PI: **RATNER, ADAM JONATHAN**	Title: Gardnerella vaginalis: toxin production and pathogenesis	
Received: 03/01/2010	FOA: PA10-067	Council: 10/2010
Competition ID: ADOBE-FORMS-B	FOA Title: Research Project Grant (Parent R01)	
1 R01 AI092743-01A2	Dual: HD	Accession Number: 3276320
IPF: 1833205	Organization: COLUMBIA UNIVERSITY HEALTH SCIENCES	
Former Number: 1R01HD061371-01A2	Department: Pediatrics	
IRG/SRG: HIBP	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Y Early Stage Investigator: Y

Senior/Key Personnel:	*Organization:*	*Role Category:*
Adam Ratner	Columbia University Medical Center (CUMC)	PD/PI
David Figurski	Columbia University Medical Center (CUMC)	Other (Specify)-Other Significant Contributor
[redacted]	[redacted]	Other (Specify)-Other Significant Contributor

Additions for Review

Supplemental Material	VLYR01A2update.pdf	05/11/2010

OMB Number: 4040-0001
Expiration Date: 06/30/2011

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION
☐ Pre-application ☐ Application ☒ Changed/Corrected Application

2. DATE SUBMITTED | **Applicant Identifier**

4. a. Federal Identifier: HD061371

b. Agency Routing Identifier:

5. APPLICANT INFORMATION

*** Organizational DUNS:** 6218898150000

* Legal Name: The Trustees of Columbia University in the City of New York
Department: Pediatrics
Division: Infectious Diseases
* Street1: 630 West 168th Street, Box 49
Street2: Sponsored Projects Administration
* City: New York
County / Parish: New York
* State: NY: New York
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 10032-3702

Person to be contacted on matters involving this application
Prefix:
* First Name: Lynette
Middle Name:
* Last Name: Arias
Suffix:
* Phone Number: (212) 305-4191
Fax Number: (212) 342-9063
Email: Grants-Office@columbia.edu

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

7. * TYPE OF APPLICANT: O: Private Institution of Higher Education
Other (Specify):
Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:
☐ 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 ☒ 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:
Gardnerella vaginalis: toxin production and pathogenesis

12. PROPOSED PROJECT:
* Start Date: 12/01/2010
* Ending Date: 11/30/2015

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT:** NY-015

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION
Prefix: Dr.
* First Name: Adam
Middle Name: Jonathan
* Last Name: Ratner
Suffix:
Position/Title: Assistant Professor
* Organization Name: Columbia University Medical Center (CUMC)
Department: Pediatrics
Division: Infectious Diseases
* Street1: 650 West 168th Street
Street2: Black Building 443
* City: New York
County / Parish: New York
* State: NY: New York
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 10032-3702
* Phone Number: (212) 305-9807
Fax Number: (212) 342-5218
* Email: ar127@columbia.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	2,012,500.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	2,012,500.00
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 ☒ 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 penalities. (U.S. Code, Title 18, Section 1001)

☒ *** 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

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: Elba Middle Name:

* Last Name: Suarez Suffix:

* Position/Title: Project Officer

* Organization: The Trustees of Columbia University in the City of New York

Department: Division:

* Street1: 630 West 168th Street, Box 49

Street2: Sponsored Projects Administration

* City: New York County / Parish: New York

* State: NY: New York Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 10032-3702

* Phone Number: (212) 305-4191 Fax Number:

* Email: Grants-Office@columbia.edu

*** Signature of Authorized Representative**

Elba Suarez

*** Date Signed**

03/01/2010

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 08/31/2011

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: Columbia University Medical Center

DUNS Number: 6218898150000

* Street1: 630 West 168th Street

Street2:

* City: New York County:

* State: NY: New York

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 10032-3702 * Project/ Performance Site Congressional District: NY-015

Project/Performance Site Location 1 ☐ 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:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Additional Location(s) Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ 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? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☒ No

IACUC Approval Date: 01/27/2010

Animal Welfare Assurance Number A3007-01

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ 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 be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. * Does this project involve activities outside of the United States or partnerships with international collaborators? ☒ Yes ☐ No

6.a. If yes, identify countries: [redacted]

6.b. Optional Explanation: [redacted]

7. * Project Summary/Abstract 1234-VLYR01A2abstract.pdf Add Attachment | Delete Attachment | View Attachment

8. * Project Narrative 1235-VLYR01A2-narrative.pdf Add Attachment | Delete Attachment | View Attachment

9. Bibliography & References Cited 1236-VLYR01A2-references.pdf Add Attachment | Delete Attachment | View Attachment

10. Facilities & Other Resources 1237-VLYR01A2facilities.pdf Add Attachment | Delete Attachment | View Attachment

11. Equipment 1238-VLYR01A2equip.pdf Add Attachment | Delete Attachment | View Attachment

12. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

Gardnerella vaginalis: toxin production and pathogenesis

PROJECT SUMMARY

Bacterial vaginosis (BV) is an exceedingly common disorder of the vaginal microflora affecting >30% of all women, with higher rates in pregnancy and among African-American populations. Women with BV are at substantially increased risk of preterm birth, which is a major cause of neonatal morbidity and mortality, as well as acquisition of sexually transmitted diseases including human immunodeficiency virus. Despite its public health importance, the pathogenesis of BV is not well understood. We have recently characterized vaginolysin (VLY), a cholesterol-dependent cytolysin from *Gardnerella vaginalis* (a bacterial species present on the vaginal mucosa in the setting of BV and thought to contribute to the pathogenesis of disease) that exhibits exquisite human specificity. We hypothesize that this species-specific toxin may be an important virulence factor of *G. vaginalis* with relevance to the pathogenesis of BV. In our preliminary data, we have characterized the receptor for VLY (human CD59) on genital tract epithelial cells. Introduction of this receptor into non-susceptible cells renders them sensitive to VLY. We have engineered a transgenic mouse expressing the hCD59 receptor and also constructed a VLY chimera that is hCD59-independent. These represent candidate in vivo models for BV. In addition, we have developed techniques for genetic manipulation of *G. vaginalis*, including transposon mutagenesis. In Aim 1, we will define genetic determinants of *G. vaginalis* virulence using new techniques for mutagenesis and assays of toxin production. In Aim 2, we will determine the role of VLY at the host-pathogen interface both in vitro and in vivo with a focus on unique aspects of the VLY-hCD59 interaction. At the conclusion of these studies, we will have expanded our knowledge of *G. vaginalis* pathogenesis, evaluated new in vivo models of BV, identified candidate strategies to inhibit toxin-host interaction, and developed new tools for continued investigation into the pathogenesis of an important disorder.

PROJECT NARRATIVE (RELEVANCE)

Bacterial vaginosis (BV) is a very common but not well-understood disease affecting women. Women with this disorder are at substantially higher risk of preterm birth and acquisition of other serious diseases, including HIV. This project investigates the role of a newly described toxin made by *Gardnerella vaginalis* in causing inflammation and damage to vaginal cells during BV and focuses on the development of new models and treatments for this important disease.

FACILITIES & OTHER RESOURCES

The recently reconstructed (November 2009) laboratory of the P.I. is more than 800 sq. ft. of dedicated space in the William Black Building at Columbia University, which also houses the research laboratories of the Departments of Pediatrics, Medicine, Physiology, Biochemistry, and others. The proximity of laboratories from several departments as well as the joint appointment and active involvement of the P.I. in the Department of Microbiology & Immunology make this a fertile environment for collaboration. The P.I.'s office space is adjacent to the laboratory. The lab contains all of the equipment necessary for molecular biology, bacteriological studies, tissue culture (dedicated laminar flow hood and CO_2 incubators in the lab), and microscopy (upright, inverted, and stereoscopic microscopes) and shares adjacent core equipment space (including dark room facilities, newly purchased Tecan fluorescent microplate reader, ultracentrifuge, and flow cytometry).

The P.I. has active vertebrate animal protocols as well as an IRB protocol for the use of primary human cells. Facilities for vertebrate animal research are managed by the Institute of Comparative Medicine at Columbia and are overseen by veterinarians and their professional staff. The animal BSL2 facility (ABSL2) was recently constructed and includes biosafety cabinets for inoculation and other procedures. The ABSL2 staff is experienced in containment practices and models of infection in rodents. The core facilities at Columbia are outstanding, including microarray and protein chemistry cores, the Genome Center, and the Irving Institute for Clinical and Translational Research (a recent recipient of a U54 CTSA award from NIH) which has core biostatistics, planning, regulatory, and execution support for translational research at Columbia.

<u>Resources Relevant to Early Stage Investigator Application</u>
The departmental and institutional commitment to the success of the P.I. is substantial. Dr. Ratner has >80% protected research time, administrative support through the Division of Pediatric Infectious Diseases, access to career development programs within both of his departments (Pediatrics and Microbiology & Immunology), and ample resources for classes, travel, and training. The Department of Pediatrics also has a formal mentorship and annual assessment program, which provides direct guidance and support to the P.I.

EQUIPMENT

In addition to standard equipment, the P.I.'s laboratory has a dedicated real-time qPCR system (ABI StepOne Plus) as well as multiple conventional and gradient thermal cyclers (Eppendorf). There is an Agilent BioAnalyzer 2100 with DNA, RNA, and protein analysis capability within the laboratory (purchased 2008) as well as an Amaxa Nucleofector II for electroporation of eukaryotic cells. The laboratory has a new (purchased in 2007) dedicated system for live-cell fluorescent imaging (Zeiss AxioObserver Z1) with 10x, 20x, 40x (long working-distance), and oil-emersion 63x fluorescent objectives. This inverted microscope has a motorized z-axis, a grid array (Apotome) system, heated stage, CCD-based and high-speed CMOS cameras, and state-of-the-art imaging software (Axiovision) running on a dedicated high-speed computer. Ample computer facilities are present in the laboratory for the proposed work (including 4 networked Intel-based Macintosh computers and 2 networked Pentium 4 PCs with up to date bioinformatics and image processing software, color laser printer, and scanner). Additional basic equipment within the lab includes freezers (-80°C, -20°C), chromatography refrigerator (+4°C), refrigerated tabletop centrifuge and microcentrifuges, laminar flow hood and two CO_2 incubators, SDS-PAGE/Western blot apparatus, agarose gel equipment and UV transilluminator with digital imaging system, microplate reader for ELISA, spectrophotometer, balances, pH meter, water baths, nutator, and incubator/shaker.

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Adam Middle Name: Jonathan
* Last Name: Ratner Suffix:
Position/Title: Assistant Professor Department: Pediatrics
Organization Name: Columbia University Medical Center (CUMC) Division: Infectious Diseases
* Street1: 650 West 168th Street
Street2: Black Building 443
* City: New York County/ Parish: New York
* State: NY: New York Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 10032-3702
* Phone Number: (212) 305-9807 Fax Number: (212) 342-5218
* E-Mail: ar127@columbia.edu

Credential, e.g., agency login: xxxxxxx

*** Project Role:** PD/PI **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** 1243-AJRbiosketch.pdf Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: * First Name: David Middle Name: H
* Last Name: Figurski Suffix:
Position/Title: Professor Department: Microbiology (5300000)
Organization Name: Columbia University Medical Center (CUMC) Division:
* Street1: 630 West 168th Street
Street2: 1516 Hammer Health Sciences Center
* City: New York County/ Parish: New York
* State: NY: New York Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 10032-3702
* Phone Number: (212) 305-3425 Fax Number: (212) 305-1468
* E-Mail: dhf2@columbia.edu

Credential, e.g., agency login: xxxxxxx

*** Project Role:** Other (Specify) **Other Project Role Category:** Other Significant Contributor

Degree Type:
Degree Year:

***Attach Biographical Sketch** 1244-figurskibio.pdf Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix: | * First Name: [redacted] | Middle Name:

* Last Name: [redacted] | Suffix:

Position/Title: Chief Scientist | Department: [redacted]

Organization Name: [redacted] | Division:

* Street1: [redacted]

Street2:

* City: [redacted] | County/ Parish:

* State: | Province: [redacted]

* Country: [redacted] | * Zip / Postal Code: [redacted]

* Phone Number: [redacted] | Fax Number:

* E-Mail: [redacted]

Credential, e.g., agency login:

*** Project Role:** Other (Specify) | **Other Project Role Category:** Other Significant Contributor

Degree Type:

Degree Year:

***Attach Biographical Sketch**	[redacted]	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support		Add Attachment	Delete Attachment	View Attachment

BIOGRAPHICAL SKETCH

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

NAME	POSITION TITLE
Ratner, Adam Jonathan	Florence Irving Assistant Professor of Pediatrics and Microbiology & Immunology
eRA COMMONS USER NAME (credential, e.g., agency login) XXXXXXX	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION		DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Yale University	New Haven, CT	B.A.	1989-1993	Biology
Columbia University	New York, NY	M.P.H.	1993-1997	Public Health
Columbia University	New York, NY	M.D.	1993-1998	Medicine
Columbia University	New York, NY	Residency	1998-2001	Pediatrics
Children's Hospital of Philadelphia		Fellowship	2001-2004	Infectious Diseases
University of Pennsylvania	Philadelphia, PA	Postdoctoral	2001-2006	Microbiology

A. Personal Statement

As a physician-scientist with broad-based training in pediatrics, infectious diseases, public health, and microbiology, I have focused my laboratory research program on bacterial diseases that are important to pediatric populations. My postdoctoral research, carried out under the mentorship of Jeffrey Weiser, examined the interplay between host cells and the airway pathogens *Streptococcus pneumoniae* and *Haemophilus influenzae*. We detailed new mechanisms of interspecies competition during colonization of mucosal surfaces, determined pathways by which mucosal epithelial cells sense sublethal concentrations of bacterial pore forming toxins, and delineated a novel link between combinations of extracellular pathogens and host cytoplasmic pattern-recognition receptors. Since starting my own laboratory in 2006, I have focused on infectious causes of premature birth, including bacterial vaginosis, a poorly understood and highly prevalent polymicrobial condition that substantially increases rates of prematurity. Our recent characterization of vaginolysin (VLY), a human-specific toxin produced by *Gardnerella vaginalis*, represents an opportunity to overcome a major roadblock in the understanding of the pathogenesis of bacterial vaginosis, the absence of reproducible in vivo model systems to understand the disease and its sequelae. Our group is at the forefront of *G. vaginalis* research, having developed new systems for its genetic manipulation as well as a variety of in vitro and in vivo assays of pathogenesis. The current application is an extension of this preliminary work, and the studies proposed will increase substantially our understanding of the pathogenesis of this organism. We have chosen collaborators carefully for these investigations. David Figurski is a leader in the fields of bacterial genetics and broad host range plasmids and has already helped us introduce bacterial expression and transposon vectors into *G. vaginalis* by conjugation with *E. coli*. [redacted] is an expert in the biology of CD59, the cell surface protein that is the receptor for VLY, and has provided us with well-characterized mice transgenic for human CD59. Thus, our group is very well positioned to carry out these studies successfully, and I believe that I am the appropriate person to lead this research effort. Thank you for your consideration.

