Descriptive Title: Regulation of mucosal immunity to respiratory viruses by Tpl2

Submission Title: Watford R21 TPL2

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:
Regulation of mucosal immunity to respiratory viruses by Tpl2

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

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

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 DW 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	Tpl2_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,

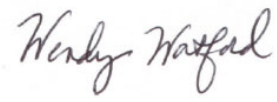
Please find enclosed a grant proposal entitled "**Regulation of mucosal immunity to respiratory viruses by Tpl2**". This R21 application addresses the major public health issue of influenza virus infection and seeks to understand mechanisms of antiviral immunity regulated by the host serine-threonine kinase, Tpl2. The rationale for these studies is that knowledge gained about the role of Tpl2 in mucosal immunity to virus infections, including Tpl2-dependent induction of protective IFN-lambda responses, may inform the design of novel anti-viral countermeasures and vaccines.

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

I request assignment to the following study section:
Immunity and host defense study section - IHD

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? [ ] Yes [X] No

1.a If YES to Human Subjects

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

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

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

IRB Approval Date:

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? [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	Tpl2_Abstract.pdf
8. Project Narrative	Tpl2_Project Narrative.pdf
9. Bibliography & References Cited	Tpl2_References_Final.pdf
10. Facilities & Other Resources	Tpl2_Facilities_Final.pdf
11. Equipment	Tpl2_Equipment_Final.pdf
12. Other Attachments	

Abstract
Respiratory viruses infect millions of people annually, causing significant morbidity and mortality and taking substantial socio-economic tolls. Approved antiviral drugs that directly target viral proteins are susceptible to virus adaptation leading to widespread resistance. Because viruses co-opt host cell machinery for their replication, host factors that regulate virus replication make attractive candidates for viral chemoprophylactics. Drugs targeting host proteins should be less susceptible to the development of viral resistance and have the added potential to cross-protect against diverse viruses. Host-encoded interferons (IFNs) are critical factors that mediate innate protection as well as modulate the adaptive immune response to viruses. Type III IFNs (IFNλs) are now appreciated to be the predominant IFNs produced during influenza virus infection, however there is limited information about the host pathways that regulate IFNλ expression. Lack of such knowledge is a barrier to improving vaccine strategies to control virus infections and transmission in susceptible populations. The host-encoded serine-threonine kinase, Tpl2, was recently demonstrated to enhance IFNλ production and host protection against influenza virus infection. Therefore, the objective of this application is to gain a better understanding of how Tpl2 coordinates the innate immune response to influenza virus. This will be examined in two Aims. In Aim 1, the biochemical mechanisms by which viruses induce Tpl2 expression and kinase activity as well as the Tpl2-dependent signaling pathways within lung epithelial cells will be determined. In Aim 2, the mechanisms by which Tpl2 induces IFNλ and amplifies the IFN response to restrict virus replication within the respiratory mucosa will be delineated. Experimental approaches will utilize murine and human lung epithelial cells as well as genetically altered mouse strains. The results of the proposed studies will lead to a more complete understanding of how lung epithelial cells generate protective mucosal responses to respiratory viruses. Information obtained from these studies will help to improve countermeasures and vaccine strategies for respiratory viruses and can likely be translated to other mucosotropic infectious diseases where IFNλs have prominent roles in immunoprotection.

Project Narrative

Respiratory viruses infect millions of people annually causing substantial morbidity and mortality. Antiviral drugs are susceptible to resistance, and current vaccine formulations are ineffective in some individuals. A more complete understanding of how host proteins regulate protective immune responses will inform the design of novel countermeasures and vaccine strategies.

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Laboratory:
Dr. Watford's (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 designated desk/computer spaces along the rear wall. There is sufficient bench and desk space for 8 investigators. The laboratory space is 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 laboratory 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 for infecting the animals with low pathogenic (mouse adapted) influenza. 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.

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). Back-up storage server systems exist for Dr. Watford's laboratory in the College of Veterinary Medicine 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.

Office: Dr. Watford has an office located in room 357 of the Veterinary Medicine Building just down the hall from her lab. Adequate office spaces are available for scientists, post-docs, technicians and students.

Scientific and intellectual environment: Dr. Watford's lab (PI) is located in the College of Veterinary Medicine within the Department of Infectious Disease. In addition, faculty with research interests similar to the PI's are located across the street in the Coverdell Building within the Center for Tropical & Emerging Global Diseases (which studies the immunology, transmission, and genetic components of infectious diseases common to tropical climates) and the Department of Cellular Biology. 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 Dr. Watford is an associate member. Members of the Watford lab attend weekly seminars and monthly research in

progress talks. 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 Watford lab attend one of these jointly with Dr. Rick Tarleton's group and Dr. Kimberly Klonowski's group. 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.

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:

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.

We also have 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).

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 just across the street in the Coverdell Center 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	
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:			
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		Tpl2_Watford_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 at the National Institutes of Health prior to establishing her own independent research group at the University of Georgia. Dr. Watford has made significant scientific contributions regarding cytokine signaling and T helper cell differentiation. In particular, in studies published in *Nature* and *JEM,* Dr. Watford identified the proprotein convertase, furin, and the serine-threonine kinase, Tpl2 (aka Map3K8 or Cot), 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. Specifically, she has shown that Tpl2 is important for TLR-mediated activation of innate cells, including the induction of proinflammatory IL-1β and the generation of reactive oxygen species. She has also demonstrated that Tpl2 promotes chemokine and chemokine receptor expression and migration of inflammatory cells to sites of inflammation. In T cells, Tpl2 promotes the generation of inflammatory Th1 (and Th17) lineages and inhibits Th2-mediated inflammation as well as iTreg differentiation and immunosuppressive functions. Collectively, these findings implicate Tpl2 in inflammation and suggest that Tpl2 blockade may be a viable treatment strategy for chronic inflammation. Of particular importance to the current application, Dr. Watford's group recently demonstrated that Tpl2 functions early during virus infection to induce IFN-lambda induction and impede influenza virus replication. The current proposal requests funds to investigate how Tpl2 is activated in response to virus sensing to promote IFNλ production by lung epithelial cells and how Tpl2 and IFNλ collectively regulate host protective immune responses. Information obtained from these studies will help inform the design of novel vaccine formulations (potentially modulating Tpl2 activity) that will enhance vaccine efficacy. Dr. Watford's training and technical expertise are well suited to conduct the proposed experiments.

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

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

OMB Number: 0925-0001

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	Tpl2_personnel budget justification_Final.pdf
Consortium Justification	
Additional Narrative Justification	Tpl2_Additional narrative justification.pdf

PERSONNEL BUDGET JUSTIFICATION

Senior Key Personnel

Wendy Watford, PhD (PI) - (1.4 summer month effort) will be responsible for providing oversight for the entire project, 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. In addition, Dr. Watford will assist in performing kinase assays in Aim 1.3 and will interface with the UGA Genomics Core Facility and Dr. Lorenz for RNA-Seq analysis in Aim 2.3.

Other Personnel

[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, *Tpl2*$^{+/-}$, *Tpl2*$^{-/-}$, *Tpl2*$^{fl/fl}$ and congenic mice used these studies thus far. Because of [redacted] exceptional diligence and reliability, [redacted] will continue to assist with animal colony maintenance for this proposal.

