## 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-1$\alpha$ 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

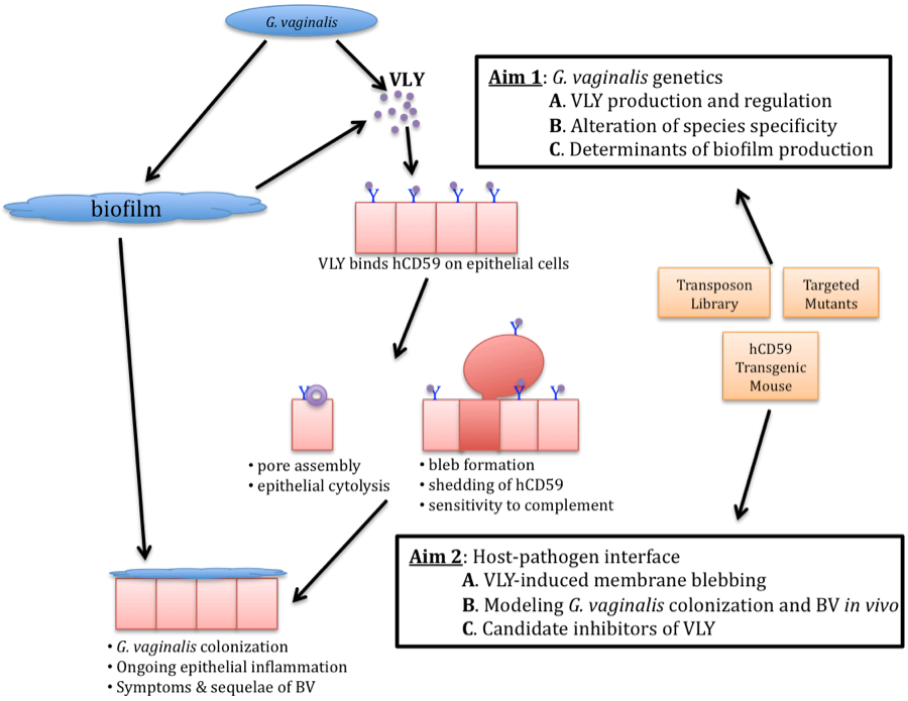
Our overall goal is to define the role of vaginolysin (VLY), a novel, human-specific toxin produced by *Gardnerella vaginalis*, in the pathogenesis of bacterial vaginosis (BV). We propose the following research plan directed at elucidating both genetic mechanisms of control of VLY production by *G. vaginalis* 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].

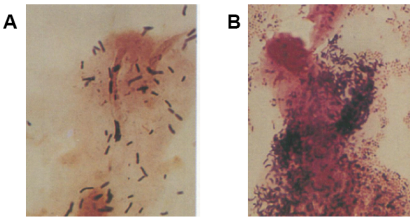



**Fig. 1**. Changes in vaginal microflora during bacterial vaginosis. (A) Normal vaginal microflora is dominated by *Lactobacillus* species (gram-positive rods). (**B**) During bacterial vaginosis, there is an abundance of gram-variable coccobacilli adherent to vaginal epithelial cells (clue cells). 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 $\alpha$-helical portion of the toxin becomes a $\beta$-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)-1$\beta$ 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-1$\beta$ 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-1$\beta$ 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.

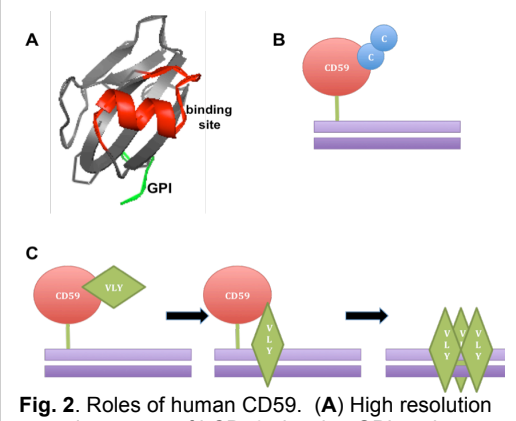



**Fig. 2**. Roles of human CD59. (**A**) High resolution crystal structure of hCD59 showing GPI anchor (green) and complement/VLY binding site (red). (**B**) hCD59 (red) binds terminal complement components (blue, C8 and C9), preventing assembly of membrane attack complex on target cells. (**C**) VLY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

