BACTERIAL AND HOST DYNAMICS OF *MYCOPLASMA GENITALIUM* INFECTION OF THE HUMAN FEMALE REPRODUCTIVE TRACT

by

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ABSTRACT

Mycoplasma genitalium (MG) is an emerging sexually transmitted pathogen that is associated with several inflammatory syndromes including cervicitis and pelvic inflammatory disease. Despite the clinical associations of MG with inflammatory sequeale, no experimental evidence was available for the capacity to establish infection of the female reproductive tract and activate host immune responses. This considered, we undertook a multifaceted approach to understanding the capacity and mechanisms of MG to cause inflammatory reproductive tract disease using cell culture models of human epithelia and a novel murine model. In our studies, MG established a long-term intracellular infection of cultured human vaginal, ecto- and endocervical epithelial cells that resulted in pro-inflammatory cytokine secretion. We determined that a significant proportion of the inflammation was activated through ligation of highly expressed Toll-like receptors (TLR) including TLR2/6 heterodimers and TLR9. Based on the responses elicited by exposure of MG to reproductive tract epithelial cells, we next tested and determined that MG was susceptible to killing by human and murine macrophages. Importantly, we then showed that intracellular localization within epithelial cells provided MG a survival niche against macrophage-mediated killing thereby providing a mechanism for evasion of the host immune response. Considering the ability of MG to exploit long-term intracellular survival within epithelial cells, we next developed a murine model of reproductive tract infection to investigate how MG establishes infection and how intact mucosa respond to MG exposure. Similar to cultured human epithelial cells, vaginal lavages of MG-inoculated mice showed significant pro-inflammatory

cytokine responses. Concomitantly, we observed intermittent vaginal shedding for up 77d PI indicating that MG is capable of long-term reproductive tract infection. Most importantly, we also determined that MG rapidly ascended to the upper reproductive tract tissues and disseminated to the knee joints following vaginal exposure. These findings were the first experimental evidence for dissemination of MG to the upper reproductive tract and provided excellent rationale for continued investigation into the capacity of MG to cause reproductive tract disease. Collectively, our results indicate that MG has the capacity to elicit reproductive tract inflammation and should heighten awareness for this emerging pathogen.

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LIST OF ABBREVIATIONS

6-his	6-histidine
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BP	Base Pair
CBA	Cytometric Bead Array
CD	Culster of Differentiation
CCU	Color Changing Unit
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic Acid
d PI	Days Post-Inoculation
EC	Epithelial Cell
ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron Microscopy
FBS	Fetal Bovine Serum
FLAG	Flagellin
FSL-1	Fibroblast-Stimulating Ligand-1
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GLN	Genital Lymph Node
G-CSF	Granulocyte-Colony Stimulating Factor

GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HSV-2	Herpes Simplex Virus-2
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
IL-	Interleukin
IPTG	Isopropyl □-D-1-thiogalactopyranoside
IRB	Institutional Review Board
IRF-3	Interferon Regulatory Factor-3
ISS	Immunostimulatory Sequences
KSFM	Keratinocyte Serum-Free Medium
LAMP	Lipid Associated Membrane Lipoprotein
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MCP-1	Monocyte Chemotactic Protein-1
MDM	Monocyte Derived Macrophages
MIP	Macrophage Inflammatory Protein
MG	*Mycoplasma genitalium*
MOI	Multiplicity of Infection
MyD88	Myeloid Differentiation Factor 88

NCBI	National Center for Biotechnology Information
NF-κB	Nuclear Factor Kappa-B
NGU	Non-gonococcal Urethritis
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEACH	PID Evaluation And Clinical Health
PI	Post-Inoculation
PID	Pelvic Inflammatory Disease
PMNL	Polymorphonuclear Leukocyte
PRR	Pattern Recognition Receptor
QPCR	Quantitative Polymerase Chain Reaction
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
rRNA	Ribosomal Ribonucleic Acid
SEAP	Secreted Alkaline Phosphatase
SEM	Scanning Electron Microscopy
STI	Sexually Transmitted Infection
TEM	Transmission Electron Microscopy
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-Alpha
UV	Ultraviolet