[redacted] (PhD candidate; 12 Calendar months, 50% effort) will serve as a Graduate Student in the Watford lab. [redacted] received [redacted] Bachelor's degree from Winthrop University in Biology. [redacted] will delineate the mechanism of regulation of Tpl2 expression and kinase activity by influenza, viral PAMPs and interferons (Aim 1.2). [redacted] will also delineate Tpl2-dependent signaling pathways in response to viruses and viral PAMPs (part of Aim 1.3) including the Tpl2-dependent mechanisms regulating IFN-lambda production (Aim 2.1) and confirm the epithelial cell-specific role for Tpl2 in host resistance against influenza (Aim 1.1).

Graduate student, TBD (PhD candidate; 6 Calendar months, 50% effort) will serve as a Graduate Student in the Watford lab. This student will primarily investigate the role of Tpl2 in IFN feedback signaling during virus infection. The student will delineate Tpl2-dependent signaling pathways in response to interferons (part of Aim 1.3). In addition, the student will determine whether exogenous IFNλ administration is sufficient to protect Tpl2-/- mice from otherwise lethal infection (Aim 2.2) and will determine how Tpl2 ablation within lung epithelial cells global alters the induction of host-protective interferon-stimulated genes (ISGs) (Aim 2.3).

ADDITIONAL NARRATIVE JUSTIFICATION

One extra module is requested in year 1, because RNA-Seq 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	Tpl2_Specific Aims.pdf
3. *Research Strategy	Tpl2_Research Strategy_Final.pdf
4. Progress Report Publication List	

Human Subjects Sections

5. Protection of Human Subjects	Tpl2_Protection of Human Subjects.pdf
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	

Other Research Plan Sections

9. Vertebrate Animals	Tpl2_VertebrateAnimals.pdf
10. Select Agent Research	
11. Multiple PD/PI Leadership Plan	
12. Consortium/Contractual Arrangements	
13. Letters of Support	Tpl2_Combined_LOS.pdf
14. Resource Sharing Plan(s)	Tpl2_ResourceSharing.pdf
15. Authentication of Key Biological and/or Chemical Resources	Tpl2_Authentication_Final.pdf

Appendix

16. Appendix

Specific Aims

Respiratory viruses infect millions of people annually causing substantial morbidity and mortality. Approved antiviral drugs that target viral proteins directly are susceptible to virus adaptation leading to widespread resistance [1-5]. Therefore, alternative strategies are needed to mitigate the impact of respiratory viral infections. Because viruses co-opt host cell machinery for their replication [6, 7], host factors that control virus replication make attractive candidates for host-targeted viral chemoprophylactics, are less susceptible to development of viral resistance and have the potential to cross-protect against diverse viruses. Host-encoded interferons (IFNs) are critical factors that mediate innate protection as well as modulate the adaptive immune response to viruses. Relative to Type I IFNs (IFNα/β), Type III IFNs (IFNλs) have only recently been described. However, they are now appreciated to be the predominant IFNs produced during influenza virus infection and are sufficient to protect the host [8]. IFNλs transduce antiviral signals preferentially in epithelial cells and induce host protective antiviral responses at barrier surfaces, including the respiratory, gastrointestinal and urogenital tracts. Despite their prominence and important immunoregulatory roles at mucosal sites, only limited information is available about the regulation of IFNλ expression. We recently demonstrated that a host serine-threonine kinase, Tpl2 (aka Map3k8 or Cot), significantly enhances IFNλ production and host protection against a respiratory virus infection *in vivo* [9]. However, it is unclear how Tpl2 expression and kinase activity are regulated within the respiratory tract by viruses and how Tpl2, in turn, controls IFNλ production and ultimately virus replication. Knowledge about how innate immune responses (including the Tpl2-dependent production of IFNλs) in the respiratory mucosa contribute to vaccine efficacy is limited. This is an important problem, because lack of such knowledge is a barrier to developing more effective disease interventions.

Our *long-term goal* is to improve vaccines and prophylactics for respiratory viral infections. The *objective* of this application is to understand how Tpl2 promotes and amplifies the antiviral IFN response within the respiratory tract to protect against a model respiratory pathogen, influenza A virus. Influenza provides an excellent model for addressing this problem due to the wealth of available reagents. Our *central hypothesis* is that Tpl2 kinase activation in response to viruses not only induces innate IFNλ production, but also amplifies the IFN response for efficient control of virus. This hypothesis was formulated on the basis of our own preliminary data that virus titers are significantly *increased* and IFNλ production is significantly *decreased* in the lungs of *Tpl2*$^{-/-}$ mice infected with influenza [9] (see also *Preliminary Studies*). The *rationale* for the proposed research is that a better understanding of mucosal immunity to respiratory viruses is needed to provide a basis for improved anti-viral interventions. We have significant and complementary expertise in evaluating innate immune responses to influenza virus infection as well as access to genetically altered mouse strains that make us especially well prepared to complete the proposed research, which is a continuation of our collaborative work [9]. To address our central hypothesis, we will test the following Specific Aims **(Figure 1)**:

Specific Aim 1: Determine the epithelial cell-intrinsic regulation and function of Tpl2 kinase during influenza infection.

Specific Aim 2: Delineate the molecular mechanism(s) by which Tpl2 amplifies the IFN response to restrict early virus replication within the respiratory mucosa.

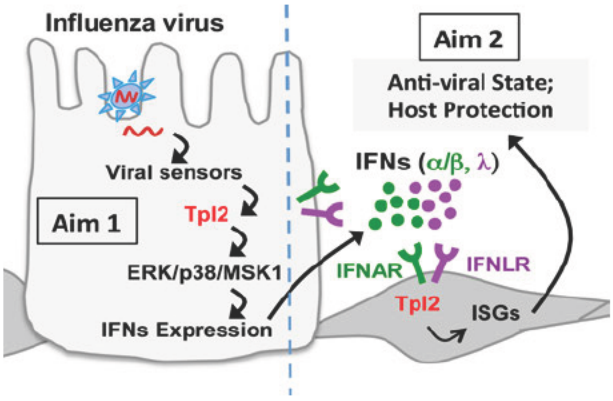


Figure 1: ***Aims.*** The overall goal of the study is to understand how Tpl2 promotes and amplifies the antiviral IFN response within the respiratory tract to protect against a model respiratory pathogen, influenza A virus.

The *expected outcomes* of the proposed studies are (1) a detailed understanding of how viruses induce Tpl2 kinase activity and IFN (especially IFNλ) production within the respiratory tract and (2) how Tpl2 contributes to IFN feedback pathways to limit virus replication. These findings will have a *positive impact* on human health, because understanding the host pathways that modulate IFNλ expression is necessary for tailoring vaccines that can exploit this pathway to enhance mucosal immunity. This proposal is appropriate for the R21 funding mechanism as it is highly novel, exploratory and takes our laboratory research in a new direction.