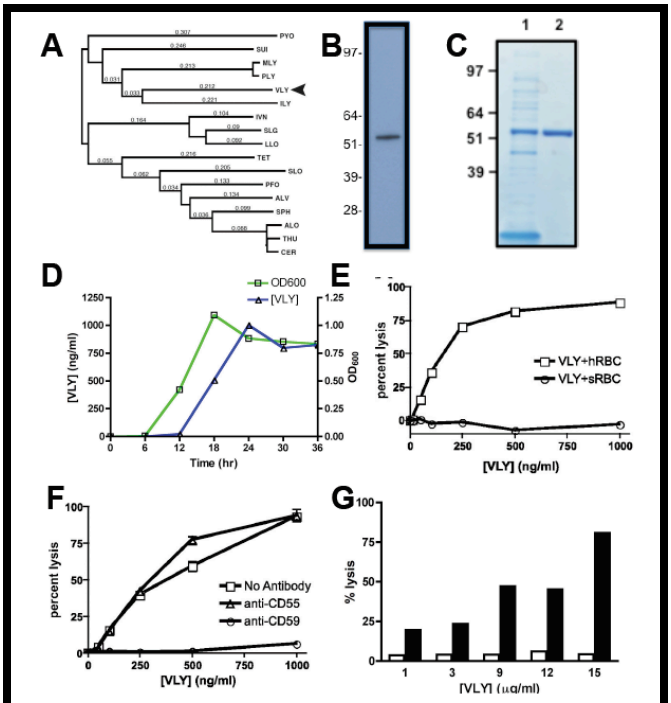



**Fig. 3**. **VLY is a human-specific, hCD59-dependent toxin**. (**A**) Neighbor-joining tree of known CDC sequences showing that VLY is most closely related to ILY. (**B**) VLY is detected as a single band in Western blot of *G. vaginalis* lysate. (**C**) Affinity purification of codon-optimized VLY 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].

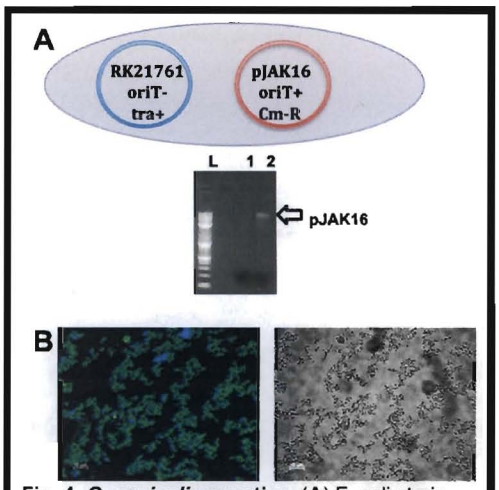



**Fig. 4. *G. vaginalis* genetics.** (**A**) E. coli strain ARE112 has the RK21761 helper plasmid and pJAK16 for mobilization. Inset gel shows alkaline lysis preparations from *G. vaginalis* without (lane 1) and with (lane 2) conjugation to *E. coli.* (**B**) *G. vaginalis* ARG25 expresses GFP from an 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.

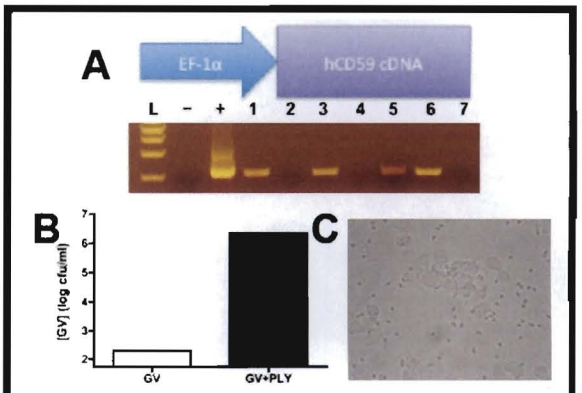



**Fig. 5. New murine models of *G. vaginalis* colonization.** (**A**) hCD59-transgenic mice produced in our laboratory. Lanes 1, 3, 5, 6 indicate mice with transgene. (**B**) Enhancement of murine *G. vaginalis* colonization by co-delivery of purified PLY. (**C**) Vaginal swab wet mount showing epithelial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