CHAPTER 1: *M. GENITALIUM* AND THE INNATE IMMUNE RESPONSE: DIAGNOSIS, CLINICAL IMPLICATIONS AND PATHOGENESIS

Intracellular reproductive tract pathogens: *C. trachomatis* and *M. genitalium*

A significant driving force in the survival, growth and evolution of microorganisms is their ability to colonize and exploit an environmental niche. Although the vertebrate host is often a secondary environment or reservoir, many microorganisms have adapted to colonize and survive within human tissues. As a consequence, these microorganisms must deal with the host response to their presence and have, in some cases, further exploited specific survival opportunities including the intracellular environment. This is most evident in the necessity of all viruses to replicate within host cells but also is cleverly utilized by a few intracellular bacterial pathogens for enhanced survival.

The Chlamydiae are a ubiquitous group of obligate intracellular pathogens. In humans, *Chlamydia trachomatis* (CT) is a pathogen of mucosal surfaces that is responsible for ocular (serovars A, B, Ba and C) and genital tract (serovars D–K and L1–L3) infections [1]. CT is the most prevalent sexually transmitted bacterium and is responsible for an estimated 89 million new cases of genital infection each year [2] making it a significant public health concern. Importantly, although CT is responsible for significant reproductive tract disease in women, up to 70% of non-chlamydial/non-gonococcal PID cases have no etiologic agent identified [3] suggesting other bacterial causes exist.

Mycoplasma genitalium (MG) is an emerging sexually transmitted bacterial pathogen [4] that, similar to CT, can exploit intracellular survival as a means to persist in the genital tract. MG is a member of the Mycoplasmataceae family of mycoplasma-like organisms (MLO). Mycoplasmas are ecologically ubiquitous as parasites of humans, mammals, reptiles, fish, arthropods and plants [5] and seem to exhibit somewhat identifiable host and tissue tropisms. For many years early last century, mycoplasmas were presumed to be viruses primarily because they are markedly smaller than other bacteria and easily pass through 0.45uM and 0.22uM filters [6]. All mycoplasmas belong to the class Mollicutes, a group of organisms recently re-classified into the phylum Tenericutes based on irregular pleomorphic cell shape and the absence of a rigid cell wall [7]. From a human disease standpoint, the most important mycoplasmas belong to the Mycoplasmataceae family in which two genera, *Mycoplasma* and *Ureaplasma*, collectively contain over 100 organisms including both pathogenic and non-pathogenic species [5]. The most notable human pathogens of this group are MG, *M. pneumoniae* and *Ureaplasma urealyticum*. Indeed, because MG and *M. pneumoniae* share so many features, MG was suspected as a human pathogen soon after its initial isolation in 1980 [8].

Isolation of *M. genitalium* and historical perspective

MG was first isolated in 1980 from 2 urethral swab specimens obtained from men with symptomatic non-gonococcal urethritis (NGU; [8]). David Taylor-Robinson transferred swabs from 13 men with NGU to Joe Tully's laboratory intending to isolate