Research Strategy

(A) Significance

Respiratory viruses infect millions of people annually, causing significant morbidity and mortality and taking substantial socio-economic tolls [10-12]. In the United States, the financial burden of seasonal influenza alone is nearly $90 billion annually [13]. Alternative strategies to both vaccines and antivirals are clearly needed for treating respiratory virus infections. Drugs targeting *host* proteins should be less susceptible to viral resistance and potentially cross-protect against diverse viruses. Interferons (IFNs) are critical host factors that mediate innate protection as well as modulate the adaptive immune response to viruses. Although structurally unrelated to the Type I IFNs (IFNα/β), Type III IFNs (IFNλs) activate a similar intracellular signaling pathway to induce largely redundant antiviral interferon-stimulated genes (ISGs) through a distinct receptor complex (IFNLR1/IL10R2) [14, 15]. Emerging evidence has demonstrated that IFNλs are the predominate interferons produced during influenza infection [8, 16]. Furthermore, they help orchestrate adaptive immune responses by promoting pro-inflammatory Th1 responses while inhibiting Th2 responses [17-21]. Accordingly, IFNλs have been suggested to inhibit humoral responses, and IFNλ expression inversely correlates with seroconversion upon vaccination [20, 22, 23]. Because of their important host protective and immunomodulatory roles at barrier surfaces, it is critical that we understand how IFNλs are regulated. We demonstrated that the host serine-threonine kinase, Tpl2 (aka Map3k8 or Cot), significantly enhances IFNλ production and host protection against influenza virus infection *in vivo* [9]. Strengths of this key study include reproducibility of the increased morbidity of *Tpl2*$^{-/-}$ mice during infection with both influenza A/X31 and influenza A/PR8 strains and in littermate control mice. The Tpl2-dependent induction of IFNλ expression was confirmed in both lung homogenates and bronchoalveolar lavage (BAL) fluid at 1 and 3 days post infection (dpi) and was further demonstrated in plasmacytoid dendritic cells [9]. However, whether Tpl2-dependent virus sensing and IFNλ expression *within lung epithelial cells* was required for inhibition of early virus replication was not directly addressed nor was the effect of gender on Tpl2-dependent IFNλ production and control of virus replication. The current proposal will not only delineate the mechanism of Tpl2-dependent anti-viral responses within the respiratory mucosa, but also address the variable of gender on this regulation. The contribution of this proposal is expected to be a detailed understanding of how Tpl2 modulates (1) initial virus sensing, (2) signal transduction and (3) IFNλ production within the respiratory mucosa to reveal how Tpl2 constrains virus replication at the respiratory mucosa. *This contribution is significant because an increased understanding of the mechanisms regulating mucosal immunity will inform the design of novel host-targeted vaccine formulations.* It is expected that the outcomes of the proposal will be broadly applicable to 'mucosa-tropic' viruses in general. Tpl2-activating adjuvants could be incorporated into vaccine formulations, whereas Tpl2 inhibitors could be exploited to increase virus replication *in vitro* to accelerate vaccine production during pandemics.

(B) Innovation

Tpl2 has been demonstrated to regulate inflammation in a variety of conditions, including sepsis [24, 25], asthma [26], inflammatory bowel disease [27], and bacterial [28-30] and parasitic [31] infections. Surprisingly, no studies (except our own preliminary study [9]) have addressed the role of Tpl2 in lung immunoregulation during respiratory viral infections, including the regulation of IFNλ. Therefore, the research proposed in this application is *conceptually innovative* because it (1) will address the expression and immunoregulatory functions of Tpl2 in the lung mucosa *within primary epithelial cells* and (2) considers Tpl2 as an intrinsic viral restriction factor, the biology of which can be exploited to derive novel viral countermeasures and vaccines. Specifically, the identification of Tpl2 as a regulator of Type III IFNs is highly *innovative,* and the delineation of this regulation will significantly advance the field of mucosal immunity. This application is *technically innovative* in that conditional deletion of Tpl2 will be used to interrogate cell-type specific contributions of Tpl2 to host protection during influenza virus infection, providing valuable insights into its functions in respiratory epithelial cells.

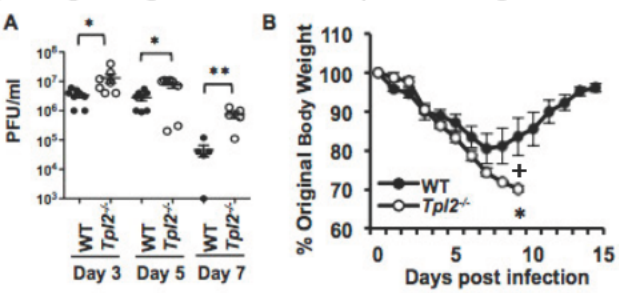
(C) Approach: Preliminary Studies

I. Tpl2 ablation enhances virus titers, morbidity and mortality to a low pathogenicity influenza virus (influenza A/X31). Wild type (WT) and *Tpl2*$^{-/-}$ mice were infected with 10^4 plaque forming units (pfu) of influenza virus A/HK-X31(H3N2, hereafter referred to as X31), and viral titers in the lungs were evaluated on 3, 5 and 7 days post infection (dpi). Lung viral titers were significantly higher in *Tpl2*$^{-/-}$ mice compared to WT mice at all time points **(Fig. 2A)** [9]. Increased virus replication was also observed in littermate control mice and correlated with increased morbidity **(Fig. 2B)** in *Tpl2*$^{-/-}$ mice [9].

II. Tpl2 functions in non-hematopoietic lung stromal cells to limit early virus replication. To distinguish whether Tpl2 functions within the hematopoietic or stromal cell compartment to limit virus replication, we assessed lung viral titers in bone marrow chimeras in which WT or *Tpl2*$^{-/-}$ bone marrow cells were transferred into lethally irradiated WT or *Tpl2*$^{-/-}$ recipients 8 weeks prior to infection with 10^4 pfu of X31 virus. On 3 dpi, lung vi-

rus titers were significantly higher in *Tpl2*$^{-/-}$ mice reconstituted with WT hematopoietic cells compared to WT mice that received WT bone marrow **(Fig. 3)** [9]. Therefore, Tpl2 signaling in non-hematopoietic lung stromal cells is necessary for limiting early virus replication. Although, in macrophages Tpl2 regulates the expression and secretion of TNF [24], which also possesses anti-viral properties [32], no difference in TNF production was observed in influenza-infected *Tpl2*$^{-/-}$ lung homogenates (data not shown), demonstrating that *Tpl2 limits virus replication independently of TNF regulation.*

Figure 2: Tpl2 restricts influenza replication *in vivo*. WT and *Tpl2*$^{-/-}$ mice were infected with 10^4 pfu of influenza A/X31. (**A**) Lung viral titers. (**B**) Morbidity assessed by weight loss. **+**, *Tpl2*$^{-/-}$ mice were euthanized because they reached human endpoints. *p<0.05, **p<0.01.



III. Tpl2 is required for optimal IFNλ production during influenza infection. Interferons are induced early during infection and are key factors initiating host protective antiviral responses, particularly at barrier surfaces [33]. To determine whether the observed increase in viral titers in *Tpl2*$^{-/-}$ mice correlated with defective induction of IFNs, WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu X31 virus, and IFNα/β/λ levels in lung homogenates or BAL fluid were measured at 1 or 3 dpi. Induction of IFNα/β was comparable between WT and *Tpl2*$^{-/-}$ mice [9], but IFNλ secretion was reduced in lung homogenates 1 dpi and in BALF 3 dpi from *Tpl2*$^{-/-}$ mice **(Fig. 4,**[9]**)**.

Figure 3: Tpl2 functions in non-hematopoietic cells to limit influenza replication. WT bone marrow cells were transferred into lethally irradiated WT or *Tpl2*$^{-/-}$ recipients 8 weeks prior to infection with 10^4 pfu of influenza A/X31, and virus titers were measured on day 3. *p<0.05.