B. Positions and Honors

Professional Positions

2004-2006 Assistant Attending Physician, Children's Hospital of Philadelphia
2006-present Assistant Attending Physician, Children's Hospital of New York-Presbyterian
2006-present Assistant Professor of Pediatrics and Microbiology & Immunology, Columbia University

Honors

1989 B.A. awarded *cum laude*, Distinction in Biology
1997 Summer Columbia P&S/NIH Short-term Research Grant (DK 39693)
1999, 2000 American Academy of Pediatrics Resident Research Grant (awarded twice)
2000 New York-Presbyterian Hospital House Staff Research Grant
2001-2006 Pediatric Infectious Disease Society-St. Jude Fellowship Program in Basic Research
2006 Keystone Symposia Travel Award
2008 Louis V. Gerstner, Jr. Scholar Award, Columbia University

2008 John M. Driscoll Children's Fund Scholar Award, Columbia University
2009 Hattie Alexander Lectureship, Columbia University
2009 Florence Irving Assistant Professorship, Columbia University
2009 Columbia University Senate (elected position)
2009 Doris Duke Charitable Foundation Clinical Scientist Development Award

Professional Societies and Committee Responsibilities

2001-present Pediatric Infectious Disease Society (PIDS)
PIDS Communications Committee (2008-present)
PIDS Program and Meetings Committee (2005-2008)
2001-present Fellow, American Academy of Pediatrics (AAP); AAP Section on Infectious Diseases (SOID)
Chair, SOID Nominations Committee (2008-2009)
Executive Subcommittee for Education (2005-present)
2006-present Infectious Diseases Society of America (IDSA)
IDSA Research Committee (2008-present)
2006-present American Society for Microbiology (ASM)
2006-present American Association for the Advancement of Science (AAAS)
2009-2010 Ad Hoc Member, Clinical Research and Field Studies in Infectious Diseases (CRFS) Study Section (NIH)
2009-present Human Microbiome Project Vaginal Strain Working Group (NIH)
2009-present Board of Directors, Pajama Program (Non-profit organization)
2009-present Society for Pediatric Research (SPR), elected member
2010-present Scientific Advisory Committee, Irving Institute for Clinical and Translational Research

Editorial Boards

2006-present Editorial Board and Section Editor for Infectious Diseases, Public Library of Science, *PLoS ONE*
2009-present Curator, Encyclopedia of Life (Smithsonian Institution), *Streptococcus* clade
2010-present Editorial Board, *Infection and Immunity*

C. Peer-reviewed publications (limited to 15 most relevant from more than 35 total)

1. DiMango E, Ratner AJ, Bryan R, Tabibi S, Prince A. Activation of NF-κB by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. **J Clin Invest** 1998; 101:2598-605. (PMID: 9616231)
2. Ratner AJ, Bryan R, Weber A, et al. Cystic fibrosis pathogens activate Ca^{2+}-dependent MAPK signaling pathways in airway epithelial cells. **J Biol Chem** 2001; 276:19267-75. (PMID: 11278360)
3. Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ, Janoff EN. Antibody-enhanced pneumococcal adherence requires IgA1 protease. **Proc Natl Acad Sci** 2003; 100: 4215-20. (PMID: 12642661)
4. Ratner AJ, Lysenko ES, Paul MN, Weiser JN. Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. **Proc Natl Acad Sci** 2005; 102: 3429-34. (PMID: 15728393)
5. Lysenko ES, Ratner AJ, Nelson A, Weiser JN. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. **PLoS Pathog** 2005; 1: e1. (PMID: 16201010)
6. Shah SS, Ratner AJ. Trends in invasive pneumococcal disease-associated hospitalizations. **Clin Infect Dis** 2006; 42: e1-5. (PMID: 16323082)
7. Ratner AJ, Hippe KR, Aguilar JL, Bender MH, Nelson AL, Weiser JN. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. **J Biol Chem** 2006; 281: 12994-8. (PMID: 16520379)
8. Ratner AJ, Aguilar JL, Shchepetov M, Lysenko ES, Weiser JN. Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. **Cell Microbiol** 2007; 9: 1343-1351. (PMID: 17474907)
9. Lysenko ES, Clarke TB, Shchepetov M, Ratner AJ, Roper DI, Dowson CG, Weiser JN. Nod1 signaling overcomes resistance of *S. pneumoniae* to opsonophagocytic killing. **PLoS Pathog** 2007; 3(8): e118. (PMID: 17722978)

10. Gelber SE, Aguilar JL, Lewis KL, Ratner AJ. Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. **J Bacteriol** 2008; 190(11): 3896-3903. (PMID: 18390664)
11. Randis TM, Kulkarni R, Aguilar JL, Ratner AJ. Antibody-based detection and inhibition of vaginolysin, the *Gardnerella vaginalis* cytolysin. **PLoS ONE** 2009; 4(4): e5207. (PMID: 19370149)
12. Parker D, Soong G, Planet P, Brower J, Ratner AJ, Prince A. The NanA neuraminidase of *Streptococcus pneumoniae* is involved in biofilm formation. **Infect Immun** 2009; 77: 3722-3730. (PMID: 19564377)
13. Lehrer RI, Jung G, Ruchala P, Wang W, Micewicz ED, Waring AJ, Gillespie EJ, Bradley KA, Ratner AJ, Rest RF, Lu W. Human α-defensins inhibit hemolysis mediated by cholesterol-dependent cytolysins. **Infect Immun** 2009; 77: 4028-4040. (PMID: 19581399)
14. Aguilar JL, Kulkarni R, Randis TM, Soman S, Kikuchi A, Yin Y, Ratner AJ. Phosphatase-dependent regulation of epithelial mitogen-activated protein kinase responses to toxin-induced membrane pores. **PLoS ONE** 2009; 4(11): e8076. (PMID: 19956644)
15. xxxxxxx

D. Research Support

xxxxxxx

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
David H. Figurski	Professor
eRA COMMONS USER NAME xxxxxxx	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
U. of Pittsburgh, Pittsburgh, PA	B.S.	1969	Molecular Biology & Biophysics
U. of Rochester, Rochester, NY	Ph.D.	1974	Microbiology
U. of California, San Diego, CA	Postdoctoral	1974-1978	Molecular Biology

A. Personal Statement

I believe the proposed research on *Gardnerella vaginalis* will be enhanced greatly by genetic analysis. My experience should be helpful. I have done research on broad-host-range plasmids for several years, and I regularly lecture medical, dental, and graduate students on the mechanisms of horizontal transfer in bacteria. Genetic approaches have been important to our own research. During the last 15 years, my laboratory has also been studying the human oral pathogen *Aggregatibacter actinomycetemcomitans*. Very little had been done genetically on this organism. We had to construct our own genetic tools, many of which have appeared in publications. We constructed a transposon, IS*903*ϕ*kan*, whose transposition can be induced and which is cryptic until transposition occurs. It was essential to our discovery of the *t*ight *ad*herence locus. This transposon may work in *G. vaginalis* as is. If not, we know several possible fixes that should make it work. Dr. Ratner and I have also discussed other genetic tools for *G. vaginalis.*

B. Positions and Honors

Positions and Employment

1978-1985 Asst. Professor, Dept. of Microbiology, College of Physicians & Surgeons, Columbia U.
1985-1992 Assoc. Professor, Dept. of Microbiology, College of Physicians & Surgeons, Columbia U.
1992-present Professor, Dept. of Microbiology & Immunology, Coll. of Physicians & Surgeons, Columbia U.
1997-2000 Assoc. Dean of Graduate Students, College of Physicians & Surgeons, Columbia U.

Awards and honors:

1964 NSF High School Student Science Fellowship, Dept. of Microbiology, Ohio University
1969-1974 NIH Predoctoral Trainee, University of Rochester
1974-1978 NIH Postdoctoral Fellowship, University of California at San Diego
1984 Lamport Award for Excellence in Basic Research, Columbia University
1985-1989 American Cancer Society Faculty Research Award
2002 Keynote speaker, Microbes and Molecules Conference, U. of Canterbury, Christchurch, NZ
2006 Bohmfalk Award for Excellence in Pre-Clinical Teaching, Columbia University
2006 Keynote speaker, Golden Anniversary Wind River Conference on Prokaryotic Biology, CO

Professional Service

Editor, *Plasmid*, 1989-2008
Member, Editorial Board, *Journal of Bacteriology*, 1985-1999
Member, ACS Advisory Committee on Microbiology and Virology, 1987-1990; Co-Chair, 1990
Member, Special NIH Review Groups, Microbial Physiology and Genetics, 1983, 1989, 1993, 1995, 1996; Bacteriology and Mycology, 1995
Member (*ad hoc*), NCI-Frederick Cancer Research & Development Center Advisory Committee, 1991

Member (*ad hoc*), NIH Microbial Physiology and Genetics Study Section, 1983
Grant Reviewer (*ad hoc*), NIH Microbial Genetics and Physiology Study Section; NIH Oral Biology and Medicine Study Section; NIH Bacteriology and Mycology Study Section; NSF Microbial Genetics; National Research Council COBASE program; US Army Research Office; ACS; The Wellcome Trust

C. Selected Publications (82 total, including 12 reviews; in chronological order)

Bechhofer, DH, Kornacki, JA, Firshein, W, and **Figurski, DH** (1986) Gene control in broad host range plasmid RK2: Expression, polypeptide product, and multiple regulatory functions of *korB*. Proc. Natl. Acad. Sci. USA 83:394-398.
Kornacki, JA, Balderes, PJ, and **Figurski, DH** (1987) Nucleotide sequence of *korB*, a replication control gene of broad host-range plasmid RK2. J. Mol. Biol. 198:211-222.
Goncharoff, P, Saadi, S, Chang, C-H, Saltman, LH, and **Figurski, DH** (1991) Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. J. Bacteriol. 173:3463-3477.
Saltman, LH, Kim, K-S, and **Figurski**, **DH** (1991) The *kilA* operon of promiscuous plasmid RK2: The use of a transducing phage (lp*klaA*-1) to determine the effects of the lethal *klaA* gene on *Escherichia coli* cells. Molec. Microbiol. 5:2673-2683.
Kornacki, JA, Chang, C-H, and **Figurski, DH** (1993) *kil-kor* regulon of promiscuous plasmid RK2: Structure, products, and regulation of two operons that constitute the *kilE* locus. J. Bacteriol. 175:5078-5090.
Ayres, EK, Thomson, VJ, Merino, G, Balderes, D, and **Figurski, DH** (1993) Precise deletions in large prokaryotic genomes by vector-mediated excision (VEX): The *trfA* gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. 230:174-185.
Thomson, VJ, Bhattacharjee, MK, Fine, DH, Derbyshire, KM, and **Figurski, DH** (1999) Direct selection of IS*903* transposon insertions by use of a broad host range vector: Isolation of catalase-deficient mutants of *Actinobacillus actinomycetemcomitans*. J. Bacteriol. 181:7298 7307.
Kachlany, SC, Planet, PJ, Bhattacharjee, MK, Kollia, E, DeSalle, R, Fine, DH, **Figurski, DH** (2000) Nonspecific adherence by *Actinobacillus actinomycetemcomitans* requires genes widespread in Bacteria and Archaea. J. Bacteriol. 182:6169-6176
Kachlany, SC, Planet, PJ, DeSalle, R, Fine, **DH, Figurski**, DH and Kaplan, JB (2001) *flp-1*, first representative of a new pilin gene subfamily, is required for nonspecific adherence of *Actinobacillus actinomycetemcomitans.* Molec. Microbiol. 40:542-554.
Bhattacharjee, MK, Kachlany, SC, Fine, DH, and **Figurski, DH** (2001) Nonspecific adherence and fibril biogenesis by *Actinobacillus actinomycetemcomitans*: TadA protein is an ATPase. J. Bact. 183:5927-5936.
Planet, PJ, Kachlany, SC, Fine, DH, DeSalle, R, and **Figurski, DH** (2003) The Widespread Colonization Island (WCI) of *Actinobacillus actinomycetemcomitans*. Nature Genetics 34:193-198.
Perez, BA, Planet, PJ, Kachlany, SC, Tomich, M, Fine, DH, and **Figurski, DH**. (2006) Genetic analysis of the requirement for *flp-2*, *tadV*, and *rcpB* in *Actinobacillus actinomycetemcomitans* J. Bacteriol. 188:6361-6375.
Tomich, M, Fine, DH, and **Figurski, DH.** (2006) The TadV protein of *Actinobacillus actinomycetemcomitans* is a novel aspartic acid prepilin peptidase required for maturation of the Flp1 pilin and TadE and TadF pseudopilins. J. Bacteriol. 188:6899-6914.
Bhattacharjee, MK, Fine, DH, and **Figurski, DH.** (2007) *tfoX* (*sxy*)-dependent transformation of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*. Gene 399:53-64.
Lim, YM, de Groof, AJC, Bhattacharjee, MK, **Figurski, DH**, and Schon, EA. (2008) Bacterial conjugation in the cytoplasm of mouse cells. Infect. Immun. 76:5110-5119.

D. Research Support

xxxxxxx

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:	Dr.
* First Name:	Adam
Middle Name:	Jonathan
* Last Name:	Ratner
Suffix:	

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:	
* First Name:	Lynette
Middle Name:	
* Last Name:	Arias
Suffix:	

* Phone Number:	(212) 305-4191
Fax Number:	(212) 342-9063
Email:	Grants-Office@columbia.edu

* Title:	Assoc. VP for Sponsored Projects Admin

* Street1:	1210 Amsterdam Avenue, Mail Code 2205
Street2:	Room 254 Engineering Terrace
* City:	New York
County/Parish:	New York
* State:	NY: New York
Province:	
* Country:	USA: UNITED STATES
* Zip / Postal Code:	10027

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ 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:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Tracking Number:GRANT10544965 Funding Opportunity Number:PA-10-067 Received Date:2010-03-01T17:05:50-04:00

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: 0925-0001

Budget Period: 1

Start Date: 12/01/2010 End Date: 11/30/2011

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Res. Federal On-Campus	61	250,000.00	152,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Robert Aaronson, (212) 264-2069

Indirect Cost Rate Agreement Date: 07/30/2009

Total Indirect Costs: 152,500.00

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

Funds Requested ($): 402,500.00

Budget Period: 2

Start Date: 12/01/2011 End Date: 11/30/2012

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Res. Federal On-Campus	61	250,000.00	152,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Robert Aaronson, (212) 264-2069

Indirect Cost Rate Agreement Date: 07/30/2009

Total Indirect Costs: 152,500.00

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

Funds Requested ($): 402,500.00

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Start Date: 12/01/2012 End Date: 11/30/2013

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Res. Federal On-Campus	61	250,000.00	152,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Robert Aaronson, (212) 264-2069

Indirect Cost Rate Agreement Date: 07/30/2009

Total Indirect Costs: 152,500.00

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

Funds Requested ($): 402,500.00

Budget Period: 4

Start Date: 12/01/2013 End Date: 11/30/2014

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Res. Federal On-Campus	61	250,000.00	152,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Robert Aaronson, (212) 264-2069

Indirect Cost Rate Agreement Date: 07/30/2009

Total Indirect Costs: 152,500.00

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

Funds Requested ($): 402,500.00

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Start Date: 12/01/2014 End Date: 11/30/2015

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Res. Federal On-Campus	61	250,000.00	152,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Robert Aaronson, (212) 264-2069

Indirect Cost Rate Agreement Date: 07/30/2009

Total Indirect Costs: 152,500.00

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

Funds Requested ($): 402,500.00

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$	1,250,000.00
Section A, Total Consortium F&A for Entire Project Period	$	0.00
*Section A, Total Direct Costs for Entire Project Period	$	1,250,000.00
*Section B, Total Indirect Costs for Entire Project Period	$	762,500.00
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$	2,012,500.00

2. Budget Justifications

Personnel Justification	1246-VLYR01A2budgetjustif.pdf	Add Attachment	Delete Attachment	View Attachment
Consortium Justification		Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

Tracking Number:GRANT10544965 Funding Opportunity Number:PA-10-067 Received Date:2010-03-01T17:05:50-04:00

PERSONNEL JUSTIFICATION

Adam Ratner, M.D., M.P.H. serves as P.I. and will devote 4.8 calendar months of effort to this project. In addition to performing hands-on experiments, he meets daily with members of the laboratory, reviews data, and prepares manuscripts for publication. He has extensive experience with bacterial and mammalian cell culture, microscopy (including live-cell fluorescent imaging), and models of infection. Dr. Ratner will oversee and provide hands-on assistance for all aspects of this project.