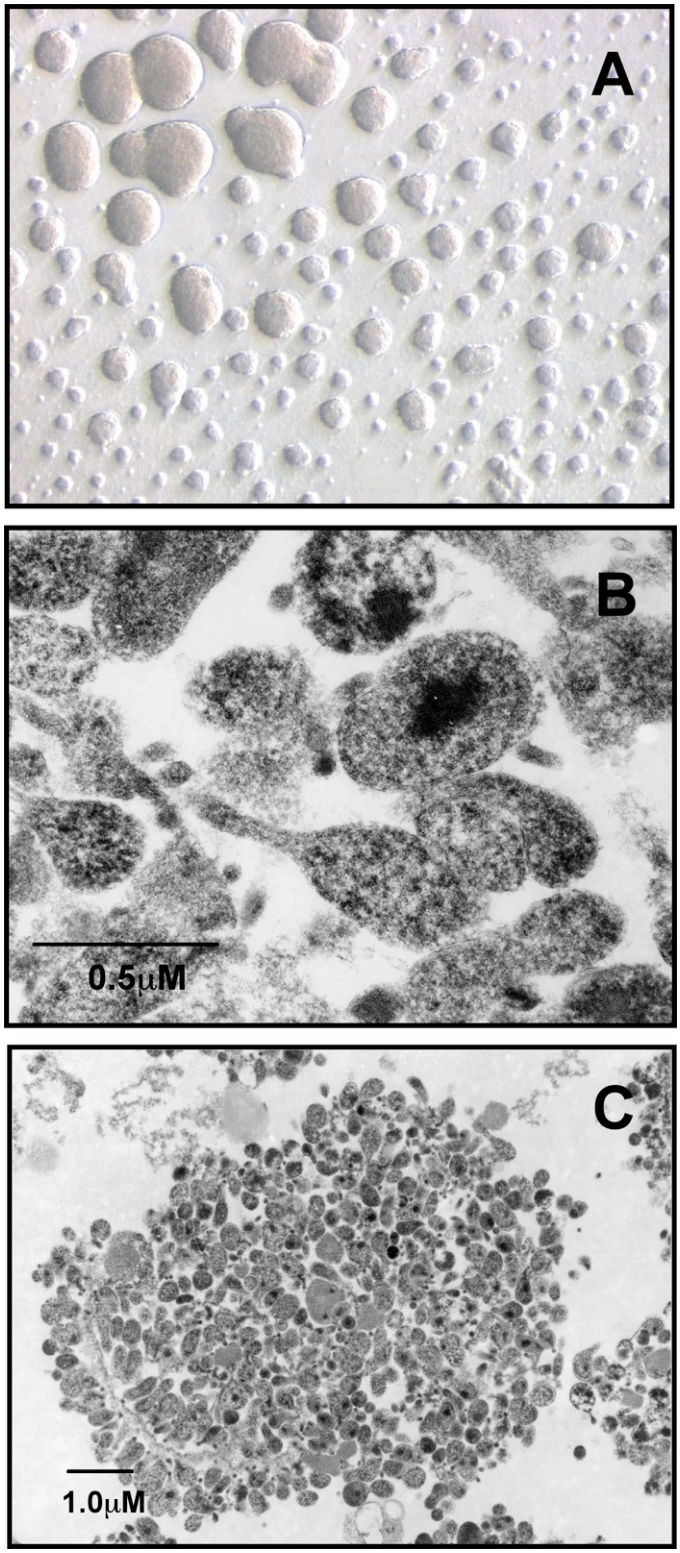


Figure 1. *In vitro* cultivation of MG. (A) Adherent microcolony formation following approximately 3-5d growth in Friis FB medium. (B) Following harvest of MG strain M2300 grown to late log phase, mycoplasmas are highly pleomorphic by TEM. (C) Gentle harvest of adherent microcolonies revealed tight association of mycoplasmas within the microcolony.

spiroplasmas, spiral shaped MLO with disputed importance as human pathogens. Using SP4 medium, microbial growth was observed from 2 specimens as indicated by a pH-mediated color change of the culture medium [8]. These isolates were denoted as G37 and M30 and were subsequently designated *Mycoplasma genitalium* because of serological characteristics distinct from other known mycoplasmas.

Since the original 1980 isolation of the G37 and M30 strains of MG only minimal progress has been made to enhance clinical isolation of the viable organism. In 1985, Taylor-Robinson's group failed to culture MG from urogenital specimens but concluded that a non-specific color change in SP4 medium qualified as a presumptive diagnosis and isolation [9]. Similar to Taylor-Robinson, Samra *et al*. observed a pH-mediated color change in several clinical specimens of variable types but were unable to culture MG or any other pathogenic microbes [10].

However, that same year, Joel Baseman and colleagues at the University of Texas-San Antonio isolated 4 MG strains from the upper respiratory tract as mixed cultures with *M. pneumoniae* [11] shaking the dogma that MG was exclusively an inhabitant of the genital tract. In 2004, Baseman *et al*. reported isolation of viable MG using SP4 medium from approximately 4% of vaginal and cervical swabs from minority women [12]. However, there is some question as to the kinetics of isolation as most isolates were detected between 14 and 20d, much faster than the more than 50d required for the original G37 isolation by Tully and colleagues. Although isolation of viable MG from male and female urogenital specimens has been cumbersome, extended co-culture

of clinical specimens with Vero cells [13] did marginally enhance isolation compared to previous methods.