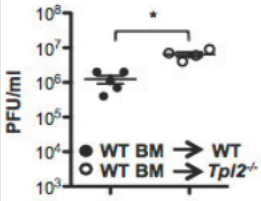
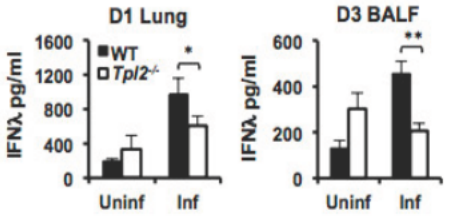


Figure 4: IFNλ production is impaired in influenza-infected *Tpl2*$^{-/-}$ mice. WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu X31 virus, and IFNλ levels in lung homogenate or BALF were measured at 1 or 3 dpi bv ELISA. * *p*<0.05. ** *p*<0.01.



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 epithelial cell-intrinsic regulation and function of Tpl2 kinase during influenza infection. *Introduction.* The main barrier to infection within the lung is the pulmonary epithelium. Cells of the upper airways (nasopharynx and bronchi) are frequently the first to be infected under physiological conditions due to their initial exposure to virions. The respiratory bronchioles contain cuboidal, non-ciliated cells known as club cells, which do not produce mucus, but secrete copious amounts of club cell secretory protein (CCSP) and limit susceptibility to respiratory viruses [34-36]. In the lower lung, type II alveolar epithelial cells (AECII) represent the primary target for influenza infection and provide a virus replication niche [37, 38]. Accumulating evidence supports an active immunological role for AECII, including pathogen sensing, induction of immune mediators, cellular recruitment and antigen presentation [39]. The serine-threonine kinase, Tpl2, mediates pathogen sensing by macrophages through TLRs and cytosolic sensors [30, 40, 41]. Furthermore, Tpl2 promotes inflammatory responses through the activation of diverse cellular pathways including MEK/ERK, p38α, NFκBp65, PI3K/Akt/mTOR and the NADPH oxidase, in response to inflammatory stimuli [41-43]. In particular, Tpl2 transduces signals in response to diverse TLR ligands and cytokines, but it does so in a cell-type and stimulus-specific manner [44]. How Tpl2 promotes pulmonary mucosal immunity has been essentially overlooked. A single study has demonstrated that Tpl2 is required for ERK activation in immortalized human airway epithelial cells in response to the TLR ligands Pam3CSK4 (TLR2), poly I:C (TLR3) and flagellin (TLR5) as well as *Pseudomonas aeruginosa* [45]. Despite the recent identification of Tpl2 as an important component of anti-viral responses [46], an understanding of how Tpl2 is regulated and functions within primary airway epithelial cells is still lacking. Bone marrow chimeras demonstrated that Tpl2 ablation within the lung stroma was permissive for increased virus titers at early time points [9]. However, whether Tpl2 ablation within epithelial

cells *per se* is sufficient to account for the early replication phenotype and/or the later morbidity and mortality in *Tpl2*$^{-/-}$ mice is unknown. The objective of this aim is two-fold: (1) to characterize Tpl2's regulation within airway epithelial cells and (2) to delineate Tpl2's contribution to virus sensing and anti-viral signaling. We will test the working hypothesis that *replicating influenza viruses activate Tpl2 kinase activity and downstream signaling pathways through RIG-I (and possibly other virus sensors) directly within epithelial cells to limit virus replication. Furthermore, Tpl2 expression in primary airway epithelial cells is amplified by virus-induced interferons.*

1.1. *Determine if Tpl2 conditional ablation within airway epithelial cells is sufficient for increased susceptibility to influenza infection.* Tpl2 is widely expressed in non-hematopoietic and hematopoietic lineages, and its pro-inflammatory functions are context-dependent [44], making it difficult to attribute specific cellular functions to Tpl2. These experiments will determine whether Tpl2 acts primarily within the pulmonary epithelium to limit both early virus replication and later morbidity. Experimental Approach: Tpl2 will be deleted from lung epithelial cells using Cre-lox technology. *Tpl2*$^{fl/fl}$ mice have been acquired from the European Mouse Mutant Archive [47] and intercrossed with surfactant protein C-cre ER^{T2} (*Sftpc*-cre ER^{T2}) and Nkx2.1-cre (*Nkx2.1*-cre) mice to generate conditional ablation of Tpl2 within the airway epithelial cells. Tamoxifen administration will drive Sftpc-cre-mediated deletion of Tpl2 within AECII [48, 49], whereas Nkx2.1-cre will spontaneously delete Tpl2 throughout the mouse lung epithelium, including club cells, AECII cells, and bronchial basal cells [50, 51]. Tpl2 deletion efficiency within lung epithelial cells will be determined by RT-PCR on purified cell populations [52] and immunohistochemistry **(See Fig. 5)**. WT, *Tpl2*$^{fl/fl}$, *Sftpc*-cre ER^{T2} *Tpl2*$^{fl/fl}$, and *Tpl2*$^{-/-}$ mice will be treated with tamoxifen as described [48, 49] and subsequently infected intranasally with 30 pfu influenza A/PR8 (H1N1). In a separate set of experiments, WT, *Tpl2*$^{fl/fl}$, *Nkx2.1*-cre *Tpl2*$^{fl/fl}$, and *Tpl2*$^{-/-}$ mice will be infected without tamoxifen pretreatment. *To examine the biological impact of sex on outcomes, male and female cohorts of 10 experimental mice (5 males and 5 females) of each genotype will be infected with influenza.* At least three such experiments will be performed. Weight loss and body condition scores will be tracked over 21 days. On 7 and 10 dpi when morbidity is observed in *Tpl2*$^{-/-}$ mice, lungs will be harvested, sectioned, stained with H&E and scored by a pathologist (TN). If pathological changes associated with Tpl2 expression in epithelial cells are noted, additional mice will be euthanized at 3 dpi to examine early innate inflammatory changes including cytokine (IFNs) production and inflammatory cell infiltrates [9].

1.2. *Characterize the regulation of Tpl2 expression within airway epithelial cells, both basally and in response to influenza infection.* We first identified Tpl2 as an IL-12-induced Stat4 target gene in CD4 T cells [31] and later as an IFNα-induced Stat4 target gene in CD8 T cells [53]. How Tpl2 expression is regulated in the pulmonary epithelium, especially during viral infection is unknown. This sub aim will test *the hypothesis that Tpl2 is prominently expressed within primary murine and human airway epithelial cells and that influenza-induced IFN signaling potentiates its expression*. Experimental Approach: WT or *Tpl2*$^{-/-}$ mice will be infected with 30 pfu influenza A/PR8 (H1N1) for 1 to 3 days. Lungs and tracheae will be removed, formalin-fixed, paraffin-embedded and sectioned separately at 4 μm for fluorescence immunohistochemistry (IHC) with a Tpl2 antibody approved for IHC (M-20, H-7; Santa Cruz Biotechnology). Sections will also be co-stained with antibodies against influenza NP (BEI Resources, NIAID), EpCAM (epithelial cells, ThermoFisher, clone 323/A3) or CD45 (hematopoietic cells, Abcam, #ab10558) and counterstained with DAPI. Influenza regulation of Tpl2 expression in *Ifnar1*$^{-/-}$, *Myd88*$^{-/-}$ and *RIG-I*$^{-/-}$ mice will be compared to that in WT mice to determine how Tpl2 expression is regulated by IFN signaling versus direct viral sensors. *Tpl2*$^{-/-}$ mice will be used as negative controls for Tpl2 IHC experiments. Staining of lung sections from naïve and influenza A/X31-infected mice demonstrate basal staining of Tpl2 primarily within cuboidal AECII cells (arrowheads) and a dramatic enhancement of Tpl2 expression in all cell types three days after influenza infection **(Fig. 5)**, with particularly strong staining seen in bronchiolar epithelial cells. As X31 infection does not typically extend into the lower airways, these data suggest that influenza induces Tpl2 expression in airway epithelial cells indirectly, perhaps via interferon feedback. Formalin-fixed paraffin-embedded healthy human lung tissue slides (Abcam, #ab4349) will be stained for basal Tpl2 expression by IHC to determine translational relevance of the murine data.