Jorge Aguilar, Research Technician (12.0 calendar months). Mr. Aguilar has worked with Dr. Ratner for more than 3 years and has experience with primary cell culture, murine models of infection, in vitro studies including ELISA, bacteriology (including quantitative culture from complex specimens), and microscopy. He assisted with the initial characterization of vaginolysin and is well positioned to perform these studies.

A **postdoctoral research scientist** (12.0 calendar months) will oversee the day-to-day aspects of the experiments for this project as well as organization and maintenance of experimental systems, data acquisition and initial analysis.

UNPAID EXTERNAL COLLABORATORS

David H. Figurski, Ph.D. will serve as a significant contributor to the project, especially with regard to bacterial genetics and transposon mutagenesis of *Gardnerella vaginalis*.

[redacted] **Ph.D.** will serve as a significant contributor to the project, providing defined hCD59 transgenic mice, expertise in complement regulatory receptors, and in vivo models.

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	1240-VLYR01A2introduction.p	Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	1241-VLYR01A2-aims.pdf	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	1242-VLYR01A2-researchstrat	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals	1247-VLYR01A2-vertebrate.pd	Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research	1248-VLYR01A2-selectagents.p	Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	1249-supportletters.pdf	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)	1250-VLYR01A2sharing.pdf	Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments | Remove Attachments | View Attachments

INTRODUCTION TO RESUBMISSION APPLICATION

This is the second (A2) resubmission of application R01 HD061371-01, "*Gardnerella vaginalis*: toxin production and pathogenesis," which was reviewed in February 2009 and then in June 2009 at the HIBP study section. The initial submission received a 35.5 percentile (priority score 203) and the A1 resubmission a 15 percentile (impact/priority score 28.) Under FY10 paylines, it was not funded by NICHD or NIAID. As a new investigator, I am grateful for the opportunity to present this revised application. I have made every effort to address the critique thoroughly, and I believe that the proposed studies have emerged considerably stronger and more focused on relevant aspects of pathogenesis. This resubmission has undergone very substantial revision, both in response to the reviewers' comments and in order to meet the new page limit guidelines for R01 applications. For that reason, changes are not marked in the text.

My impression from the summary statement for the A1 application was that the reviewers found the subject matter of interest, and there was substantial enthusiasm for studies of pathogenic mechanisms of *G. vaginalis* focused on the new human-specific cytolysin (vaginolysin, VLY) and its receptor, human CD59. The summary of discussion described the strengths of the application as "*the expertise and productivity of the investigator in the field, the supportive preliminary data ensuring feasibility, the innovative approach, the adequate response to previous critiques, and the significance of this understated female problem.*" The weaknesses identified were "*the ambitious nature of the project, the lack of a transgenic model, and the relevance to humans.*" However, the proposed research was felt to be *"potentially very important with a high probability of it being successful.*"

The major concern of the reviewers surrounded the hCD59-transgenic murine lines for the in vivo studies in Aim 2. I have approached this problem in three ways. First, I provide data demonstrating that we have generated C57BL/6 lines with hCD59 under the control of the ubiquitously expressed EF-1α promoter. Second, we have initiated a collaboration with [redacted] is an expert in the biology of CD59 and other complement regulatory molecules, and he has created and characterized several hCD59-transgenic murine lines. He has generously agreed to share his expertise as well as these mice with us for our studies. Third, we provide new data in this resubmission that even if the hCD59-transgenic colonization studies are unsuccessful we will still be able to study the role of the toxin/host interaction in the initiation and maintenance of *G. vaginalis* colonization. We have developed a new model of murine *G. vaginalis* vaginal colonization using coinoculation of a non-species-specific cholesterol-dependent cytolysin (PLY), which unlike VLY does not require hCD59 for its activity. Codelivery of this toxin with *G. vaginalis* enhances colonization, allowing high-level murine colonization with *G. vaginalis* for the first time. This both lends credence to our hypothesis that toxin function is crucial to establishment of colonization and provides a novel platform with which we can test various knockout strains and potential inhibitors of colonization in case there are unforeseen technical issues with the hCD59 transgenic approach.

We have addressed the other concerns of the reviewers as well. We provide additional evidence of our ability to manipulate *G. vaginalis*, including the construction of a GFP-expressing strain and streptomycin-resistant mutants useful in vivo. We include plans for correcting for growth defects in mutant strains, testing biofilm mutants both for changes in VLY production and in the in vivo models, addressing LPS contamination more rigorously, and preparing targeted mutations of the most interesting genetic loci as suggested in Critique 1. We also describe a more detailed approach to our analysis plan for the bleb studies and to ensuring that candidate inhibitors do not sensitize human cells to complement attack, as suggested in Critique 2. I believe that this research plan will help us to understand the pathogenesis of bacterial vaginosis and will lead to new therapeutic strategies for this important and difficult to treat disease. Thank you for your consideration.

Gardnerella vaginalis: toxin production and pathogenesis

SPECIFIC AIMS

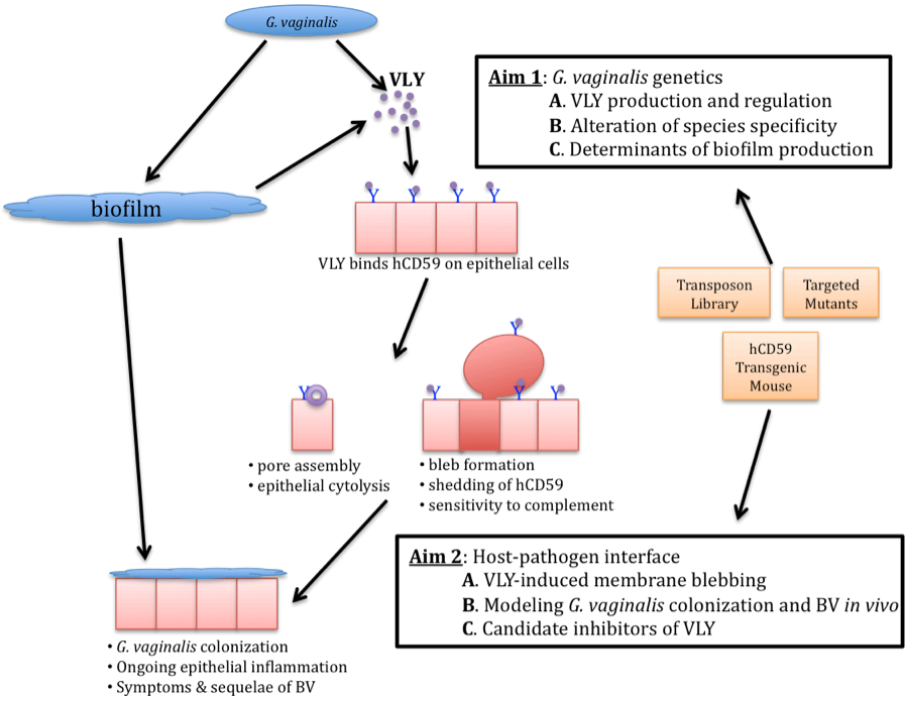
Our overall goal is to define the role of vaginolysin (VLY), a novel, human-specific toxin produced by *Gardnerella vaginalis*, in the pathogenesis of bacterial vaginosis (BV). We propose the following research plan directed at elucidating both genetic mechanisms of control of VLY production by *G. vaginalis* and the specific role of VLY in the interaction of *G. vaginalis* with host cells. Using techniques developed in our laboratory, we will perform the first genetic screens in *G. vaginalis*, define genes required for toxin production and other virulence properties, and construct defined mutants of *G. vaginalis* that express a validated, non-species-selective VLY toxin chimera (**Aim 1**). We will also determine the role of VLY at the host-pathogen interface by characterizing VLY-specific responses of vaginal epithelial cells, including bleb formation and increased host cell sensitivity to complement. Both of these responses are unique to VLY and its interaction with the hCD59 receptor, and we hypothesize that they are important to the pathogenesis of BV (**Aim 2A**). Finally, we will take advantage of our knowledge of the VLY-hCD59 interaction to develop novel in vivo models of *G. vaginalis* pathogenesis. Using defined mouse lines transgenic for hCD59 as well as VLY mutants that lack species selectivity, we will manipulate both host and pathogen to perform a detailed analysis of the role of VLY in vivo (**Aims 2B-C**). In addition to vastly expanding our knowledge of *G. vaginalis* pathogenesis and of VLY, this research program will address the potential to manipulate the VLY-hCD59 interaction in order to develop therapeutic strategies and in vivo models for bacterial vaginosis.

<u>Aim 1</u>: Define determinants of *Gardnerella vaginalis* virulence using new genetic techniques.

A. Determine genes required for production and regulation of VLY.
B. Construct and evaluate specific *G. vaginalis* strains with altered species specificity.
C. Determine genes required for biofilm formation in *G. vaginalis*.

<u>Aim 2</u>: Determine the role of VLY in *G. vaginalis* at the host-pathogen interface in vitro and in vivo.

A. Determine the role of VLY-induced membrane blebbing as a mechanism for protection of vaginal epithelial cells from toxin pores and as a pathway sensitizing cells to complement.
B. Define the role of the VLY-hCD59 interaction in *G. vaginalis* pathogenesis in vivo.
C. Evaluate candidate inhibitors of the VLY-hCD59 interaction in vivo.



RESEARCH STRATEGY

SIGNIFICANCE

Bacterial vaginosis, sexually transmitted infections, and preterm birth Bacterial vaginosis (BV) is an exceedingly common and poorly understood disorder associated with significant adverse sequelae. Nationwide point-prevalence estimates of BV among reproductive aged women are roughly 30%, corresponding to 21 million women with BV [2]. Rates are higher in pregnant women and in African-American populations [2]. Although the symptoms associated with BV are not life threatening, BV substantially increases the risk of a number of significant health outcomes. Chief among these is preterm birth (PTB). The nationwide rate of PTB (parturition at less than 37 weeks gestation) is 12.7% (March of Dimes Peristats). BV causes 90,000 excess preterm births per year (at an overall cost in excess of $1 billion) and accounts for at least 30% of the racial difference in PTB rates [3]. In pregnancy, women with BV are at increased risk for chorioamnionitis, post-operative wound infections, and post-partum endometritis. BV increases both acquisition and shedding of a number of sexually transmitted infections, including HIV [4]. BV increases the risk of heterosexual acquisition of HIV at least 2-fold [5,6], and exposure of HIV-infected cells to vaginal secretions from women with BV [7] or to pure cultures of *Gardnerella vaginalis* [8,9] increases production of HIV transcripts and viral shedding. Synergistic interactions between BV and other sexually transmitted infections including herpes simplex, gonorrhea, and chlamydia have been detailed [10,11].

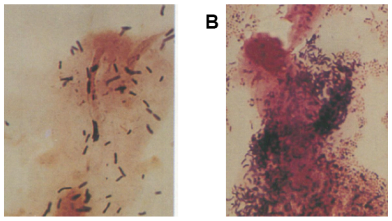


Fig. 1. Changes in vaginal microflora during bacterial vaginosis. (A) Normal vaginal microflora is dominated by *Lactobacillus* species (gram-positive rods). (**B**) During bacterial vaginosis, there is an abundance of gram-variable coccobacilli adherent to vaginal epithelial cells (clue cells). Figure adapted from [1].

Given its public health importance, it is striking that the pathogenesis of BV is not well understood. BV is a pathological state characterized by loss of the normal *Lactobacillus*-dominated vaginal microflora and overgrowth of other species, especially *Gardnerella vaginalis* (**Fig. 1**). The disturbance to the vaginal microenvironment during BV is complex, involving alterations in pH, deficiency of components of host immunity [12], and a marked expansion of microbial diversity [13]. BV can be exceedingly difficult to eradicate, even with targeted antimicrobial therapy, and relapse rates are high, often exceeding 50% [14]. Thus, the pathogenesis of BV represents a substantial hole in public health-focused microbiological research. New approaches to prevention and treatment of BV are urgently needed. The focus of this proposal is on a novel approach to the pathogenesis of BV.

Gardnerella vaginalis*: an enigmatic bacterial species with a controversial history* In the 1950s, Leopold [15] and then Gardner and Dukes [16] noted the presence of small, pleomorphic gram-variable organisms in the genital tracts of women with "non-specific vaginitis," an early name for BV. Believing it to be a Gram-negative, they named the species *Haemophilus vaginalis* (I will continue to refer to it by its current name, *Gardnerella vaginalis* here.) In an elegant series of papers, Gardner, Dukes, and others cultivated this fastidious organism, demonstrated that it was present in essentially all women with BV, and established a causal link between the organism and the clinical syndrome [16]. The last of these goals was the most problematic. Using human volunteers, they showed that vaginal washes from women with BV were sufficient to cause BV in unaffected women [16]. Likewise, using pure cultures of *G. vaginalis*, Criswell et al. showed that the organism itself could cause BV in healthy women, albeit only in a minority of those tested [17]. From these early studies, it seemed that a causal link between *G. vaginalis* and BV had been established. Subsequent bacteriological characterization revealed that *G. vaginalis* is not a member of the *Haemophilus* genus and is, in fact, a gram-positive bacterium, lacking endotoxin and having a Gram-positive cell wall structure [18]. In addition, the failure to establish a model of BV by introducing cultures of *G. vaginalis* into laboratory animals dealt a blow to the notion that the *G. vaginalis*-BV link had fulfilled Koch's postulates. A variety of experimental models have been tried without success, including mice, rabbits, mares, and primates [19].