Unfortunately, this method is still too labor-intensive and requires significant time from collection of clinical specimens to detectable MG growth. Collectively, cultivation of viable organisms from clinical specimens has proven ineffective for clinical MG diagnosis and thus a number of alternative approaches including PCR and serology have been investigated (Refer to Chapter 2).

Epidemiology and establishment of *M. genitalium* as a sexually transmitted pathogen

Following the initial isolation of MG, very few epidemiologic studies were undertaken largely because of difficulties in culturing the organism. Using PCR-based methodologies, an extensive evaluation of MG prevalence in healthy young adults (i.e. those not attending an STD clinic) was undertaken. This study utilized urine specimens from 1714 women and 1218 men who participated in Wave III of the National Longitudinal Study of Adolescent Health study and showed that the prevalence of MG was approximately 1% compared with 0.4%, 4.2%, and 2.3% for *N. gonorrhoeae*, CT, and trichomonas infections, respectively [4]. These findings indicated that MG is present in the general American population at a prevalence similar to other genital pathogens and provided rationale for investigation into whether MG is a cause of urogenital disease.

The earliest evidence for MG's role in urogenital disease came from several studies addressing whether MG was an independent cause of NGU in men. Of 5455

patients examined in 23 published manuscripts, the overall MG prevalence in men with NGU was 20.8% (470/2261) compared to men without urethritis that had a prevalence of 5.9% (124/2107) (Reviewed in [14]). Importantly, MG was detected in more patients with urethritis than those without symptoms in each of the studies. This data is striking considering the wide-ranging case definitions of urethritis that were employed among these independent studies. Furthermore, MG DNA burdens were found to positively correlate with increasing severity of NGU symptoms [15]. It is important to note that infection by MG is largely independent of CT [16], a notable cause of NGU, and is estimated to be symptomatic in greater than 70% of infected males [17]. After considerable investigation, it has been concluded that MG is an independent cause of inflammatory urogenital disease in men.

Importantly, a growing body of literature has addressed the question of whether MG is transmitted through sexual contact and, collectively, has provided strong supportive evidence (Reviewed in [14]). Compared to CT with transmission frequencies between 26% [18] and 68% [19], MG appears to be transmitted with similar efficiency (up to 58%; [16]) using PCR-based detection methods. Thus, several studies have focused since on the role of MG in various female reproductive tract syndromes including cervicitis, urethritis, pelvic inflammatory disease (PID) and tubal-factor infertility.

Cervicitis (formerly termed mucopurulent cervicitis, see [20]) is defined clinically by the presence of either mucopurulent discharge, friability at the external os (easily-induced bleeding), edematous ectopy or the presence of elevated polymorphonuclear

leukocytes (PMNL) typically detected by Gram staining of endocervical swab material [20]. The case definition of cervicitis is variable among clinicians therefore making interpretation and comparison of clinical data difficult. For example, measuring PMNL in cervical secretions has varied from the simple observation of more PMNL than epithelial cells but often include a quantitative threshold ranging from >10 to >30 PMNL/high powered field (hpf). Using varied case definitions, MG has been implicated as an independent cause of cervicitis in several studies [21-23]. In the largest and most recent study, Manhart and colleagues found that MG was strongly associated with cervicitis (>30 PMNL/hpf) as it was detected in 7% of all endocervical specimens but in approximately 11% of women with cervicitis [21]. After removing individuals co-infected with CT or *N. gonorrhoeae*, the presence of MG was still associated with a 3.3-fold greater risk of cervicitis. Taken together, these studies indicate strongly that MG could be a cause of cervical inflammation and should be considered an etiologic agent, particularly when endocervical swabs are negative for other known pathogens.