1.3. *Delineate Tpl2 kinase-dependent activation and signal transduction pathways within airway epithelial cells.* Tpl2 is widely expressed in both hematopoietic and non-hematopoietic tissues, and it regulates MAPK signaling in a cell-type and stimulus-specific manner [44]. In particular, TNF requires Tpl2 for the activation of ERK, JNK and NFκB in mouse embryonic fibroblasts (MEFs) but only for ERK and p38α activation in macrophages [44, 54]. To fully appreciate the contribution(s) of Tpl2 to pulmonary antiviral responses, it is necessary to understand the breadth and scope of signaling pathways regulated by Tpl2 in airway epithelial cells. Therefore, the goal of this sub aim is to delineate Tpl2-dependent signal transduction pathways within lung epithelial cells in response to influenza virus, key viral PAMPs and interferons. *Our hypothesis is that Tpl2 is critical for virus-induced epithelial cell signaling through TLRs RLRs, and even IFNs*. Experimental Ap-

proach: ***Primary cell isolation:*** Functional studies will examine Tpl2-dependent anti-viral responses in airway cell types showing high Tpl2 positivity in Aim 1.2, including AECII and bronchiolar epithelial cells such as club cells. Primary AECII ($CD45^{-}EpCAM^{hi}$) will be isolated from WT and *Tpl2*$^{-/-}$ mice by enrichment of $CD45^{-}EpCAM^{+}$ cells from dispase-digested lungs as described [55]. We can reliably isolate primary AECII from WT and *Tpl2*$^{-/-}$ mice at greater than 96% purity based on the epithelial marker EpCAM and the AECII marker, surfactant protein A **(Fig. 6A)**. Our preliminary data reveal that Tpl2 is highly expressed in AECII basally, more so than in immune cells, including macrophages (adherent peritoneal exudate cells, PECs), B cells and natural killer (NK) cells **(Fig. 6B)**. Similar studies will be conducted to isolate primary club cells as described [56]. Because these cell types extend into the lower airways, the more pathogenic influenza-A/PR8 (H1N1) strain will be used for all *in vivo* and functional studies. ***Tpl2 kinase assay:*** Because of the large cell number required, murine and human AECII cell lines (MLE-12 and A549, respectively) will be used for Tpl2 kinase assays. Cells will be grown to 80% confluence and infected with influenza A/PR8 in the presence of TPCK-trypsin at a multiplicity of infection (MOI) of 5 to ensure synchronous infection. Tpl2 expression and Tpl2 kinase activation (assessed by *in vitro* kinase assay) [57] will be measured over one replication cycle using the following time points: 0.5, 1, 2, 4, 6 and 8 h. We have established the Tpl2 kinase assay in our laboratory **(Fig. 7)**. To determine whether virus replication is required for Tpl2 kinase activation, we will inactivate PR8 by beta-propiolactone treatment [58] and test the ability of non-replicating virus to induce Tpl2 kinase activation. The ability of individual viral ligands, including single-stranded RNA (transfected 5'ppp RNA, RIG-I ligand or non-transfected synthetic ligand R848, TLR7 ligand), dsRNA (poly I:C;TLR3 ligand), DAMPs (extracellular ATP, NLRP3 ligand) or IFNs to induce Tpl2 kinase activity will also be assessed. ***Tpl2-dependent signal transduction:*** WT or *Tpl2*$^{-/-}$ AECII will be infected with 5 MOI influenza A/PR8 (0.5, 1, 2, 4, 6 and 8 h) or stimulated with key model ligands or inflammatory cytokines over a brief time course (15, 30, 60 or 120 min). Activation of signaling pathways will be evaluated by immunoblotting and/or phospho-flow staining as previously described [43]. Ligands to be tested include LPS (TLR4, control), poly I:C (TLR3), R848 (TLR7), 5'-triphosphate RNA (5'ppp RNA;RIG-I), type I IFNs (IFNα/β), TNF and IL-1β, because they have been shown to induce Tpl2-dependent signaling in other cell types [24, 40, 41, 44, 59] and/or mediate critical anti-viral functions. Key phosphorylation events will be analyzed according to published methods, including phosphorylation of IRFs 3 and 7 [60, 61], ERK1/2 [24, 41], p38α [54], NFκBp65 [44], Akt [62], S6 ribosomal protein [43, 62, 63], and Stat1 [9]. Cells will be stimulated for 15, 30 or 60 min with TLR ligands or IFNs or for 1, 2 or 4 h upon cytosolic delivery of 5'ppp RNA (InvivoGen) [9]. Tpl2 activation (measured by pERK) occurs within 2 h in macrophages [9].

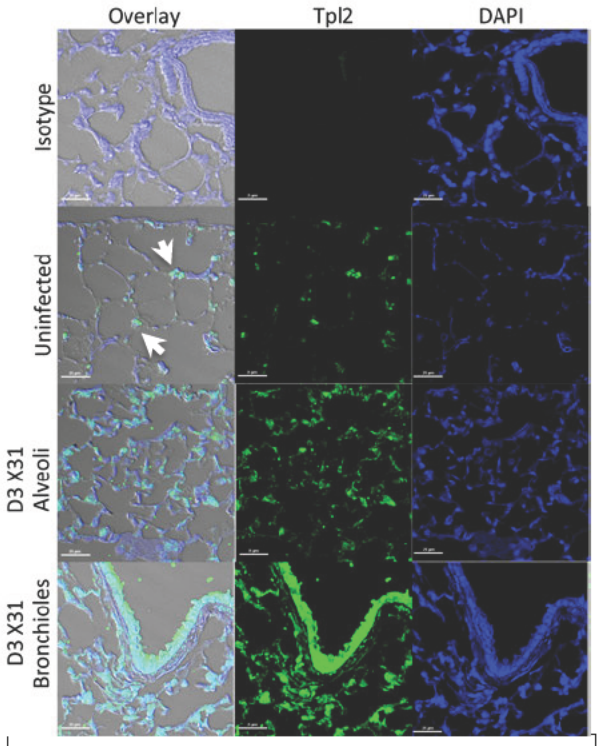


Figure 5: Tpl2 expression in influenza infected lungs. Immunofluorescence of *in vivo* influenza infection within WT mouse lungs at D3, stained with Tpl2 M20 or isotype control antibody. N=2 expts.