Why has it been so difficult to establish an animal model of this important disease? One hypothesis is that the cause of BV is not *G. vaginalis* alone — rather, that a confluence of specific microbes and host factors come together to cause the syndrome of BV. Indeed, this polymicrobial hypothesis is logical for a number of reasons: a number of women in the Criswell trials did not develop BV despite intravaginal inoculation of *G. vaginalis*, the diversity of microorganisms in BV is high and many species (both cultivatable and uncultivatable) are potential causative factors, and *G. vaginalis* can, on occasion, be found in the genital tracts of women without other signs of BV [20]. However, because introduction of pure cultures of *G. vaginalis* can cause BV in

some women and because *G. vaginalis* is, even in the age of molecular testing for uncultivatable bacterial species, the *sine qua non* of BV [21,22], we hypothesize that a major factor in the failure to develop an animal model of BV is that the primary virulence determinant of *G. vaginalis* is specific to human cells. *G. vaginalis* produces a number of potential virulence factors, including sialidase, prolidase, and a protein toxin [19].

Cholesterol-dependent cytolysins: a widespread family of bacterial toxins with major roles in pathogenesis The cholesterol-dependent cytolysin (CDC) family of protein toxins has more than 20 distinct toxins from at least six gram-positive genera. The properties of this toxin family have been reviewed [23]. CDCs are secreted as monomers and bind to cholesterol-containing host membranes through a mechanism dependent on a conserved 11 aa region (the undecapeptide) and on short loops present in the structure of domain 4 [24,25]. After binding, the CDCs oligomerize into a pre-pore conformation, and subsequently undergo a striking rearrangement by which a previously α-helical portion of the toxin becomes a β-barrel and inserts into the membrane, forming a pore [26,27,28,29].

The CDCs play a major role in the pathogenesis of their cognate organisms. These toxins are produced by major human pathogens, including *Streptococcus pneumoniae*, *S. pyogenes*, *S. intermedius*, *Listeria monocytogenes*, *Clostridium perfringens*, and *Bacillus anthracis*. Isogenic bacterial strains lacking the CDC gene have been studied in vivo and support a major role in pathogenesis. Bacteria seem to utilize CDCs for a variety of purposes. Listeriolysin O (LLO) from *L. monocytogenes* aids in escape of *Listeria* from the vacuole following activation by a host factor [30]. Streptolysin O (SLO) from *S. pyogenes* lyses target cells but also functions as a protein secretion system, delivering streptococcal NAD+ glycohydrolase to the host cytosol [31]. Pneumolysin (PLY) is essential to the pathogenesis of pneumococcal disease [32] and has several functions — pore formation, complement binding, and modulation of the host immune system in models of interbacterial competition [33,34]. Because host membrane cholesterol is necessary for CDC function and because preincubation of CDCs with cholesterol inhibits pore formation, it was hypothesized that cholesterol acts as the CDC receptor [35]. While cholesterol is important to CDC activity, the situation is more complicated than that. Intermedilysin (ILY), the CDC from *Streptococcus intermedius*, exhibits species specificity inconsistent with the use of cholesterol as a sole receptor [36]. The activity of ILY on human cells but not those of closely related species, including primates, prompted a search for an additional receptor. The mechanism of ILY species restriction depends on the ability of ILY to bind human CD59 (hCD59), a complement-regulatory molecule expressed on all human cells [37].

***Vaginolysin: a novel, human-specific, CD59-dependent toxin* from G. vaginalis** We recently characterized vaginolysin (VLY), a human-specific, CD59-dependent CDC from *G. vaginalis* [38]. Hemolysin production from *G. vaginalis* has been described, and some reports have demonstrated an IgA-mediated immune response to a *Gardnerella* hemolysin during BV [39,40]. However, until our studies (described below) detailed genetic and functional information were lacking. Among the CDCs, VLY is most closely related to ILY from *S. intermedius*, despite the distant phylogenetic relationship between these species. This relationship suggests a possible lateral transfer event as the mechanism of acquisition of VLY by an ancestor of *Gardnerella* [41]. We have initiated detailed investigations of VLY and its contribution to *G. vaginalis* virulence. As the second species-specific CDC, VLY provides an opportunity to expand our knowledge about this important group of toxins. In addition, as a human-specific virulence factor, VLY may afford a more detailed and nuanced understanding of the pathogenesis of BV and its adverse sequelae. The role of VLY in *G. vaginalis* pathogenesis and BV is the main focus of this proposal.

Host immunity, cytokines, and pathogenesis of bacterial vaginosis Immune responses are of paramount importance in BV, and dysregulation of cytokine production in response to microbial challenge is thought to be a major driving force in BV-associated pathogenic mechanisms. In particular, an abundance of mature interleukin (IL)-1β is thought to play a role in the sustained immune activation that mediates adverse sequelae of BV [42,43,44]. Loss of epithelial integrity as a result of chronic inflammation may also be important in BV-related increases in susceptibility to HIV and other sexually transmitted infections. Several genetic association studies have addressed risk factors for BV, and specific polymorphisms in cytokine genes (IL-1β and IL-8) have been associated with increased risk of BV [39,40]. These data demonstrate the importance of local host immunity in the pathogenesis of BV. Our data demonstrate a link between VLY and IL-1β production, consistent with VLY acting as an agent of epithelial stimulation at the vaginal mucosal surface.

Human CD59: more than a toxin receptor It is striking that a bacterial toxin would use hCD59 as its receptor. hCD59 is a small, glycophosphatidylinositol (GPI) anchored protein that is ubiquitously expressed on human cells. It functions as a complement restriction factor, binding directly to terminal complement components C8 and C9, thus preventing membrane attack complex (MAC) deposition on the surface of human cells (**Fig. 2**) [45,46]. Deficiency of hCD59, either as a component of paroxysmal nocturnal hemoglobinuria or as an autosomal recessive hCD59-specific deficiency, leads to complement-mediated lysis of erythrocytes [47,48,49]. hCD59 has remarkable species specificity; it restricts human complement components but not those of other species, even other primates [50]. Unexpectedly, ILY and VLY appear to have co-opted this selective mechanism and have, as a result, drastically narrowed their host range compared to related CDCs. This is of particular interest because it has recently been proposed that CDC toxins, complement components, and other pore-forming molecules may be part of a large superfamily with shared structural motifs [51,52]. In addition to acting as a complement restriction factor, hCD59 localizes to lipid rafts and has signaling capacity [53]. Ligation of hCD59 by antibodies induces calcium signaling and tyrosine phosphorylation of src family kinases [54,55,56], and this has been hypothesized to play a role in initiation and maintenance of immune responses. Based on our preliminary studies, we will test the hypothesis that, in addition to using hCD59 as a receptor to initiate pore-formation, VLY induces signaling through hCD59 that mediates proinflammatory effects and ultrastructural changes in epithelial cells. In addition, we propose that by sequestering hCD59 or causing its removal on membrane blebs, VLY removes a major protective mechanism, leaving vaginal epithelial cells vulnerable to attack from human complement components.

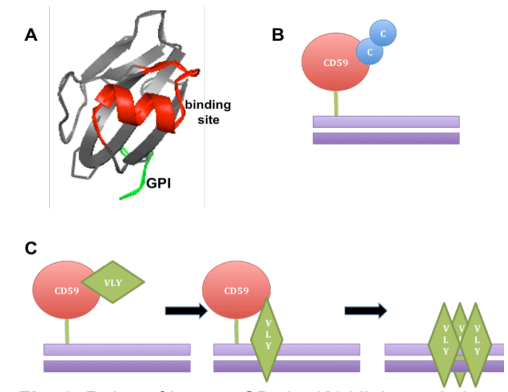


Fig. 2. Roles of human CD59. (**A**) High resolution crystal structure of hCD59 showing GPI anchor (green) and complement/VLY binding site (red). (**B**) hCD59 (red) binds terminal complement components (blue, C8 and C9), preventing assembly of membrane attack complex on target cells. (**C**) VLY (green) binds hCD59 prior to interaction with membrane cholesterol and oligomerization/pore formation.

***Broadening the host range of* G. vaginalis** We will use our knowledge of the VLY-hCD59 interaction to characterize new in vivo models of *G. vaginalis* pathogenesis and BV. We have produced mice transgenic for the hCD59 receptor and constructed a chimeric toxin consisting of the first three domains of VLY fused to the fourth domain of pneumolysin, a CDC that does not exhibit species selectivity. We have shown that co-delivery of a non-selective toxin enhances *G. vaginalis* colonization. By altering both bacteria and host, we will produce murine models that can be colonized by a variety of *G. vaginalis* isolates and will construct *G. vaginalis* strains that can colonize a wide range of mouse strains. **These two strategies will address the major barrier to understanding the pathogenesis of BV, the absence of an animal model.**

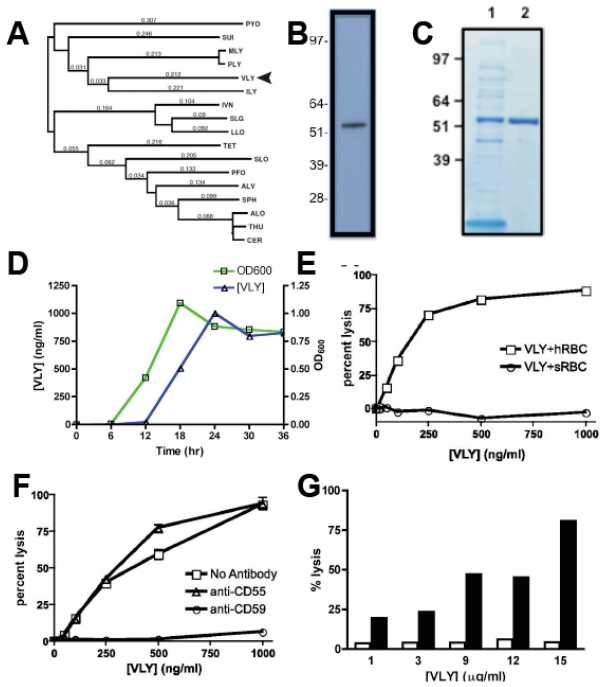


Fig. 3. **VLY is a human-specific, hCD59-dependent toxin**. (**A**) Neighbor-joining tree of known CDC sequences showing that VLY is most closely related to ILY. (**B**) VLY is detected as a single band in Western blot of *G. vaginalis* lysate. (**C**) Affinity purification of codon-optimized VLY from *E. coli*. (**D**) Production of VLY as measured by sandwich ELISA during growth of *G. vaginalis*. (**E**) VLY lyses human (hRBC) but not sheep (sRBC) erythrocytes. (**F**) Antibody to hCD59 but not hCD55 inhibits VLY-mediated hemolysis. (**G**) VLY lyses CHO cells transfected with hCD59 (black bars) but not control (vector alone) CHO cells (white bars). Figure adapted from [38,57] and unpublished data.

INNOVATION

Our characterization of a human-specific toxin from *G. vaginalis* and its receptor on the surface of human cells represents an opportunity to overcome the narrow host range of this bacterial species. By genetically manipulating both microbe and host, we will create new animal models for BV and understand the specific role of *G. vaginalis* in its pathogenesis. We have used innovative methods to generate the first descriptions of genetic manipulation of *G. vaginalis*, chimeric toxins with altered species specificity, and the first successful model of murine colonization with this organism. Our planned approach will use these tools

to will efficiently generate valuable information about the pathogenesis of this poorly understood condition.

APPROACH

PRELIMINARY STUDIES

Identification and characterization of vaginolysin (VLY) We identified a *G. vaginalis* open reading frame with 54% sequence identity to pneumolysin. The predicted primary amino acid sequence demonstrated significant similarity to known CDCs. Biochemical and functional studies indicated that VLY protein is produced by growing *G. vaginalis* and that it is a species-specific, cholesterol-dependent toxin that requires human CD59 (hCD59) for activity (**Fig. 3**) [38]. We have tested a variety of *G. vaginalis* strains, including both long-term laboratory cultures and freshly collected clinical specimens, and all produce VLY [57].

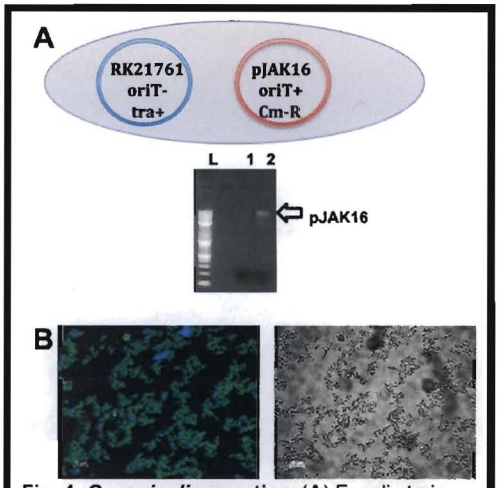


Fig. 4. *G. vaginalis* genetics. (**A**) E. coli strain ARE112 has the RK21761 helper plasmid and pJAK16 for mobilization. Inset gel shows alkaline lysis preparations from *G. vaginalis* without (lane 1) and with (lane 2) conjugation to *E. coli.* (**B**) *G. vaginalis* ARG25 expresses GFP from an inducible promoter (right, GFP; left, phase). Scale bar 10 μm.

***Genetic manipulation of* G. vaginalis**
One limitation to working with *G. vaginalis* is the lack of available genetic systems. No reports describing genetic engineering or even plasmid replication in this organism are available. However, the ability to manipulate *G. vaginalis* will be essential to the long-term progress of studies of the pathogenesis of BV. In collaboration with Dr. David Figurski, we have successfully transferred IncQ family plasmids into multiple *G. vaginalis* strains by conjugation with an *E. coli* strain carrying a "helper plasmid," which is an RK2 derivative encoding the necessary machinery for plasmid transfer (**Fig. 4**). The pJAK16 plasmid confers chloramphenicol resistance to *G. vaginalis*, allowing for selection of plasmid-containing colonies, and contains an inducible promoter as well as a multiple cloning site for insertion of heterologous sequences. We have recently used this strategy to create strains of *G. vaginalis* expressing GFP (**Fig. 4**). This discovery also permits transposon-mediated mutagenesis of *G. vaginalis* using the pJAK derivative, pVJT128, which encodes an inducible IS903 transposase and the IS903ϕ*kan* transposon. We have mobilized this plasmid into *G. vaginalis* successfully as well. These studies are the first report of genetic manipulation of *G. vaginalis* and open up substantial new possibilities for understanding its pathogenesis.