Because MG is associated with cervicitis, long-term infection of these tissues also could lead to ascending infection of upper reproductive tract tissues. Indeed, MG has been a suspected cause of secondary sequelae due to ascending infection such as endometritis, PID and infertility. In the PID Evaluation and Clinical Health (PEACH) study that included 831 women with suspected PID, the MG prevalence was similar to CT and *N. gonorrhoeae* (CL Haggerty, personal communication). In addition, 5 other PCR-based studies have associated MG with PID or endometritis [3, 24-27]. In a study of 115 women at a STD clinic in Nairobi, Kenya with histologically-confirmed

endometritis, MG was found significantly more often in women with endometritis (16%) compared to women without symptoms (2%) [24]. With regard impaired fertility, only indirect serological associations with MG exist and additional studies are necessary [28, 29]. Clinically, MG has been detected by PCR in human upper genital tract specimens [3, 30] but the prevalence values were low and will require additional studies to firmly show that MG can ascend to the upper reproductive tract tissues. In Chapters 6 and 7, we employ a novel mouse model to investigate the capacity of MG to establish vaginal infection and disseminate to upper genital tract tissues following vaginal exposure.

Treatment of *M. genitalium* and chronic reproductive tract disease

Evaluation of MG treatment efficacy has been a subject of significant interest but lacks experimental data largely because only a few randomized controlled clinical trials have been undertaken. In female patients diagnosed with PID, the Centers for Disease Control and Prevention guidelines recommend therapy with ofloxacin, levofloxacin, ceftriaxone plus doxycycline or cefoxitin and probenecid plus doxycycline [31]. However, several reports in both men and women suggest these treatment regimens would be ineffective for eradicating MG as NGU persists in a significant proportion of patients treated with tetracyclines [32-35] and levofloxacin [32, 36]. In addition, treatment failure in men with NGU using azithromycin has resulted in the resistance of MG to macrolide antibiotics [37] suggesting that partially effective regimens of antibiotic therapy may promote the development of resistance. Finally, ineffective treatment of

patients with undiagnosed MG infection could also contribute to MG's development of antibiotic resistance.

Although limited data is available, treatment failure in women is of utmost importance as prolonged inflammation at upper genital tract sites may lead to significant morbidity and impaired fertility [38]. To this end, a better understanding of how MG establishes infection and interacts with the human reproductive mucosa will serve to provide mechanistic rationale for targeted development of antimicrobial therapeutics and preventative measures.

THE MUCOSAL INNATE IMMUNE RESPONSE

The female reproductive tract

The female reproductive tract includes the normally sterile upper genital tract composed of the uterus, fallopian tubes and ovaries, and the polymicrobial environment of the lower genital tract including the vagina and transitional zone of the ecto- and endocervix [38]. Epithelial cells (ECs) of the reproductive mucosa provide a physical barrier against invading pathogens and are regarded as non-traditional immune cells capable of activating the early innate immune response [39]. The female reproductive tract mucosa, most notably at the vagina, must be highly sensitive to pathogen exposure and simultaneously tolerant to the diverse microflora normally present at these sites. Currently, the mechanisms for maintaining this tightly regulated balance are largely unknown. However, reproductive tract ECs are known to recognize several classes of conserved pathogen-associated molecular patterns (PAMP) via the Toll-like receptors

(TLR; described in more detail below) that activate the innate response through secretion of various inflammatory mediators. Throughout this dissertation, we investigate how MG interacts with the vaginal and cervical epithelia and, specifically, the mechanisms by which MG causes inflammation. Continued investigation of TLR responses provides a greater understanding of the early events following pathogen exposure and how MG establishes and maintains urogenital infection in reproductive tract tissues.

Toll-like receptors

The TLR are a highly conserved family of type I transmembrane receptors first identified through molecular investigations of Toll, a gene necessary for protection of *Drosophila melanogaster* from fungal infection during embryogenesis [40]. More recent studies in mammalian host cells have established a direct relationship between specific TLR and well-defined microbial compounds responsible for activation of the innate immune response. Activation of these molecules ultimately results in inflammation through secretion of cytokines and other immunostimulatory molecules. Thirteen TLR proteins have been identified in mammals that work alone or in combinations to recognize a growing list of TLR agonists produced by microbial infection. Human TLR are expressed selectively by many cell types including EC and immune cells and recognize highly conserved PAMPs either as single complexes (e.g. lipopolysaccharide is recognized by TLR4/CD14 alone) or in combinations (e.g. bacterial lipoproteins are recognized by TLR2 and 6 in tandem).