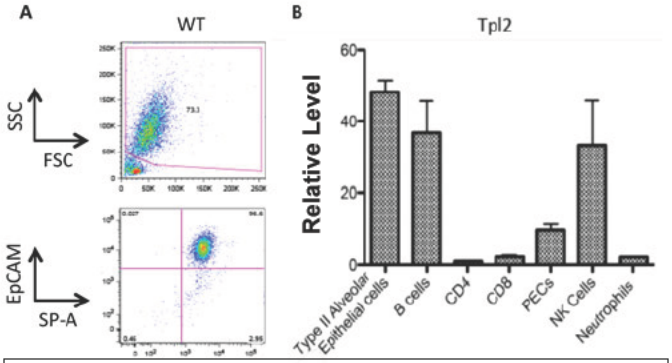


Figure 6: Tpl2 is highly expressed in primary AECII. (A) Flow cytometry of murine AECII from WT mice based upon surface EpCAM and intracellular SP-A staining. (B) Tpl2 expression in murine cells determined by RT-PCR; N=2

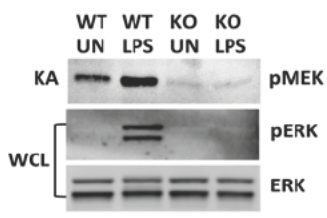


Figure 7: Tpl2 kinase assay. WT and *Tpl2*$^{-/-}$ thioglycollate-elicited PECs were stimulated with LPS for 30 min. Tpl2 was immunoprecipitated from whole cell lysates. Immunoprecipitates were subjected to kinase assay (KA) against kinase-dead GST-MEK substrate for 30 min, 30°C.

Aim 1 expected outcomes, potential pitfalls and alternative approaches:

Completion of Aim 1 will provide an understanding of how Tpl2 functions within respiratory epithelial cells to sense viruses and to transduce anti-viral signals. These experiments should reveal (1) how Tpl2 expression

and activation is regulated within the radio-resistant lung epithelial cells where influenza replicates, (2) the extent to which viral infection induces Tpl2 expression through feed-forward IFN signaling and (3) the signal transduction roles for Tpl2 in response to viral PAMPs and IFNs. Because Tpl2 is highly expressed basally in AECII and promotes influenza-induced IFNλ, we suspect that Tpl2 plays an integral role in regulating the immune status of the respiratory mucosa. The proposed experiments are expected to reveal that Tpl2 kinase is activated in response to viral RNA sensing by RIG-I (and possibly other sensors [9, 30, 41]) and, like IFNλ, requires viral replication intermediates for activation. This would suggest that live-attenuated influenza vaccines (LAIV) would be superior activators of Tpl2 (and IFNλs) compared to inactivated vaccines and may support the incorporation of Tpl2-activating adjuvants within inactivated vaccine formulations. Because we are well versed in the proposed techniques, and necessary reagents are available, we do not anticipate any barriers to completing this Aim. One unknown is the Tpl2 deletion efficiency within the cre strains. In the unlikely event that neither construct efficiently deletes Tpl2, one allele of Tpl2 would be preemptively deleted from the germ-line to yield cre+ $Tpl2^{Fl/-}$ mice and/or the tamoxifen regimen could be optimized to improve deletion efficiency.

Specific Aim 2: Delineate the molecular mechanism(s) by which Tpl2 amplifies the IFN response to restrict early virus replication within the respiratory mucosa. *Introduction.* We identified the serine-threonine MAP kinase, Tpl2, as an antiviral host factor for influenza [9]. Increased virus replication, morbidity and mortality correlated with nearly abrogated production of IFNλ3 (IL-28B) in $Tpl2^{-/-}$ mice infected with a normally *low pathogenic* influenza strain (X31) [9]. IFNλs are regarded as the principal IFNs induced during influenza virus infection [8, 16], and their protective role has been well established [15, 64, 65]. They function similarly to the Type I IFNs (IFNα/β), exerting powerful antiviral effects through the induction of interferon-stimulated genes (ISGs). Consistent with the preferential expression of IFNLR1 by epithelial cells [66], IFNλ promotes host resistance to viruses that infect mucosal sites, including the respiratory, gastrointestinal and urogenital tracts (reviewed in [67]). Because of their important host protective and immunomodulatory roles at barrier surfaces, it is critical that we understand how IFNλs are regulated. The *objective* of this aim is to determine precisely how Tpl2 influences IFNλ production and anti-viral effects within lung epithelial cells. Based on our own preliminary data, we will test the *working hypothesis* that *Tpl2 is essential for mucosal IFNλ production and potentiates paracrine IFNα/β/λ signal transduction by airway epithelial cells, the combined effect of which is to induce the expression of interferon-stimulated genes (ISGs) that limit virus replication.*

2.1. *Define the molecular mechanism(s) by which Tpl2 promotes the expression of Type III IFNs by virus-infected epithelial cells.* Experimental Approach: Primary AECII (WT and $Tpl2^{-/-}$) will be infected with influenza A/ X31 (H3N2) and influenza A/ PR8 (H1N1) at an MOI of 0.1, 1 or 10 in the presence of TPCK-trypsin for 4, 8, 24, and 48 h. Serum will be added back after 2 hours of infection. Supernatants will be harvested and stored at –80ºC for IFN quantitation by ELISA (murine IL-28A and IL-28B, ThermoFisher). Total RNA will be extracted from cell pellets, and expression of IFNs will be measured by RT-PCR (Applied Biosystems). Expression of IFNα and IFNβ will be analyzed for comparison as described [9]. Preliminary data demonstrate that influenza-induced IFNλ secretion is abrogated in $Tpl2^{-/-}$ AECII **(Fig. 8)**. Molecular mechanism. Accumulating evidence supports the hypothesis that Tpl2 promotes IFNλ production by inducing the phosphorylation of $NFkBp65^{S276}$ and recruitment of the transcriptional co-activator, CBP, to the IFNλ promoter. Murine IFNλ2-3 genes contain NFκB transcription binding sites, and transcriptional regulation of IL-28A/B is considerably more dependent upon NFκB signaling than IFNα/β [68]. Tpl2 promotes NFκB activity via MSK1-dependent NFκBp65 phosphorylation on Serine 276 in TNF-stimulated mouse embryonic fibroblasts [44]. This modification is cell-type and stimulus-specific and enhances NFκBp65 transcriptional activation without altering DNA binding. The human protein atlas reveals that MSK1 (aka RPS6KA5), like Tpl2, is highly expressed in tracheal and bronchiolar epithelial cells. Experiments will examine whether $NF\kappa B^{S276}$ phosphorylation is important for IFNλ expression using inhibitors of $NF\kappa B^{S276}$ phosphorylation. PKA and MSK1 have both been shown to induce $NF\kappa Bp65^{S276}$ phosphorylation [69, 70]. H-89, a PKA/MSK1 inhibitor (InvivoGen, 10 μM), will be used to inhibit $NF\kappa Bp65^{S276}$, followed by infection with influenza A/PR8 virus as described above. After 24 or 48 h, IFNλ secretion will be quantified by ELISA. Because this inhibitor also inhibits PKA, siRNA knock-down of MSK1 will be used to confirm inhibitor studies. Immunoblotting will be used to distinguish whether MSK1, PKA or both kinases are activated by influenza infection. If $NF\kappa Bp65^{S276}$ phosphorylation is necessary for IFNλ induction,

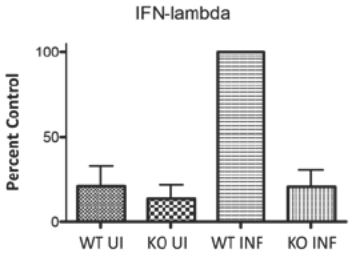


Figure 8: Influenza-induced IFN-λ expression is Tpl2-dependent. WT and $Tpl2^{-/-}$ AECII cells were uninfected (UI) or infected (INF) for 2 h with influenza A/X31 at an MOI of 10 prior to the addition of serum. Data pooled from 2 expts.

experiments will investigate whether influenza-induced NFκBp65^{S276} phosphorylation is Tpl2-dependent using immunoblotting or phospho-flow for anti-phospho-NFκBp65^{S276} (Cell Signaling Technologies) in WT and *Tpl2*$^{-/-}$ AECII. MSK1 is activated by both ERK and p38 stress-activated MAPK pathways. While Aim 1.3 will determine if influenza infection induces Tpl2-dependent activation of these MAPKs, this sub aim will further determine whether inhibition of those MAPK pathways impact IFNλ production via a Tpl2-dependent mechanism by pre-treating WT or *Tpl2*$^{-/-}$ AECII with ERK or p38 inhibitors prior to influenza infection. Finally, by comparing IFNλ production in response to different virus subtypes, namely H3N2 (X31) and H1N1 (PR8), we will determine whether viral surface antigens influence IFNλ antiviral responses, which may inform vaccine design.