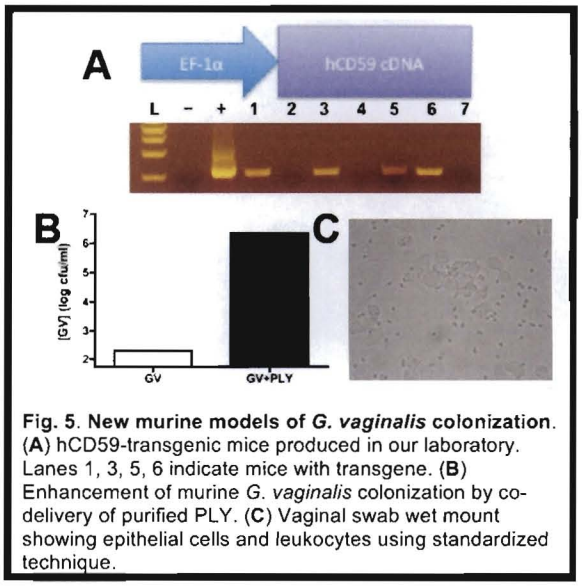


Fig. 5. New murine models of *G. vaginalis* colonization. (**A**) hCD59-transgenic mice produced in our laboratory. Lanes 1, 3, 5, 6 indicate mice with transgene. (**B**) Enhancement of murine *G. vaginalis* colonization by co-delivery of purified PLY. (**C**) Vaginal swab wet mount showing epithelial cells and leukocytes using standardized technique.

New murine models of* G.vaginalis *colonization
Because our central hypothesis is that VLY species specificity is a major impediment to the study of bacterial vaginosis, we have engineered a mouse expressing human CD59 under the control of a ubiquitous promoter. This strategy (**Fig. 5**) has resulted in multiple distinct murine lines with germline transmission of the hCD59 gene and expression in screening studies. The transgene has been bred to the C57/BL6 background for several generations. The mice have no overt phenotype, and we do not expect to find such phenotypes even on detailed examination as CD59-deficient mice are viable and have only moderate hemolytic phenotypes [58]. In addition, other groups have produced both global and tissue-specific hCD59 transgenic mice either for transplantation studies or for targeted cell ablation with ILY [59,60]. Through our collaboration with [redacted] we now have multiple well-characterized hCD59 transgenic lines to use as adjuncts to our generated ones. Detailed characterization of the hCD59-transgenic mice is ongoing in our laboratory, and this model represents one of our two approaches to the generation of a robust, reproducible animal model of BV and its associated sequelae.

In order ensure the feasibility of these studies, we have used ARG20, a streptomycin-resistant *G. vaginalis* strain generated in our lab, to colonize the lower genital tract of C57BL/6 mice in the presence or absence of purified PLY, a non-species-specific toxin. Coadministration of PLY substantially increased the density of *G.*

vaginalis as assessed by serial dilution and quantitative cultures (on streptomycin-containing HBT agar) of vaginal swabs collected in a standardized fashion (**Fig. 5**). This strategy appears to represent a marked improvement over prior animal models for *G. vaginalis* colonization, and will allow us to evaluate factors important in the establishment and maintenance of colonization. In addition, we have confirmed that we can isolate murine vaginal epithelial cells with a standardized swab technique (**Fig. 5**) in order to determine the current stage of estrus and to evaluate the presence of clue cells and other signs of BV.

VLY domain 4 binds hCD59 and mediates species selectivity
Mucosal epithelial cells sense bacterial pore-forming toxins and can initiate immune signaling while bacterial densities remain low [61]. Purified VLY recapitulates the proinflammatory effects of whole *G. vaginalis* on epithelial cells (**Fig. 6**), raising the possibility that the VLY:hCD59 interaction might be an important step in pathogenesis. The hCD59 receptor is expressed on the surface of human genital tract epithelial cells, the target cell type during *G. vaginalis* colonization and BV [62]. We hypothesized that VLYD4 was required for hCD59 binding. Using overlap-extension PCR, we generated a toxin chimera, containing domains 1-3 of VLY and domain 4 of PLY, a non-species-selective CDC. Unlike the parent VLY, the VLY:PLYD4 chimera lysed human and non-human cells with equal efficacy (**Fig. 6**). This result indicates that D4 plays a major role in species-selectivity among the CDCs.

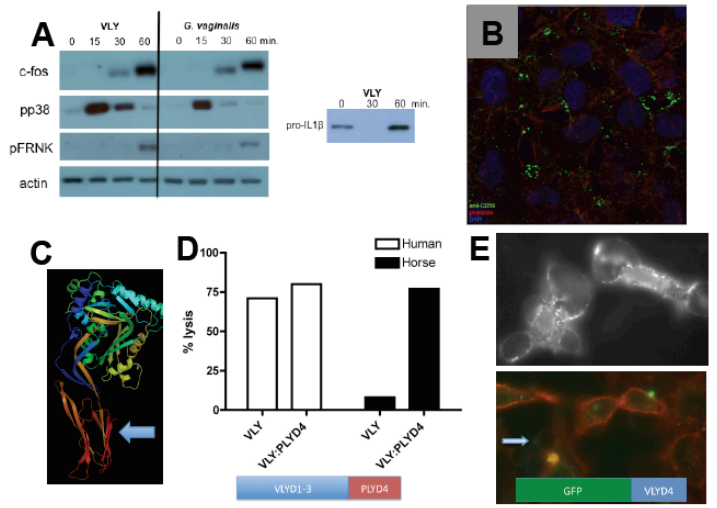


Fig. 6. **VLYD4 and host cell responses**. (**A**) Purified VLY and whole *G. vaginalis* induce expression of c-fos and phosphorylation of p38 MAPK and FAK-related non-kinase (FRNK). VLY induces maturation of IL-1β. (**B**) Human cervical epithelial cells express surface hCD59 (green). Nuclei (blue) and actin (red) shown for cellular architecture. (**C**) Homology model of VLY structure with D4 indicated. (**D**) The VLY:PLYD4 chimera lyses human and equine erythrocytes, while native VLY is human-specific. (**E**) Exposure of human cervical epithelial cells to VLY (upper) or GFP:VLYD4 (lower) induces rapid bleb formation. Co-localization of GFP:VLYD4 and bleb structure indicated at arrow. Membranes stained with wheat-germ agglutinin-Alexa Fluor 594 (red).

hCD59 signaling initiates plasma membrane blebbing In order to generate a probe for studies of toxin-hCD59 interactions, we created a GFP:VLYD4 fusion protein. This protein binds to hCD59-expressing human epithelial cells but does not form pores and is a valuable tool for studies of hCD59-specific responses. Ligation of epithelial hCD59 by native VLY, hCD59 antibody, or GFP:VLYD4 fusion protein rapidly induces membrane blebbing (**Fig. 6**). Blebs remain intact and present over the course of hours prior to resolution. We hypothesize that this observation reflects a unique response of host cells to hCD59-dependent signaling pathways. Bleb formation represents a potential means for removal of toxin from the surface of epithelial cells and is a previously uncharacterized host response to bacterial toxins. Because bleb formation may also lead to removal of hCD59 from the cell surface, we will test the hypothesis that this mechanism sensitizes epithelial cells to lysis from autologous complement.

VLY antiserum and toxoids inhibit cytolysis In light of our findings of the importance of VLY to *G. vaginalis*-mediated cytotoxicity and host responses, we explored methods to inhibit the VLY:hCD59 interaction. Rabbit polyclonal antiserum against VLY efficiently protects human erythrocytes from VLY-mediated lysis (**Fig. 7**). We also generated a genetic toxoid of VLY, VLY(V471R/K473C/P480W) by repeated rounds of site-directed mutagenesis. This toxoid, which has CDC consensus residues restored for 3 amino acids in D4, does not cause lysis of human cells. However, it can interfere with ILY-mediated lysis of human erythrocytes (**Fig. 7**). Interference may occur by binding to (and occupying) the hCD59 receptor or by forming non-functional hetero-oligomers with ILY. We will investigate both antibody and toxoids as VLY inhibitors in Aim 2.

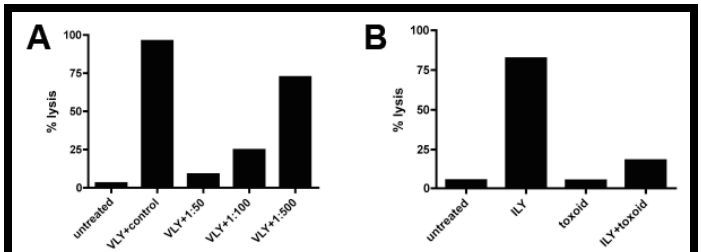


Fig. 7. **Inhibition of the VLY:hCD59 interaction**. (**A**) Polyclonal anti-VLY antiserum but not control serum inhibits VLY-mediated cytolysis in a dose-dependent manner. (**B**) VLY toxoid inhibits ILY-mediated lysis of human erythrocytes.

***Biofilm formation by* G. vaginalis** While the focus of this application is on the role of VLY in the pathogenesis of *G. vaginalis*, other virulence factors may also play a significant role. For example, *G. vaginalis* is known to form adherent biofilms in vivo [63]. Recent data suggest that even following targeted antimicrobial treatment, such biofilms can persist and likely contribute to the high recurrence rate of BV following treatment [64]. These effects can be modeled in vitro, as *G. vaginalis* forms biofilms on polystyrene, allowing quantification using a safranin-based assay (**Fig. 8**). In addition, fluorescent live-cell imaging of *G. vaginalis* grown in a glass bottomed chamber reveals the formation of complex, multicellular collections of *G. vaginalis*, consistent with biofilm formation (**Fig. 8**). We will screen the library of transposon-insertion mutants generated in Aim 1A for mutants defective in biofilm formation. Based on findings in other systems, we predict a role for *G. vaginalis luxS* in this phenotype as well. Our approach is also amenable to determination of genes encoding other putative virulence factors of *G. vaginalis* (e.g. adhesins, sialidase and prolidase).

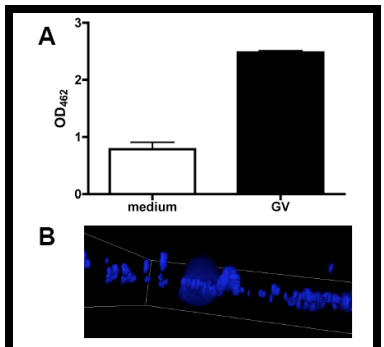


Fig. 8. ***G. vaginalis* biofilms.** (**A**) *G. vaginalis* forms biofilms on polystyrene plates as evaluated by the safranin assay. (**B**) Live cell imaging of *G. vaginalis* biofilms in glass-bottomed dishes. *G. vaginalis* communities were multicellular and spanned >20 μm in height.

Aim 1: **Define determinants of *Gardnerella vaginalis* virulence using new genetic techniques.**

Aim 1A: Determine genes required for production and regulation of VLY.

Experimental Design Our hypothesis is that the interaction between VLY and hCD59 is crucial to the host range and epithelial response to *G. vaginalis*. By genetically manipulating *G. vaginalis* we may be able to alter epithelial detection and host range, setting the stage for a novel in vivo model of bacterial vaginosis.

Creation of transposon mutant library We will generate a library of transposon mutants of *G. vaginalis* using the pJAK16-derivative plasmid pVJT128. This construct has been used to mutagenize otherwise refractory bacterial species and was created by our collaborator, Dr. David Figurski [65]. *G. vaginalis* strains carrying the plasmid will be selected on HBT agar supplemented with chloramphenicol. Plasmid transconjugants will be grown in selective broth, followed by induction of the transposase with IPTG. This treatment mobilizes the IS903ϕ*kan* transposon, which inserts randomly into the *G. vaginalis* genome. Genome-wide insertion will be confirmed by Southern blot, as described [65]. Because the cryptic *kan* gene on the transposon is only expressed when inserted into a recipient gene that is being expressed (and not from the plasmid itself), the library will be enriched for insertions in coding regions, as described [65]. Candidate mutants will be selected on kanamycin and screened for inactivation of the *vly* gene. As described below, we will also prepare *vly* mutants by targeted disruption. However, the transposon library will provide an unbiased screen for regulators of VLY production and other potential virulence determinants.

Creation of targeted mutations In addition to generating a VLY-deficient *G. vaginalis* strain using transposon mutagenesis, we will perform targeted disruption of the *vly* gene by homologous recombination. A targeting construct consisting of the first 500 bp of the *vly* gene followed by an antibiotic resistance cassette (kanamycin resistance), followed by the terminal 500 bp of the *vly* gene will be cloned into the polylinker of pJAK16, which we have demonstrated can be mobilized into *G. vaginalis*. We will mobilize this plasmid into *G. vaginalis* by conjugation, selecting initially for plasmid-encoded resistance (chloramphenicol), and subsequently for resistance encoded by the insert (kanamycin). Kanamycin-resistant, chloramphenicol-sensitive recombinants should have a disruption of the *vly* gene and have been cured of plasmid — these colonies will be screened for VLY-deficiency as above. We will employ a similar strategy to generate a *luxS* deficient mutant (and complemented strain) to explore its role in VLY regulation as well.

Phenotypic analysis of mutants We will screen for transposon insertion into the *vly* gene in several ways. Qualitatively, we will look for *G. vaginalis* colonies that are non-hemolytic on human blood agar. The hemolytic phenotype of *G. vaginalis* is human-specific and is likely the result of VLY production. Supernatants from overnight growth of individual transformants in 96-well plates will be assayed in at least triplicate for VLY production by ELISA and compared statistically (using ANOVA with appropriate post-tests to compare individual mutants to the wild-type — this will allow correction for multiple comparisons and will decrease false positives). Finally, colonies will be screened by PCR for the *vly* gene, as transposon insertion should lead to an additional ~900 bp of sequence. We anticipate screening ~4,000 colonies, depending on transposition efficiency. Similar numbers of colonies were screened in a pVJT128 library of *A. actinomycetemcomitans* and

generated multiple hits in the catalase gene [65]. If non-hemolytic colonies (or strains that produce either more or less VLY than the wild-type are encountered on the ELISA assays) have insertion sites outside the *vly* locus, the insertion site will be determined by inverse PCR as described [65] using available draft *G. vaginalis* genome sequences. Importantly, this library of insertion mutants will represent a valuable resource for the study of *G. vaginalis* pathogenesis and will be used to screen for other factors linked to virulence.

Genetic complementation A VLY-deficient strain of *G. vaginalis*, cured of plasmid but retaining the transposon insertion, will act as a host for introduction of the pJAK16 plasmid carrying either wild-type or mutant *vly* genes. Genetic complementation studies will establish that phenotypes seen with the VLY-deficient strain are truly the result of lack of VLY. Introducing mutant *vly* sequences will allow dissection of the importance of various VLY functions (hCD59 binding, pore-formation) in vivo. Finally, we propose that a VLY-deficient *G. vaginalis* expressing the VLY:PLYD4 chimeric toxin, which is species non-selective, would represent the first step toward a non-human model for the study of BV (described further in Aims 1B and 2C). We hypothesize that the lack of species selectivity of this chimera might allow for colonization of the murine genital tract — these studies represent the logical extension of those proposed here and are supported by our preliminary data showing enhancement of *G. vaginalis* colonization of the murine vagina in the setting of PLY co-inoculation. Importantly, we hypothesize that genes other than the *vly* gene may regulate toxin production either during growth or in response to environmental conditions. As described above, *luxS* represents such a candidate — one that we believe may be non-essential and may play a role in the density-dependent regulation of VLY production. The impact of specific genes (including VLY and regulatory genes such as *luxS*) will be confirmed via genetic complementation, with correction for any differences in growth rates in mutant strains, as assessed by replicate growth curves with monitoring by both OD_{600} and quantitative culture. In the complementation and pJAK16 expression studies, it will be important to evaluate the expression levels of VLY and other targets (using Western blot and ELISA) in order to ensure that they are at wild-type levels.