Following ligation of the TLR, an intracellular signaling cascade is activated through MyD88 or an alternate MyD88-indpendent pathway involving interferon response factor 3 (IRF-3; [41]). MyD88 pathways lead to activation and nuclear translocation of the transcription factors NF-κB and JNK/AP-1 that initiate a transcription cascade designed to increase innate resistance to invading pathogens. IRF-3 directly initiates transcriptional cascades that produce similar anti-microbial outcomes. Importantly, TLR activation results in secretion of potent cytokines and chemokines from stimulated cells that are important for recruitment of other immune cells to the site of infection [41].

ECs of the vagina and cervix express robust levels of TLR2, 3, 5, 6 and CD14 with low levels of TLR1, 4 and 7-9 and are responsive to TLR stimulation with several bacterially-derived agonists [42]. The TLR2/6 heterodimer is hypothesized to be important for recognition of bacterial ligands by reproductive tract ECs because of the increased expression level and immunologic responsiveness relative to other TLR [42]. Considering the intimate extra- and intracellular association of MG with reproductive tract ECs, we have investigated the role of TLR2, 6 and 9 for recognition of the viable MG organism and specific MG components including several antigenic proteins.

M. genitalium outer membrane & adhesion to host cells

Both MG and *M. pneumoniae* have been described to have a flask- or club-like morphology with an electron-dense tip structure believed to be involved in attachment to eukaryotic cells (Reviewed in [5]). Some mycoplasmas utilize a cytoskeleton for motility

and therefore are able to form rigid shapes despite the lack of an outer membrane structure [6]. Interestingly, complete genomic analyses of MG and *M. pneumoniae* have failed to show any homology among eukaryotic or other bacterial actin-related genes [43, 44]. The single exception noted to date is a tubulin-like protein that was identified in MG [45]. More than 10 other cytoskeletal proteins have been identified in *M. pneumoniae* [5] but similar analyses of the MG genome have not been reported. Importantly, MG is a motile organism that uses a novel but poorly understood gliding-type motility (Reviewed in [46]) suggesting that some cytoskeleton system is employed.

An apparent compensation for the lack of a cell wall, MG and other MLO encode a large number of membrane lipoproteins in their small genome. The dense layer of lipoproteins, that span the single lipid bi-layer, is the outermost cellular structure that interacts with the environment. Consequently, a number of membrane proteins have been identified as immunogens in humans [47] and are likely targets of the host innate and adaptive immune responses as MG intimately interacts with host cells. To this end, we investigate whether viable MG and 3 representative antigenic membrane proteins activate highly expressed TLR of the female reproductive tract to evaluate their capacity to elicit inflammation (Refer to Chapter 4).

TLR recognition of microbial membrane proteins

It is well documented that membrane proteins from other pathogenic *Mycoplasma spp*. can interact with TLR leading to inflammatory cytokine production [48]. Like other MLO, MG contains a significant proportion of lipoproteins in the cell membrane [49]

consistent with the identification of 21 genes that encode N-terminal lipidation signals throughout the 580kb genome [43]. It has been shown recently that Triton X-114 preparations of detergent-soluble components from MG upregulate transcription of TNF-α, IL1-β and IL-6 mRNA in THP-1 human monocytic cells [48, 50] suggesting that lipoproteins may, at least in part, be responsible for immune activation. A recent report showing that MG149 activates NF-κB via TLR1/2 [51] further supports the hypothesis that MG is capable of eliciting innate immune responses via cell surface TLR. However, TLR1 expression in female genital ECs [42] and male penile urethral ECs (MM Herbst-Kralovetz and RB Pyles, unpublished finding) is very low so the relevance of TLR1/2 heterodimers for MG recognition is not clear. Importantly, no previous studies have addressed TLR activation by MG. In Chapter 4, we investigate EC recognition of MG and the inflammatory capacity of 3 TLR-activating MG membrane proteins. Then, we extend our findings from Chapter 4 to evaluate the utility and efficacy of select TLR-activating MG components as vaginally applied immunomodulating microbicide candidates (Refer to Chapter 8).