2.2. *Determine if reduced IFNλ production is responsible for increased virus titers, morbidity and mortality in Tpl2*$^{-/-}$ *mice.* This sub aim will test the hypothesis that Tpl2 restricts early virus replication through its induction of paracrine IFNλ. Experimental Approach: WT and *Tpl2*$^{-/-}$ mice will be infected with 30 pfu of influenza-A/PR8 and treated with recombinant IFNλs (IL-28A/B, 1 μg/mouse each; R&D Systems) or control protein on day 0 as described [21] to determine if exogenous IFNλs are sufficient to reverse the susceptibility of *Tpl2*$^{-/-}$ mice to influenza. Mouse weights and body condition scores will be assessed daily. At 3 and 7 dpi, groups of 5 or more mice will be euthanized, and BAL cell numbers, cellular composition, inflammatory cytokines, and lung pathology will be measured as described previously [9]. A separate cohort of animals will be used exclusively for quantitation of lung virus titers by plaque assay.

2.3. *Determine how Tpl2 ablation within airway epithelial cells globally alters early innate responses to influenza infection.* To gain a global and unbiased view of which components of the influenza innate immune response are regulated by Tpl2, we will perform RNA sequencing (RNA-Seq) analysis on WT and *Tpl2*$^{-/-}$ primary AECII infected with influenza A/PR8. This sub aim will test the hypothesis that influenza-infected *Tpl2*$^{-/-}$ epithelial cells fail to establish a dominant anti-viral IFN transcriptional signature and should provide valuable information about the potential mechanisms responsible. Experimental Approach. WT, *Tpl2*$^{-/-}$, *Ifnar1*$^{-/-}$ or *Tpl2*$^{-/-}$ *Ifnar1*$^{-/-}$ AECII will be uninfected or infected with influenza A/PR8 for 6, 12 or 24 h in the presence of TPCK trypsin with serum addition 2 h after infection or stimulated with type I IFN (IFNβ). MOI will be based on results from Aim 2.1. Total RNA will be extracted using an E.Z.N.A.™ kit (Omega). RNA-Seq will be performed in-house at UGA's Georgia Genomics Facility in collaboration with Dr. Magdy Alabady (See Magdy letter of support). The RNA sequencing library will be prepared from isolated RNA and sequenced on an Illumina NextSeq 500 instrument as described previously [71]. 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 Lorenz letter of support). Ingenuity pathway analysis (IPA) will also be performed to identify specific biological pathways that are regulated in a Tpl2-dependent manner within AECII. Specific targets will be validated using real-time PCR analysis of sorted AECII. We expect that a preponderance of Tpl2-dependent genes will be those typical of an interferon signature, including interferon-stimulated genes (ISGs). If Tpl2 regulates IFN signal transduction in epithelial cells (Aim 1.3), Tpl2-dependent genes that are indirect targets of autocrine IFN signaling will be defined using *Ifnar1*$^{-/-}$ and *Tpl2*$^{-/-}$ *Ifnar1*$^{-/-}$ mice. It is also likely that additional unanticipated Tpl2-regulated genes will also be identified to provide novel molecular insights.

Aim 2 expected outcomes, potential pitfalls and alternative approaches:

Completion of this Aim is expected to reveal multiple insights about IFNλ regulation, including a possible mechanism for their strong NFκB-dependence compared to IFNα/β - i.e., Tpl2- and NFκB-dependent recruitment of the histone acetyltransferase CBP to IFNλ promoters. We therefore believe that Tpl2 will be important in regulating other genes whose transcription is sensitive to NFκB-dependent co-transactivation by CBP. Consequently, we expect to identify subsets of Tpl2-dependent ISGs that are severely impaired in expression during influenza infection *in vitro* and *in vivo*. However, in the event that influenza-induced NFκBp65^{S276} phosphorylation fails to explain Tpl2-dependent IFNλ regulation, Tpl2-dependent phosphorylation and nuclear translocation of IRF1 and IRF7, will be examined using immunoblotting and confocal microscopy, although normal induction of IFNα/β during influenza virus infection of *Tpl2*$^{-/-}$ mice argues against Tpl2-dependent IRF7 activation as a dominant mechanism *in vivo*. Furthermore, since MAVs localization to peroxisomes is specifically associated with IFNλ (but not type I IFNs) production [72, 73], we will examine MAVs localization to peroxisomes versus mitochondria in *Tpl2*$^{-/-}$ AECII as described [73]. We are well versed in performing the proposed techniques, all reagents are available, and we have strong preliminary data to support our overarching hypothesis and do not anticipate any barriers to completing the objectives of this Aim.

Proposal summary: Our preliminary data implicate Tpl2 as a critical regulator of IFNλ production and viral replication. Proposed studies will provide mechanistic insights into how Tpl2 regulates virus sensing pathways, IFN production and autocrine IFN signaling for broadly improving vaccines against mucosotropic infections.

Protection of Human Subjects

No human subjects research is proposed, but human lung tissue will be purchased commercially from a vendor. No identifying information will be provided.

VERTEBRATE ANIMALS

Introduction

The purpose of the proposed research with vertebrate animals is to determine how the serine-threonine kinase, Tpl2, alters the host immune response, particularly how it regulates the production of IFN–lambdas, during respiratory viral infection. This knowledge could be exploited to enhance anti-viral vaccines, perhaps by adjuvanting Tpl2 activation within epithelial cells. We have recently demonstrated that Tpl2 ablation leads to significantly reduced IFN-lambda production and increased virus replication in *Tpl2*$^{-/-}$ mice. By utilizing murine models of influenza infection, we can determine which cell types utilize Tpl2 for host protection against acute influenza infection. Furthermore, the use of primary alveolar type II epithelial in experiments *in vitro* will also reveal mechanistic information about the regulation of mucosal immune responses. Results from these studies may provide a basis for the development of urgently needed alternative strategies for treating virus-related illnesses and enhancing vaccine production. Wild type (WT) and genetically mutated mice must be used for these experiments.

1. Description of Procedures

Tissue harvesting. For harvesting of lung cells and tissue, mice will be anesthetized by i.p. administration of Tribromoethanol (TBE, aka Avertin 180-250mg/kg). To verify death, exsanguination will be performed prior to tissue removal. 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 150 control mice (C57BL/6J) and 110 *Tpl2*$^{-/-}$ mice will be needed to perform *in vitro* experiments and maintain breeding colonies. Approximately 20 control mice (*Tpl2*$^{fl/fl}$), 20 *Sftpc*-cre ER^{T2}-expressing *Tpl2*$^{fl/fl}$ mice, 20 *Nkx2.1*-cre expressing mice, 10 *Ifnar1*$^{-/-}$ mice and 10 *Tpl2*$^{-/-}$ *Ifnar1*$^{-/-}$ mice will be needed to maintain breeding colonies. Therefore, 340 mice of various genotypes will be required for *in vitro* studies and breeders over the project period.