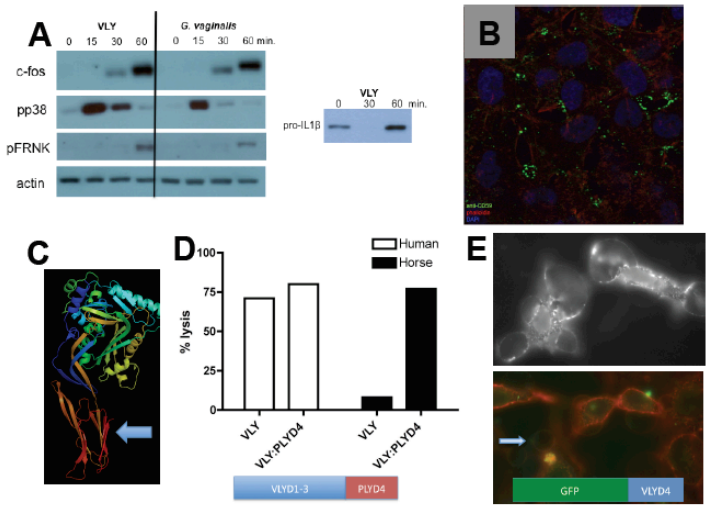



**Fig. 6**. **VLYD4 and host cell responses**. (**A**) Purified VLY and whole *G. vaginalis* induce expression of c-fos and phosphorylation of p38 MAPK and FAK-related non-kinase (FRNK). VLY induces maturation of IL-1β. (**B**) Human cervical epithelial cells express surface hCD59 (green). Nuclei (blue) 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.

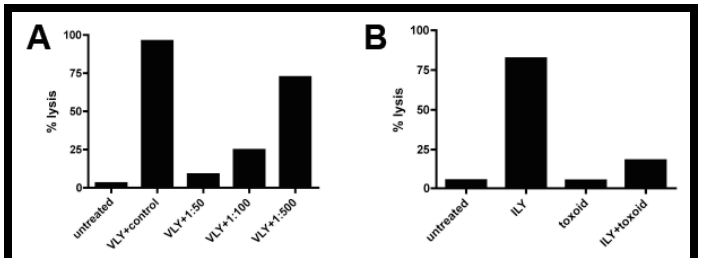



**Fig. 7**. **Inhibition of the VLY:hCD59 interaction**. (**A**) Polyclonal anti-VLY antiserum but not control serum inhibits VLY-mediated cytolysis in a dose-dependent manner. (**B**) VLY toxoid inhibits ILY-mediated lysis of human erythrocytes.

***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).

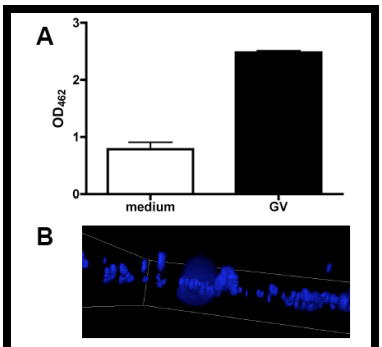



**Fig. 8**. ***G. vaginalis* biofilms.** (**A**) *G. vaginalis* forms biofilms on polystyrene plates as evaluated by the safranin assay. (**B**) Live cell imaging of *G. vaginalis* biofilms in glass-bottomed dishes. *G. vaginalis* communities were multicellular and spanned >20 μm in height.

**<u>Aim 1</u>: 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 <u>library of transposon mutants of *G. vaginalis*</u> 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 <u>targeted disruption of the *vly* gene by homologous recombination</u>. 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 <u>*luxS* deficient mutant</u> (and complemented strain) to explore its role in VLY regulation as well.

***Phenotypic analysis of mutants*** We will <u>screen for transposon insertion</u> 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.