Anticipated Results and Interpretation We anticipate that by screening several thousand mutants, we can identify mutants with insertions in the *vly* gene. Such mutants should be non-hemolytic on human blood agar, should not secrete VLY into their supernatants, and should allow us to probe the role of VLY in *G. vaginalis*-epithelial cell interactions more fully. Mutants generated in this series of experiments will also be used in studies described above. The ability to compare a VLY-deficient mutant with its complemented derivative will be particularly powerful in advancing our understanding of the role of VLY in pathogenesis.

Potential Pitfalls, Alternative Approaches, and Future Directions Based on our success in mobilizing pJAK16 and pVJT128 into *G. vaginalis* by conjugation from *E. coli*, we do not anticipate difficulty in performing the experiments with pVJT128. CDC-deficient strains of many Gram-positive species have been generated, making it less likely that *vly* is an essential gene [32,36,66]. Construction of a transposon-mutagenesis library of *G. vaginalis* has uses beyond those detailed in this Aim. There are other putative virulence factors of *G. vaginalis* (prolidase, sialidase, adhesins) that can be investigated in future studies using this tool. Even if we are unable to generate a VLY-deficient mutant using transposon mutagenesis, we will still have constructed a useful library for the future study of non-essential genes in *G. vaginalis*.

Aim 1B: Construct and evaluate specific G. vaginalis strains with altered species specificity.

Experimental Design

Expression of chimeric and mutant toxins in trans The overall goal of this series of experiments is to construct and evaluate strains of *G. vaginalis* that express defined VLY mutant toxins. We will approach this goal in two distinct ways. First, we will express wild-type VLY or defined mutants in trans, using plasmid-driven expression. Expression will be from the IPTG-driven P*tac* promoter in pJAK16 or from the cloned *vly* promoter region. These strains will be generated in the background of *vly*-deficient mutants of *G. vaginalis* generated in Aim 1A and will overlap to some extent with the genetic complementation studies described above. However, it is important that these experiments not depend entirely on our success in Aim 1A, so we can also perform these manipulations in a wild-type *G. vaginalis* background. The disadvantage of the latter approach is that there would be expression of VLY from the chromosomal locus. The advantage is that useful in vitro and in vivo data (especially with the VLY:PLYD4 chimera) may still be obtained.

Generation of unmarked chromosomal mutations at the vly locus The use of insertion mutants in the *vly* locus is not ideal, as such constructs may have polar effects. The complementation studies will address

that possibility, but such effects may still be important. For that reason, we will engineer targeted, unmarked, in-frame mutations of the *vly* locus. Several such strains will be generated, including an unmarked, in-frame deletion of the *vly* coding region, a replacement of the *vly* coding region with one coding for the VLY:PLYD4 chimeric toxin, and one coding for the VLY-P480W mutant that binds hCD59 but does not form pores due to a mutation in the undecapeptide. As a control, we will make a strain in which the wild-type *vly* coding region is reintroduced in order to control for potential changes induced through targeting. To generate these strains, we will employ a combined positive- and negative-selection strategy. We have successfully used such methods in the gram-positive pathogen *Streptococcus pneumoniae* to generate wild-type, deletion, and toxoid-expressing mutants at the pneumolysin locus [67]. We will use the bicistronic "Janus cassette" first described by Sung et al. [68], based on its successful application in related gram-positive organisms. This strategy of selection and counterselection yields first a *G. vaginalis* strain in which the *vly* locus has been replaced by the Janus cassette (conferring Km-resistance and dominant Sm-sensitivity). In subsequent steps, defined mutants (mutant *vly* genes or an in-frame deletion of the coding sequence) are constructed, as loss of the Janus cassette can be selected for by reversion to Sm resistance. We have generated multiple Sm-resistant *G. vaginalis* strains without difficulty. Once the targeted strains have been constructed, we will confirm the structure of the modified locus using PCR and sequencing. As the native promoter will be retained, we do not anticipate changes in expression of mutant VLY. The Janus cassette insertion and the in-frame deletion should be devoid of VLY expression.

Phenotypic analysis Strains will be compared for *vly* transcription and VLY production by RT-PCR and ELISA respectfully. Supernatants will be tested for hemolytic activity against human, sheep, and horse blood to compare species selectivity index (HD_{50}(human)/HD_{50}(sheep or horse)) calculated and compared by ANOVA with post-tests. Stimulatory activity on vaginal epithelial cells will be assessed (examining pp38 phosphorylation, c-fos upregulation, IL-1β maturation, and IL-8 induction). Western blot, real-time RT-PCR, and ELISA techniques will be used with quantification (densitometry for westerns) and statistical comparisons appropriate for each.

Anticipated Results and Interpretation These defined mutants will allow for detailed dissection of the importance of VLY to species selectivity and various epithelial inflammatory responses. Specifically, we anticipate that strains expressing the non-selective VLY:PLYD4 chimera will induce cytolysis and signaling in non-human cells. VLY-deficient strains should lack or have severely attenuated lytic and inflammatory consequences. We hypothesize that pore formation by VLY will be crucial to some responses (p38 MAPK phosphorylation), but that hCD59 ligation will be required for others (such as FRNK phosphorylation, which requires signaling through src family kinases downstream of hCD59). The CDCs may act as ligands for TLR4 independent of pore-formation [69,70]. These engineered strains will permit elucidation of pathways that are independent of both pore-formation and hCD59 binding.

Potential Pitfalls, Alternative Approaches, and Future Directions Alternative Gram-positive counterselection strategies include the upp gene used in *Enterococcus faecalis* [71] and sucrose counterselection, successfully used in *Bacillus* and other species [72]. At the conclusion of this Aim, we will have constructed new strains of *G. vaginalis* with defined alterations at the *vly* locus, including insertion mutants, in-frame deletion, replacement with mutant toxin sequences, and the important control, reinsertion of the wild-type *vly* sequence. We have already shown that VLY is sufficient to induce lytic and inflammatory responses in vitro. However, the use of these targeted mutants will allow us to confirm that VLY is necessary. In addition, the use of non-*E. coli* derived materials will minimize possible effects of LPS. In addition, we will have generated extremely useful strains for in vivo studies (Aim 2). Those studies can proceed without these engineered strains, but the ability to assess the relative contribution of various aspects of the VLY:hCD59 interaction in vivo will be very valuable.

***Aim 1C: Determine genes required for biofilm formation in* G. vaginalis.**

Experimental Design While we hypothesize that VLY production is crucial for *G. vaginalis* colonization, it is clear that there are other factors that may contribute to virulence. Among these is the ability to form biofilm, which appears to be very important in BV pathogenesis and clinical treatment failures [63,64,73].

Phenotypic screen for biofilm-deficient mutants We will use the mutant library generated in Aim 1A to determine genes required for biofilm production. As a positive control, the targeted *luxS* mutants represent a

candidate gene that we hypothesize will have a substantial effect on biofilm formation. Individual transposon insertion mutants will be grown in at least triplicate in 96-well polystyrene plates as described above and compared to the parental strain. At 24 and 48 hr (duplicate plates, triplicate wells), liquid will be removed for colony counts in order to ensure that mutants with overall growth phenotypes are not mischaracterized as biofilm mutants. Wells will be washed and adherent organisms stained with safranin. After removal of excess dye and washing with water, adherent safranin will be solubilized and OD_{462} measured. Mutants will be compared to wild-type by ANOVA with Tukey post-test, and strains with significant differences will have the transposon insertion site determined by inverse PCR as in Aim 1A. While growth on polystyrene may be useful as an initial screen, it is not an ideal model of biofilm formation in vivo. We will also examine all putative mutants (as well as the wild-type and a subset of strains without a phenotype on the safranin assay) in additional measures of biofilm formation. These will include growth on glass-bottomed dishes with live-cell imaging and quantitative analysis of community structure (mean height and diameter of organism groups per field — at least 5 fields per strain, compared statistically) as well as growth in the presence of human epithelial cells. Mutants will also be assessed for VLY production by ELISA and compared statistically.

Targeted mutation and complementation Candidate *G. vaginalis* mutants deficient in biofilm formation will be genetically complemented as described above for *vly*-deficient strains, and the specificity of the phenotype determined using assays of biofilm formation. It may be advantageous to construct unmarked, in-frame deletions using the Janus cassette in order to minimize polar effects. The mutants identified in these experiments will be tested in vivo using the hCD59-transgenic mouse (Aim 2) to determine the effect of biofilm phenotypes in colonization and pathogenesis.

Anticipated Results and Interpretation Based on our preliminary data and on the presence of putative homologues of known quorum-sensing genes in the draft *G. vaginalis* genomes, we hypothesize that there will be non-essential genes regulating biofilm production and that these will be identified using our transposon mutagenesis approach. Because of the importance of biofilms to *G. vaginalis* pathogenesis and antibiotic resistance [64,73], we hypothesize that such pathways will be of substantial utility in murine models. As above, it is important to also test a priori hypotheses of involved pathways. Thus, we will use targeted mutant strains deficient in the production of *luxS*, the gene for AI-2 synthesis, as initial candidates.

Potential Pitfalls, Alternative Approaches, and Future Directions Redundant or essential genes may not be identified using this approach. However, based on findings in other Gram-positive organisms, we anticipate that we will find mutants with altered phenotypes in this screen and that these will be useful both in further in vitro studies (e.g. of antimicrobial susceptibility) and in murine models. These same techniques are applicable to identification of other putative virulence factors of *G. vaginalis* such as sialidase, prolidase, and adhesins, each of which would be useful to test in vitro and in vivo.

Aim 2: Determine the role of VLY in *G. vaginalis* at the host-pathogen interface in vitro and in vivo.

Aim 2A: Determine the role of VLY-induced membrane blebbing as a mechanism for protection of vaginal epithelial cells from toxin pores and as a pathway sensitizing cells to complement.

Experimental Design In addition to activating immune pathways, mucosal epithelial cells can undergo ultrastructural rearrangements in response to microbial products. Examples of this include actin pedestal formation in intestinal epithelial cells in response to pathogenic *E. coli* [74] and epithelial extrusion in response to *L. monocytogenes* [75]. Although actin rearrangement in response to toxins has been described [76], we have characterized a novel epithelial response to a pore-forming toxin — the rapid formation of blebs in response to VLY. This response does not occur with other toxins that we have tested and is recapitulated by either antibody against hCD59 or the GFP:VLYD4 fusion. We propose that this response requires hCD59 signaling following VLY binding, representing a novel pathway for mucosal recognition of toxins.

Live cell imaging of hCD59-dependent bleb formation In this Aim, we will use cultured epithelial cells in order to understand the response of physiologically relevant cell types to *G. vaginalis* and VLY. We will use both HeLa cells and VK2/E6E7, which is a vaginal epithelial cell line transformed by human papilloma virus proteins [77]. We will take advantage of the recent commercial availability of a three-dimensional human primary vaginal epithelial model (EpiVaginal; MatTek). This model preserves native architecture and polarity due to culture at an air-liquid interface. In addition, we have extensive experience with primary cell culture, including the growth and characterization of primary ciliated murine epithelial cells [78]. Thus, we will grow and

validate primary murine vaginal epithelial cells as described [79,80]. These cells will be derived from hCD59 transgenic mice or their transgene-negative littermates.

Epithelial cells will be treated with VLY at sublytic concentrations, as we have demonstrated that significant blebbing occurs at concentrations well below those required for cytolysis. A range of concentrations will be used and will be based on preliminary experiments for each cell line. Cell death will be assessed in at least two ways. First, duplicate wells for each condition will be inspected visually for monolayer integrity and then assessed by trypan blue exclusion for estimation of cell death. Second, supernatants will be assayed for lactate dehydrogenase (LDH) release and compared to control wells and to 100% lysis (treatment of cells with 1% Triton X-100) for quantification of cell death. In order to exclude the potential confounding effects of LPS contamination of recombinant protein preparations, we will use proteins that have been run over polymixin columns (buffers for vehicle controls will be treated identically) and will confirm that purified *E. coli* LPS does not induce blebbing. We will also use heat-inactivated preparations in order to further assess the contribution of heat-stable contaminants such as LPS. *Lactococcus* expression systems would represent another strategy to reduce the potential effects of LPS. We will follow the dynamics of epithelial bleb formation in real-time using fluorescence microscopy (**Fig. 6**). Epithelial cells will be stimulated with *G. vaginalis* (wild-type, mutant, and complemented strains), purified VLY (both wild-type and hCD59 binding-deficient mutants), purified complement components, and antibody to hCD59 at various concentrations. Prior to stimulation, the cells will have been labeled with two fluorescent dyes as above. Bleb formation will be monitored over at least a 4 hr period. Parameters to be assessed in at least 10 fields per condition and compared statistically will include percentage of cells with visible blebs per high-power field, mean bleb diameter (ImageJ; NIH), and number of blebs per cell. We anticipate that each of the ligands that bind and crosslink hCD59 will lead to bleb formation that can be monitored in real-time. In order to determine the specificity of this effect for hCD59, we will compare CHO-hCD59 with control CHO-IRES cells as well as control vs. hCD59-transgenic murine cells and evaluate the rapid bleb response. We will also transfect vaginal cells with siRNA targeting hCD59 (and scrambled controls), assess efficiency and knockdown, and compare bleb formation among transfected cells.

hCD59 loss and complement sensitivity We hypothesize that bleb formation will lead not only to removal of toxin monomers from vaginal epithelial cells but also to loss of hCD59 from the cell surface. This could potentially serve both a protective function (cells with less hCD59 may be less sensitive to the VLY that remains in solution) and dangerous one (cells lacking hCD59 may be more sensitive to complement-mediated lysis.) Antibody methods can detect surface hCD59 (**Fig. 6B**). We will induce blebbing as above and allow the cells to recover for various periods. Cells will be fixed and surface hCD59 availability assessed by flow cytometry using anti-hCD59 monoclonal antibodies. Many hCD59 antibodies have been used for flow cytometry, including some that bind at sites distinct from the complement/VLY binding site, and several will be used in independent experiments to distinguish lack of reactivity due to an occupied binding site from absence from the cell surface. In order to assess the physiologic relevance of loss of hCD59, we will carry out VLY-sensitivity and complement lysis assays. Following blebbing, cells will be washed and allowed to recover for various periods (1-24 hr). At regular intervals, cells will be exposed to various doses of either VLY or delipidated human serum (with complement components but without cholesterol due to inhibitory effects on toxin activity). Cytolysis will be measured and evaluated statistically by LDH assay as above.

Anticipated Results and Interpretation Signaling through hCD59 may represent a novel pathway of toxin recognition and a link between VLY binding and rapid bleb formation. We hypothesize that hCD59-binding toxin preparations and whole *G. vaginalis* will initiate epithelial bleb formation and that VLY-deficient bacteria or hCD59-independent toxin mutants will not. In addition, we propose that shedding of VLY-hCD59-containing blebs may render cells less sensitive to immediate re-exposure to VLY (due to loss of receptor) but more sensitive to autologous complement (due to the loss of an inhibitor of membrane attack complex deposition). Either of these findings would represent a new understanding of toxin-induced epithelial response with implications beyond VLY and *G. vaginalis*.