Influenza infections.

For conditional ablation of Tpl2 in AECII, Tamoxifen-induced deletion will be performed using Tpl2 floxed mice crossed onto Sfptc-cre ER^{T2} mice, such that tamoxifen administration will induce deletion of Tpl2 specifically within AECII. Tamoxifen (Sigma) will de dissolved in a 10:1 mixture of sunflower seed oil/ethanol at 10 mg/ml, and 1 mg will be injected intraperitoneally daily for five days to induce deletion of Tpl2 in AECII. Deletion efficiency will be confirmed by immunofluorescence immunohistochemistry for Tpl2 and SP-C on paraffin-embedded lung sections and by RT-PCR in purified AECII. Five to 10 days after the last tamoxifen treatment, mice will be infected with influenza. Mice will be anesthetized by i.p. administration of TBE (180-250 mg/kg), then intranasally instilled with 0.05 ml of PBS containing 30 PFU PR8 using a pipette and disposable tips. Influenza infection induces rapid weight loss due to the induction of inflammatory cytokines. WT mice will experience flu-like symptoms and will recover fully, whereas Tpl2-deficient mice will show signs of more severe disease. In order to minimize their pain or distress, mice 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 10 mice per group (5 males and 5 females) per experimental condition will be used to obtain statistically significant findings, and three such experiments will be performed. Therefore, 70 WT, 120 *Tpl2*$^{fl/fl}$, 130 *Tpl2*$^{-/-}$, 60 Sftpc-cre ER^{T2} *Tpl2*$^{fl/fl}$ mice, 60 Nkx2.1-cre *Tpl2*$^{fl/fl}$ mice, 20 *Ifnar1*$^{-/-}$ mice, 20 *Tpl2*$^{-/-}$ *Ifnar1*$^{-/-}$ mice, 10 *MyD88*$^{-/-}$ mice and 10 *Rig-I*$^{-/-}$ mice will be required. A total of 500 mice are needed for influenza infection studies.

Total numbers of animals required over the project period: 840

2. Justification

Some experiments are only possible using non-transformed cells from living animals in which specific genetic mutations have been introduced. For example, understanding how Tpl2 coordinates immune responses to mediate immunity against viruses is only possible by performing studies in genetically-engineered animals, such as *Tpl2*$^{-/-}$ mice. Where possible, suitable human and murine lung type II epithelial cell lines have been proposed. However, it is essential that initial experiments first be conducted and validated in normal cells in which signaling and cell behavior are unaltered by transformation. Primary type II alveolar epithelial cells (AECII) will be isolated directly from WT and *Tpl2*$^{-/-}$ mice for analysis.

Mice are the accepted small animal model for studies of immunity and disease pathogenesis caused by respiratory virus infections, including influenza A viruses. By using mice, we will be able to determine the effect of Tpl2 *in vivo* under conditions that mimic human respiratory virus infection. In this regard, *Tpl2*$^{-/-}$ and Tpl2-floxed 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. The 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.

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 "**Regulation of mucosal immunity to respiratory viruses by Tpl2**." 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. All resources and software required for bioinformatics 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 responses in Tpl2 ablated AECII epithelial cells subjected 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

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

The University of Georgia
Georgia Genomics Facility

Te ephone (706) 542-6409
Fax (706) 542-6414
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 "Regulation of mucosal immunity to respiratory viruses by Tpl2."

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 Tpl2-/- primary AECII infected with influenza A/PR8. 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

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 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: C57BL/6, relevant CD45 congenics, and *Ifnar1*$^{-/-}$ mice have been purchased from The Jackson Laboratory and bred and maintained in house. *Myd88*$^{-/-}$ mice will also be obtained from The Jackson Laboratory. Genotypes of experimental mice will be confirmed by PCR. The CD45 status of congenic mice is verified using the appropriate antibodies by flow cytometry. *Tpl2*$^{-/-}$ mice were generated and kindly provided by Philip Tsichlis. *Tpl2*$^{fl/fl}$ mice were purchased from Alexander Fleming Biomedical Sciences Research Center and the colony has being intercrossed with Sftpc cre ER^{T2} and Nkx2.1 cre transgenic mice purchased from The Jackson Laboratory. Sftpc cre ER^{T2} mice are used to generate tamoxifen-induced deletion of floxed alleles specifically within type II alveolar epithelial cells. Nkx2.1-cre induces more generalized lung-specific deletion within in AECII, club cells, and bronchial basal cells. Tpl2 floxed colonies will be maintained by intercrossing *Cre- Tpl2*$^{fl/fl}$ mice with *Cre+ Tpl2*$^{fl/fl}$ mice so that littermate controls are always available. Every effort is made to ensure that mice used in these studies remain specific pathogen-free and are well controlled to investigate the specified gene's functions. 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. To ensure that experimental results are reflective of Tpl2 ablation and not differences in the underlying background, confirmatory experiments are also conducted in WT and *Tpl2*$^{-/-}$ littermates generated from intercrossing *Tpl2*$^{+/-}$ mice. Wild type C57BL/6 mice are used as a control in all validation experiments. A 'back-up' of the *Tpl2*$^{-/-}$ line has been created in the form of cryopreserved embryos that are stored at Emory.

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. Large stocks of X31 and PR8 have been grown in chicken eggs and aliquoted in order to minimize virus stock-dependent differences during this study. Virus titers were tested by plaque assay in Madin-Darby Canine Kidney Cell line (MDCK).

Antibodies: All antibodies to be used in our experimental plan are commercially available and validated by the companies that provide them. IFNλ antibodies will be purchase from R&D Systems, and Tpl2 antibodies will be purchased from Santa Cruz Biotechnology. The M-20 clone, a rabbit polyclonal antibody that has been routinely used in the literature and in this lab for immunoprecipitations and Western blotting, has been discontinued. However, we purchased a stockpile of that antibody to have on hand for completing the proposed experiments. The M-20 clone has been replaced by a mouse monoclonal antibody (H-7), also validated by the company. We have also had success with the H-7 clone, which will be used to validate key experiments. We also routinely include *Tpl2*$^{-/-}$ cells for controls in Western blot and immunoprecipitation experiments and we use appropriate isotype controls to ensure that the antibody reactivity is specific to Tpl2.

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. MLE-12 cells were purchased from ATCC (CRL-2110) and were originally generated from 5 month-old female mouse lungs and transformed by SV40. These cells are considered to be representative of lower airway type II epithelial cells and validated by the vendor to express lung surfactant proteins B and C. A549 cells will be purchased from ATCC (CCL-185) and were originally derived from a lung carcinoma of a 58 year old human male. These epithelial cells are widely used to model lower lung epithelial cell functionality during viral infection. All cell lines will be propagated according to manufacturer's instructions.

Recombinant proteins**:** Mouse IL-28A/IFNλ2 and IL-28B/IFNλ3 are *E. coli*-derived recombinant proteins and are validated to contain <0.1 EU endotoxin per 1 ug or protein. They are functionally validated to inhibit virus replication within a human cell line by the manufacturer.

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. We determine which lot produces expected results (i.e. reflective of previous results from our own lab and published results of others) in various cell culture applications, including *in vitro* polarization of T cells and LPS-induced cytokine production by macrophages.

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*	IHD		
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*					