mice are used as a control in all validation experiments.

Viruses:
HKx31 virus (A/Hong Kong/1/1968 hemagglutinin and neuraminidase with remaining six segments from PR8) (H3N2) and A/Puerto Rico/8/34 (PR8) virus (H1N1) were obtained from Dr. Mark Tompkins who received the viruses from Dr. Peter Doherty. The identity of the influenza viruses has been confirmed by sequencing and in addition, for the influenza viruses, virus-specific Abs.

Antibodies:
All Plac8 antibodies to be used in our experimental plan are commercially available (Bioss and Cell Signaling Technologies) and validated by the companies that provide them. We have also validated the anti-human Plac8 antibody (Cell Signaling) for its use in immunoprecipitations studies in Aim 2.3 **(Figure 8)**. In addition, we maintain an inventory of all antibodies, vendor name and validation data provided at time of purchase. For all flow cytometry and microscopy, appropriate isotype control antibodies will be used.

Cell lines:
Madin-Darby Canine Kidney (MDCK) cells will be used for virus plaque assays. Our MDCK stocks were provided by Dr. Mark Tompkins who originally acquired them from a commercial vendor (ATCC) and has the original documentation. These cells will be passaged, maintained and stored under appropriate Biosafety Level 2 conditions.

Fetal bovine serum:
One reagent that has the potential of introducing variability into *in vitro* experiments is fetal bovine serum (FBS), as every lot is different with respect to immune-regulatory factors such as cytokines. Therefore, we test at least 3-5 lots prior to purchasing a large lot of FBS, which is stored frozen in aliquots until use or expiry. Lot testing for our lab includes preparing media for various applications and determining which lot produces expected results that are reflective of previous results from our own lab as well as published results of others. Assays typically include *in vitro* polarization of T cells to Th1, Th2, Th17 and iTreg lineages followed by intracellular cytokine staining and LPS stimulation of macrophages followed by measurement of inflammatory cytokine secretion.

Other:
Other resources used in this proposal will be standard laboratory reagents. If we need to generate or obtain additional unique resources they will be authenticated using methods similar to those described above.

PHS Assignment Request Form

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

Funding Opportunity Number: PA-16-161

Funding Opportunity Title: NIH Exploratory/Developmental Research Grant Program (Parent R21)

Awarding Component Assignment Request *(optional)*

If you have a preference for an Awarding Component (e.g., NIH Institute/Center) assignment, please use the link below to identify the most appropriate assignment then enter the short abbreviation (e.g., NCI for National Cancer Institute) in "Assign to/Do Not Assign To Awarding Component" sections below. Your first choice should be in column 1. All requests will be considered; however, locus of review is predetermined for some applications and assignment requests cannot always be honored.

Information about Awarding Components can be found here: https://grants.nih.gov/grants/phs_assignment_information.htm#Awarding Components

	1	2	3
Assign to Awarding Component:	NIAID		
Do Not Assign to Awarding Component:			

Study Section Assignment Request *(optional)*

If you have a preference for a study section assignment, please use the link below to identify the most appropriate study section then enter the short abbreviation for that study section in “Assign to/Do not Assign to Study Section” sections below. Your first choice should be in column 1. All requests will be considered; however, locus of review is predetermined for some applications and assignment requests cannot always be honored.

For example, you would enter “CAMP” if you wish to request assignment to the Cancer Molecular Pathobiology study section or enter “ZRG1 HDM-R” if you wish to request assignment to the Healthcare Delivery and Methodologies SBIR/STTR panel for informatics. Be careful to accurately capture all formatting (e.g., spaces, hyphens) when you type in the request.

Information about Study Sections can be found here: https://grants.nih.gov/grants/phs_assignment_information.htm#Study_Section

	1	2	3
Assign to Study Section: *Only 20 characters allowed*	CMI-A		
Do Not Assign to Study Section: *Only 20 characters allowed*			

PHS Assignment Request Form

List Individuals who should not review your application and why *(optional)* *Only 1000 characters allowed*

Identify Scientific areas of expertise needed to review your application *(optional)*
Note: Please do not provide names of individuals

	1	2	3	4	5
Expertise: *Only 40 characters allowed*					