Potential Pitfalls, Alternative Approaches, and Future Directions A variety of cellular mechanisms to respond to microbial toxins have been described, but none involving hCD59. Because of the importance of hCD59 in complement restriction and its ubiquitous expression, such a pathway may be of considerable relevance. One immediate future direction will be determination of specific hCD59 signals linking receptor ligation to bleb formation. hCD59 signaling involves initiation of calcium fluxes and activation of src-family

tyrosine kinases. We can determine the role of calcium fluxes by preloading cells with BAPTA/AM, a cell-permeant calcium chelator and by live-cell calcium imaging using Fluo-4/AM. We have successfully used both of these techniques in similar settings [81]. We will use both pharmacologic and siRNA-mediated approaches with appropriate controls to inhibit specific members of the Src kinase family (primarily Src and Lck, for which specific inhibitors are available). These studies will assist us in further delineating the signaling pathways linking hCD59 binding to bleb formation. One potential pitfall is potential redundancy within signaling pathways such as the src kinase family. As a result of this, partial effects (such as decreased bleb size or number) may be observed. This is the reason for the quantitative and statistical approach to bleb assessment and for the use of general as well as specific inhibition strategies. hCD59 is a GPI-anchored protein and, as such, lacks transmembrane and cytoplasmic domains. Thus, in order to initiate intracellular signaling, hCD59 must complex with other proteins, analogous to LPS sensing mediated by GPI-anchored CD14, which complexes with Toll-like receptors [53]. If the pathway responsible for linking hCD59 to bleb formation remains unclear, we will perform immunoprecipitation of hCD59 and attempt to identify novel binding partners of hCD59.

Aim 2B: Define the role of the VLY-hCD59 interaction in* G. vaginalis *pathogenesis in vivo.

Experimental Design We have generated hCD59 transgenic mice, as described in Preliminary Studies above. Having confirmed appropriate expression and cell-surface localization of hCD59, we will perform murine vaginal colonization with *G. vaginalis*. This protocol has been approved by the Columbia University IACUC (Protocol AC-AAAB0279, *Development of a murine model of bacterial vaginosis*). In order to determine the necessity for hCD59 transgene expression, transgenic animals will be compared with their transgene-negative littermates in each of the experiments described.

Murine vaginal colonization Adult female estrus-synchronized mice (7 animals per group — see power calculation in Vertebrate Animal section) will be anesthetized, and PBS-washed, log-phase Sm-resistant *G. vaginalis* will be instilled into the vagina in 10 μl total volume. Total organism burden to be initially evaluated will range from 10^5-10^8 cfu/10 μl PBS. Control animals will receive 10 μl of sterile PBS in identical fashion. *G. vaginalis* strains to be evaluated will include laboratory strains and clinical isolates. Vaginal samples will be collected every 24 hr for 7 days in initial experiments. Sampling will consist of collection of a 100 μl vaginal lavage to be processed for quantitative culture on selective media, ELISA (for VLY as well as selected murine cytokines including IL-1β), and pH. ELISA values will be normalized to total protein to account for differences in collection efficiency. In addition, a swab will be collected using the standardized methodology described above, and Gram-stained smears will be scored for the presence of clue cells. These gram stained slides will also be evaluated by two blinded reviewers who have been trained in the Nugent scoring system for BV [1]. Scores will be tabulated, interrater reliability assessed (κ statistic), and disagreement >2 points on the Nugent scale adjudicated by a third blinded reviewer. At each time point, groups will be compared for *G. vaginalis* load, local VLY concentration, local cytokine levels, pH, and Nugent score using appropriate non-parametric statistics (Kruskal-Wallis) and post-tests. It is important to note that the lavage specimens will be limited in volume, and it may not be possible to perform all testing proposed during the initial experiments. In that case, we will prioritize quantitative culture, pH, VLY ELISA, and Nugent scoring (which does not require liquid sample). In order to confirm that the wash procedure does not alter the duration of colonization, selected groups of animals will be sampled only at later time points. At the conclusion of the experiment, animals will be euthanized and blood collected for analysis of IL-1β and IL-8 levels, antibody titers to VLY, and histopathology.

Determination of the role of VLY and biofilm in colonization Once we have established optimal conditions for murine colonization, we will confirm the requirement for VLY in *G. vaginalis* colonization by comparing VLY-deficient and complemented strains for efficiency of colonization in this model. The VLY toxoid-expressing strain and its control will be useful to determine the role of pore formation by VLY. Strains will also be compared at the level of induction of inflammation and pathology (Nugent score, pH changes). The requirement for biofilm formation in vivo in *G. vaginalis* colonization and induction of disease can be assessed using mutants (and complemented derivatives) identified in Aim 1C (as well as the *luxS*-deficient strain, though that may have both VLY and biofilm defects). As rigorous proof that the VLY species specificity dictates host range and pathology, we will test strains of *G. vaginalis* expressing the hCD59-independent toxin chimera, VLY:PLYD4. These strains should colonize and induce pathology in wild-type mice and hCD59 transgenics.

Anticipated Results and Interpretation We hypothesize that VLY is a major determinant of host range and pathogenesis. Thus, both of these approaches (wild-type *G. vaginalis* in the hCD59-transgenic mouse and

VLY:PLYD4-expressing *G. vaginalis* in the wild-type mouse) should produce colonization, immune responses, and pathology. We will gain information about the relative importance of pore-formation and hCD59 signaling and will have developed an extremely valuable small animal model of BV.

Potential Pitfalls, Alternative Approaches, and Future Directions If it is difficult to colonize either wild-type or hCD59-transgenic mice under these conditions, we will undertake two alternative strategies. First, we will use mouse-passaged strains of *G. vaginalis*. The advantage of this procedure is that it would enrich the sample for more adherent, possibly more robust, colonizers. The disadvantage is that the differences between the passaged strains and the original wild-type would be unknown and might require substantial effort to characterize. The second approach would be to use intravaginal instillation of purified VLY (in the case of hCD59-transgenics) or PLY or VLY:PLYD4 (in the case of non-transgenic mice) in various concentrations. We have used this strategy to enhance *G. vaginalis* colonization in the past. In addition, depending on the specific results obtained in these experiments, we should be able to progress to understanding specific components of immunity required for control of *G. vaginalis* colonization and disease. We can cross the hCD59 transgene into murine lines deficient in various components of innate or adaptive immunity in order to determine the specific requirements for each of these in colonization or pathogenesis. Alternatively, the VLY:PLYD4-expressing organisms could be used in those hosts directly.

Aim 2C: Evaluate candidate inhibitors of the VLY-hCD59 interaction in vivo.

Experimental Design We will use the techniques described in Aim 2B for in vivo modeling of *G. vaginalis* colonization and bacterial vaginosis. Importantly, though this Aim builds on the results of the prior experiments, it is not dependent on the establishment of a long-term colonization model with *G. vaginalis*.

Antibody and toxoid-based inhibition studies We will use our existing polyclonal anti-VLY antibodies, which block cytolysis and epithelial toxin detection in vitro, to prevent VLY-mediated pathology in vivo. In addition, we will use dominant-negative toxoid VLY, which interferes with VLY-mediated pore formation. Using the colonization model developed in Aim 2B (or purified toxin delivery if the colonization model is unsuccessful), we will deliver candidate inhibitors in various concentrations prior to (and, in subsequent experiments, concurrently with or following) colonization with *G. vaginalis*. For wild-type *G. vaginalis*, we will use the hCD59-transgenic mouse. For VLY:PLYD4-expressing *G. vaginalis,* the experiments will take place in wild-type mice. Outcomes will be evaluated as in Aim 2B, with *G. vaginalis* quantitative culture, local cytokine production, and altered pH. IC_{50} values will be compared, and non-parametric statistical analysis among groups will account for multiple comparisons.

Anticipated Results and Interpretation We anticipate that local delivery of either antibody targeting VLY or dominant-negative toxoid will be effective in inhibiting the *G. vaginalis*- or VLY-mediated pathology in our in vivo models. In addition, we hypothesize that if VLY is itself necessary for colonization that colony counts and colonization duration will be shortened as well. Either of these findings would represent a major advance, as the current therapeutic options for BV are limited and are associated with frequent treatment failures.

Potential Pitfalls, Alternative Approaches, and Future Directions While we hypothesize that VLY is essential for *G. vaginalis* to cause disease, candidate inhibitors may be ineffective in vivo despite disrupting toxin function in vitro. Mucosal delivery of biomolecules can be difficult, and it is possible that not enough toxoid or antibody will be retained to be effective. There are various physical methods that we could use to try to address that situation, including the use of gels already formulated for use in vaginal microbicides. Future directions will include screening for small molecule inhibitors of VLY using cytolysis assays in vitro (with subsequent testing of lead compounds in vivo) and generation of monoclonal antibodies against VLY. It is crucial to ensure that these inhibitors do not inhibit hCD59 binding to C8/C9, as this could sensitize host cells to complement attack. Further mutant studies to determine the precise binding site for VLY on hCD59 (and whether it is identical to the C8/C9 site) would be important for such investigations.

At the conclusion of these Aims, we will have gained new information regarding the role of VLY in *G. vaginalis* pathogenesis and will have generated new in vivo models and candidate therapeutics. Our long-term goal, understanding and preventing the adverse sequelae of BV, will be greatly aided by the program of research described here, and we thank you for your consideration.

VERTEBRATE ANIMALS INFORMATION

Description of the Proposed Use of Animals

A major goal of this research is to develop and characterize murine models of bacterial vaginosis. Based on our prior work involving murine infection models and a power analysis, we have calculated that 7 mice per group will be required to detect differences of 0.5 $\log_{10}$ cfu/ml, assuming 80% power, a desired P value of 0.05, a standard deviation of 0.25 $\log_{10}$ cfu/ml, and a failure rate of 10%. These calculations are based on quantitative culture to be performed at the conclusion of the experimental time course. Specific groups to be tested are delineated above and include control (PBS-treated) mice and mice receiving intravaginal *G. vaginalis* at various concentrations. Experiments will be performed in hCD59-transgenic and non-transgenic animals and will use strains of *G. vaginalis* described above.

Justification of Animal Use, Species Selected, and Numbers

Bacterial vaginosis is an important cause of preterm birth and a risk factor for sexually transmitted diseases in humans. To date, there have been no animal models of this disease. For this reason, it is crucial to understand whether the species selectivity of the major *G. vaginalis* toxin, vaginolysin (VLY), is involved in this limited host range. In vitro models have proven inadequate for studying the complex ecosystem of the vaginal mucosa, particularly with regard to microbial infection. Currently available models are limited with respect to their ability to evaluate spatial and temporal changes in bacterial and host cell distributions. We plan to use C57BL/6 mice and matched transgenics expressing the hCD59 receptor to determine the role for VLY in this setting.

Animals are not involved in the experiments in Aim 1 or Aim 2A. In Aim 2B, we calculate a need for 19 groups of 7 mice each for studies involving hCD59-transgenic animals (3 concentrations for each of 6 *G. vaginalis* strains, 1 PBS control group) as well as 7 groups of 7 mice each for studies involving wild-type mice and chimera-expressing *G. vaginalis* (3 concentrations for each of 2 *G. vaginalis* strains, 1 PBS control group). In Aim 2C, the precise numbers will depend to some extent on our findings in Aim 2B. At a minimum, this would include 13 groups of 7 mice each (3 concentrations of 2 inhibitors, 2 *G. vaginalis* strains, 1 PBS control group). This leads to an estimated need for 39 (19+7+13=39) groups of 7 mice, for a total of 273 mice over the study period.

Veterinary Care

Mice will be housed in the barrier ABSL2 facility at Columbia University College of Physicians & Surgeons. This facility is attended by a full-time veterinary and support staff that is experienced in providing high quality care to laboratory animals. C57BL/6 mice and transgenic models are in wide use at Columbia, and the veterinary staff is experienced and equipped to house and care for them. All animals will be in a specific pathogen-free setting, receive food and water ad libitum, and otherwise receive standard care.

Procedures for Limitation of Pain, Discomfort, and Injury

Intravaginal inoculation and lavage/culture are performed in anesthetized (ketamine/xylazine) mice in order to limit discomfort. This is not a dangerous procedure, and sampling takes less than 1 minute per animal. Mice are immediately returned to their cages and generally do not have ill effects. Animals are monitored daily, and mice that become ill during these experiments will be euthanized. Blood drawing and obtaining material for histopathologic analysis will only be done at the conclusion of the time course, following euthanasia.

Method of Euthanasia
Euthanasia will be performed by CO_2 inhalation, in accordance with institutional and AMVA guidelines. The professional veterinary staff will assist the investigators in the performance of euthanasia.

SELECT AGENTS

Select agents are not involved in this research.

G. vaginalis and *E. coli* are BSL-2 organisms. Our laboratory is equipped for BSL-2 research and certified by Columbia University Environmental Health and Safety. All laboratory members have received specific training in laboratory safety and the proper use and disposal of infectious agents.

REFERENCES

1. Nugent RP, Krohn MA, Hillier SL (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol 29: 297-301.
2. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, et al. (2007) The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. Sex Transm Dis 34: 864-869.
3. Koumans EH, Kendrick JS (2001) Preventing adverse sequelae of bacterial vaginosis: a public health program and research agenda. Sex Transm Dis 28: 292-297.
4. Schmid G, Markowitz L, Joesoef R, Koumans E (2000) Bacterial vaginosis and HIV infection. Sex Transm Infect 76: 3-4.
5. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS (2008) Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. AIDS 22: 1493-1501.
6. Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, et al. (1997) HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. Lancet 350: 546-550.
7. Al-Harthi L, Roebuck KA, Olinger GG, Landay A, Sha BE, et al. (1999) Bacterial vaginosis-associated microflora isolated from the female genital tract activates HIV-1 expression. J Acquir Immune Defic Syndr 21: 194-202.
8. Hashemi FB, Ghassemi M, Roebuck KA, Spear GT (1999) Activation of human immunodeficiency virus type 1 expression by Gardnerella vaginalis. J Infect Dis 179: 924-930.
9. Zariffard MR, Sha BE, Wang QJ, Chen HY, Bremer J, et al. (2005) Relationship of U1 cell HIV-stimulatory activity to bacterial vaginosis and HIV genital tract virus load. AIDS Res Hum Retroviruses 21: 945-948.
10. Allsworth JE, Lewis VA, Peipert JF (2008) Viral sexually transmitted infections and bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. Sex Transm Dis 35: 791-796.
11. Peipert JF, Lapane KL, Allsworth JE, Redding CA, Blume JD, et al. (2008) Bacterial vaginosis, race, and sexually transmitted infections: does race modify the association? Sex Transm Dis 35: 363-367.
12. Witkin SS, Linhares IM, Giraldo P, Ledger WJ (2007) An altered immunity hypothesis for the development of symptomatic bacterial vaginosis. Clin Infect Dis 44: 554-557.
13. Fredricks DN, Fiedler TL, Marrazzo JM (2005) Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med 353: 1899-1911.
14. Larsson PG, Stray-Pedersen B, Ryttig KR, Larsen S (2008) Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. BMC Womens Health 8: 3.
15. Leopold S (1953) Heretofore undescribed organism isolated from the genitourinary system. U S Armed Forces Med J 4: 263-266.
16. Gardner HL, Dukes CD (1954) New etiologic agent in nonspecific bacterial vaginitis. Science 120: 853.
17. Criswell BS, Ladwig CL, Gardner HL, Dukes CD (1969) Haemophilus vaginalis: vaginitis by inoculation from culture. Obstet Gynecol 33: 195-199.
18. Sadhu K, Domingue PA, Chow AW, Nelligan J, Cheng N, et al. (1989) Gardnerella vaginalis has a gram-positive cell-wall ultrastructure and lacks classical cell-wall lipopolysaccharide. J Med Microbiol 29: 229-235.
19. Catlin BW (1992) Gardnerella vaginalis: characteristics, clinical considerations, and controversies. Clin Microbiol Rev 5: 213-237.
20. Ratnam S, Fitzgerald BL (1983) Semiquantitative culture of Gardnerella vaginalis in laboratory determination of nonspecific vaginitis. J Clin Microbiol 18: 344-347.
21. Josey WE, Schwebke JR (2008) The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. Int J STD AIDS 19: 152-154.
22. Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D (2008) Molecular quantification of Gardnerella vaginalis and Atopobium vaginae loads to predict bacterial vaginosis. Clin Infect Dis 47: 33-43.
23. Tweten RK (2005) Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infect Immun 73: 6199-6209.
24. Farrand AJ, Lachapelle S, Hotze EM, Johnson AE, Tweten RK (2010) Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface. Proc Natl Acad Sci U S A.
25. Soltani CE, Hotze EM, Johnson AE, Tweten RK (2007) Structural elements of the cholesterol-dependent cytolysins that are responsible for their cholesterol-sensitive membrane interactions. Proc Natl Acad Sci U S A 104: 20226-20231.

26. Czajkowsky DM, Hotze EM, Shao Z, Tweten RK (2004) Vertical collapse of a cytolysin prepore moves its transmembrane beta-hairpins to the membrane. EMBO J 23: 3206-3215.
27. Dang TX, Hotze EM, Rouiller I, Tweten RK, Wilson-Kubalek EM (2005) Prepore to pore transition of a cholesterol-dependent cytolysin visualized by electron microscopy. J Struct Biol 150: 100-108.
28. Ramachandran R, Tweten RK, Johnson AE (2004) Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment. Nat Struct Mol Biol 11: 697-705.
29. Ramachandran R, Tweten RK, Johnson AE (2005) The domains of a cholesterol-dependent cytolysin undergo a major FRET-detected rearrangement during pore formation. Proc Natl Acad Sci U S A 102: 7139-7144.
30. Singh R, Jamieson A, Cresswell P (2008) GILT is a critical host factor for Listeria monocytogenes infection. Nature.
31. Madden JC, Ruiz N, Caparon M (2001) Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. Cell 104: 143-152.
32. Berry AM, Yother J, Briles DE, Hansman D, Paton JC (1989) Reduced virulence of a defined pneumolysin-negative mutant of Streptococcus pneumoniae. Infect Immun 57: 2037-2042.
33. Marriott HM, Mitchell TJ, Dockrell DH (2008) Pneumolysin: a double-edged sword during the host-pathogen interaction. Curr Mol Med 8: 497-509.
34. Ratner AJ, Lysenko ES, Paul MN, Weiser JN (2005) Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. Proc Natl Acad Sci U S A 102: 3429-3434.
35. Johnson MK, Geoffroy C, Alouf JE (1980) Binding of cholesterol by sulfhydryl-activated cytolysins. Infect Immun 27: 97-101.
36. Nagamune H, Ohnishi C, Katsuura A, Taoka Y, Fushitani K, et al. (1997) Intermedilysin. A cytolytic toxin specific for human cells of a Streptococcus intermedius isolated from human liver abscess. Adv Exp Med Biol 418: 773-775.
37. Giddings KS, Zhao J, Sims PJ, Tweten RK (2004) Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. Nat Struct Mol Biol 11: 1173-1178.
38. Gelber SE, Aguilar JL, Lewis KL, Ratner AJ (2008) Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from Gardnerella vaginalis. J Bacteriol 190: 3896-3903.
39. Goepfert AR, Varner M, Ward K, Macpherson C, Klebanoff M, et al. (2005) Differences in inflammatory cytokine and Toll-like receptor genes and bacterial vaginosis in pregnancy. Am J Obstet Gynecol 193: 1478-1485.
40. Cauci S, Di Santolo M, Casabellata G, Ryckman K, Williams SM, et al. (2007) Association of interleukin-1beta and interleukin-1 receptor antagonist polymorphisms with bacterial vaginosis in non-pregnant Italian women. Mol Hum Reprod 13: 243-250.
41. Planet PJ, Ratner AJ (2008) Phylogenetic analysis of the cholesterol-dependent cytolysins: lateral transfer and purifying selection within a widespread family of bacterial pore-forming toxins. Keystone Symposium on Molecular Evolution as a Driving Force in Infectious Diseases. Breckenridge, CO.
42. Cauci S, Guaschino S, De Aloysio D, Driussi S, De Santo D, et al. (2003) Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. Mol Hum Reprod 9: 53-58.
43. Genc MR, Witkin SS, Delaney ML, Paraskevas LR, Tuomala RE, et al. (2004) A disproportionate increase in IL-1beta over IL-1ra in the cervicovaginal secretions of pregnant women with altered vaginal microflora correlates with preterm birth. Am J Obstet Gynecol 190: 1191-1197.
44. Mattsby-Baltzer I, Platz-Christensen JJ, Hosseini N, Rosen P (1998) IL-1beta, IL-6, TNFalpha, fetal fibronectin, and endotoxin in the lower genital tract of pregnant women with bacterial vaginosis. Acta Obstet Gynecol Scand 77: 701-706.
45. Davies A, Simmons DL, Hale G, Harrison RA, Tighe H, et al. (1989) CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. J Exp Med 170: 637-654.
46. Ninomiya H, Sims PJ (1992) The human complement regulatory protein CD59 binds to the alpha-chain of C8 and to the "b"domain of C9. J Biol Chem 267: 13675-13680.
47. Motoyama N, Okada N, Yamashina M, Okada H (1992) Paroxysmal nocturnal hemoglobinuria due to hereditary nucleotide deletion in the HRF20 (CD59) gene. Eur J Immunol 22: 2669-2673.

48. Yamashina M, Ueda E, Kinoshita T, Takami T, Ojima A, et al. (1990) Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. N Engl J Med 323: 1184-1189.
49. Zalman LS, Wood LM, Frank MM, Muller-Eberhard HJ (1987) Deficiency of the homologous restriction factor in paroxysmal nocturnal hemoglobinuria. J Exp Med 165: 572-577.
50. Zhao XJ, Zhao J, Zhou Q, Sims PJ (1998) Identity of the residues responsible for the species-restricted complement inhibitory function of human CD59. J Biol Chem 273: 10665-10671.
51. Rosado CJ, Buckle AM, Law RH, Butcher RE, Kan WT, et al. (2007) A common fold mediates vertebrate defense and bacterial attack. Science 317: 1548-1551.
52. Rosado CJ, Kondos S, Bull TE, Kuiper MJ, Law RH, et al. (2008) The MACPF/CDC family of pore-forming toxins. Cell Microbiol 10: 1765-1774.
53. Kimberley FC, Sivasankar B, Paul Morgan B (2007) Alternative roles for CD59. Mol Immunol 44: 73-81.
54. Murray EW, Robbins SM (1998) Antibody cross-linking of the glycosylphosphatidylinositol-linked protein CD59 on hematopoietic cells induces signaling pathways resembling activation by complement. J Biol Chem 273: 25279-25284.
55. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H (1991) GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 254: 1016-1019.
56. Suzuki KG, Fujiwara TK, Sanematsu F, Iino R, Edidin M, et al. (2007) GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. J Cell Biol 177: 717-730.
57. Randis TM, Kulkarni R, Aguilar JL, Ratner AJ (2009) Antibody-based detection and inhibition of vaginolysin, the Gardnerella vaginalis cytolysin. PLoS One 4: e5207.
58. Qin X, Hu W, Song W, Grubissich L, Hu X, et al. (2009) Generation and phenotyping of mCd59a and mCd59b double-knockout mice. Am J Hematol 84: 65-70.
59. Hu W, Ferris SP, Tweten RK, Wu G, Radaeva S, et al. (2008) Rapid conditional targeted ablation of cells expressing human CD59 in transgenic mice by intermedilysin. Nat Med 14: 98-103.
60. Diamond LE, Oldham ER, Platt JL, Waldmann H, Tone M, et al. (1994) Cell- and tissue-specific expression of a human CD59 minigene in transgenic mice. Transplant Proc 26: 1239.
61. Ratner AJ, Hippe KR, Aguilar JL, Bender MH, Nelson AL, et al. (2006) Epithelial cells are sensitive detectors of bacterial pore-forming toxins. J Biol Chem 281: 12994-12998.
62. Sood R, Zehnder JL, Druzin ML, Brown PO (2006) Gene expression patterns in human placenta. Proc Natl Acad Sci U S A 103: 5478-5483.
63. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, et al. (2005) Adherent biofilms in bacterial vaginosis. Obstet Gynecol 106: 1013-1023.
64. Swidsinski A, Mendling W, Loening-Baucke V, Swidsinski S, Dorffel Y, et al. (2008) An adherent Gardnerella vaginalis biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. Am J Obstet Gynecol 198: 97 e91-96.
65. Thomson VJ, Bhattacharjee MK, Fine DH, Derbyshire KM, Figurski DH (1999) Direct selection of IS903 transposon insertions by use of a broad-host-range vector: isolation of catalase-deficient mutants of Actinobacillus actinomycetemcomitans. J Bacteriol 181: 7298-7307.
66. Sukeno A, Nagamune H, Whiley RA, Jafar SI, Aduse-Opoku J, et al. (2005) Intermedilysin is essential for the invasion of hepatoma HepG2 cells by Streptococcus intermedius. Microbiol Immunol 49: 681-694.
67. Ratner AJ, Aguilar JL, Shchepetov M, Lysenko ES, Weiser JN (2007) Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. Cell Microbiol 9: 1343-1351.
68. Sung CK, Li H, Claverys JP, Morrison DA (2001) An rpsL cassette, janus, for gene replacement through negative selection in Streptococcus pneumoniae. Appl Environ Microbiol 67: 5190-5196.
69. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, et al. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A 100: 1966-1971.
70. Park JM, Ng VH, Maeda S, Rest RF, Karin M (2004) Anthrolysin O and other gram-positive cytolysins are toll-like receptor 4 agonists. J Exp Med 200: 1647-1655.
71. Kristich CJ, Manias DA, Dunny GM (2005) Development of a method for markerless genetic exchange in Enterococcus faecalis and its use in construction of a srtA mutant. Appl Environ Microbiol 71: 5837-5849.
72. Wu SS, Kaiser D (1996) Markerless deletions of pil genes in Myxococcus xanthus generated by counterselection with the Bacillus subtilis sacB gene. J Bacteriol 178: 5817-5821.

73. Patterson JL, Girerd PH, Karjane NW, Jefferson KK (2007) Effect of biofilm phenotype on resistance of Gardnerella vaginalis to hydrogen peroxide and lactic acid. Am J Obstet Gynecol 197: 170 e171-177.
74. Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, et al. (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91: 511-520.
75. Pentecost M, Otto G, Theriot JA, Amieva MR (2006) Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion. PLoS Pathog 2: e3.
76. Iliev AI, Djannatian JR, Nau R, Mitchell TJ, Wouters FS (2007) Cholesterol-dependent actin remodeling via RhoA and Rac1 activation by the Streptococcus pneumoniae toxin pneumolysin. Proc Natl Acad Sci U S A 104: 2897-2902.
77. Fichorova RN, Rheinwald JG, Anderson DJ (1997) Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 57: 847-855.
78. Antunes MB, Woodworth BA, Bhargave G, Xiong G, Aguilar JL, et al. (2007) Murine nasal septa for respiratory epithelial air-liquid interface cultures. Biotechniques 43: 195-196, 198, 200 passim.
79. Fernandez S, Gillgrass A, Kaushic C (2007) Differential responses of murine vaginal and uterine epithelial cells prior to and following herpes simplex virus type 2 (HSV-2) infection. Am J Reprod Immunol 57: 367-377.
80. Yao XD, Fernandez S, Kelly MM, Kaushic C, Rosenthal KL (2007) Expression of Toll-like receptors in murine vaginal epithelium is affected by the estrous cycle and stromal cells. J Reprod Immunol 75: 106-119.
81. Ratner AJ, Bryan R, Weber A, Nguyen S, Barnes D, et al. (2001) Cystic fibrosis pathogens activate Ca2+-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. J Biol Chem 276: 19267-19275.

COLUMBIA UNIVERSITY
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DEPARTMENT OF
Microbiology & Immunology

February 18, 2010

Dr. Adam Ratner
Departments of Pediatrics and Microbiology & Immunology
Columbia University
650 West 168th Street (BB 421)
New York, NY 10032

Dear Adam,

I am delighted that you are continuing your genetic studies on the virulence of the human pathogen *Gardnerella vaginalis*. I should be able to help you.

I studied broad-host-range plasmids, most self-transmissible or mobilizable, for nearly 30 years. About 12 years ago, my laboratory constructed and successfully used an inducible and cryptic Tn*903*-based transposon on a promiscuous and efficiently mobilizable IncQ plasmid for easy and convenient mutagenesis in a wide variety of bacterial genera, especially those that have no or few genetic tools. This was significant to our own research on and our discovery of an adherence locus in the genetically recalcitrant human pathogen *Aggregatibacter actinomycetemcomitans*. Since then we have designed other genetic tools for use with this organism.

The unstudied nature of *Gardnerella vaginalis* presents a delicious challenge that I am sure can be overcome. I am very happy to provide you with any advice and to make available any plasmids, reagents, and equipment from my laboratory that you may find relevant. You and the members of your laboratory are very welcome anytime.

I have enjoyed talking with you, and I look forward to more exciting developments from your research.

With best regards,

David H. Figurski, Ph.D.
Professor of Microbiology
& Immunology

RESOURCE SHARING PLAN

Resources developed within the scope of this application will be made available to the scientific community. This includes publications (through PubMed Central and/or publication in open-access journals), raw data, and reagents.

PHS 398 Checklist

OMB Number: 0925-0001

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier: HD061371

2. Change of Investigator / Change of Institution Questions

☐ 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:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☐

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

* Previously Reported: Yes ☐ No ☐

4. * Program Income

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

☐ Yes ☒ 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)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☒ Yes ☐ No