Immune evasion by *M. genitalium* via antigenic variation

Considering that MG encodes a number of antigenic membrane proteins [43], it is implied that these proteins could be targets of the host immune system. Despite the relatively small size, approximately 4% of the MG genome represents repeated genetic elements [52] including regions within the MgPa operon, specifically in the *mgp*B and *mgp*C genes [43]. MgPa is a major immunodominant adhesin protein [47, 49, 53, 54]

and, similar to P1 of *M. pneumoniae*, has an important role for attachment of MG to host cells [53]. The gene encoding MgPa is within an operon containing 3 genes: MG190 (*mgp*A), MG191 (*mgp*B) and MG192 (*mgp*C). Transcription of these genes occurs only at a single site within the genome but 9 truncated copies of MG191 and MG192 are located throughout the genome at sites designated MgPa repeats or MgPar regions. The sequence identity among the repeated elements and the coding operon ranges from 78-90% [43] and have been shown to be involved in homologous recombination with the MgPa coding region [55-58]. These findings suggest that outer membrane proteins such as MgPa are under immune-mediate pressure and that recombination could be an efficient strategy to generate antigenic variation.

It has been demonstrated previously that significant sequence diversity within the MgPa operon exists within and among cultured MG strains, [55-58] clinically-obtained reproductive tract specimens [55-57] and from samples collected following experimental infection of chimpanzees [59]. In turn, variability of the immunogenic epitopes of outer membrane proteins might allow for evasion of host immune responses and facilitate long-term reproductive tract infection. However, additional evidence is needed to understand whether MgPa and other membrane proteins are targeted by the immune system. In Chapter 4, we select several antigenic membrane proteins that are expressed during MG genital tract infection and investigate whether they are recognized by relevant human TLR. In addition, animal studies in Chapter 7 determine which MG proteins elicit the most robust antibody responses following long-term reproductive tract infection in mice.

Animal models of *M. genitalium* reproductive tract infection

As epidemiological data have convincingly implicated MG as a cause of reproductive tract disease in women, the lack of a reproducible small animal model has been an impediment to the continued investigation of pathobiology and evaluation of therapeutic interventions. Only 5 years after the initial clinical isolation of MG, several large animal species were found susceptible to experimental urogenital MG infection [60]) providing early evidence that MG could be a pathogen of the human genital tract (Refer to Chapters 6 and 7). Studies in the early 1990's by David Taylor-Robinson and colleagues provided preliminary evidence that the type strain of MG (G37) could establish lower reproductive tract infection in female balb/c mice [61]. These studies provided essential evidence that MG could colonize the reproductive tract of female mice but, to date, no subsequent reports have been made using the described model.

To address the need for a small animal model of genital MG infection, we set out to develop a novel murine model to investigate MG reproductive tract pathogenesis and immunobiology. Specifically, a model was necessary to better understand the early immunological events following sexual transmission and how MG evades the host immune response to disseminate and establish long-term infection of reproductive tract tissues. The animal studies conducted in this body of work were designed to establish a reproducible platform to study MG reproductive tract infection and to confirm, when possible, many observations made previously in cell culture models. Utilizing the animal model allowed for an extension of the reductionist approaches used in cell culture- and molecular biology-based experiments to better understand the dynamic interaction of this

intracellular STI pathogen with tissues of the reproductive tract. The animal model facilitated a better understanding of how the cervical and vaginal mucosa respond to MG, and together with specific studies of cultured vaginal and cervical ECs, allowed us to dissect the role of ECs for initiation of the innate immune response following MG exposure.

SCOPE OF THESIS

The overarching hypothesis of this project is that *M. genitalium*, an emerging sexually transmitted pathogen, is capable of long-term intracellular infection of reproductive tract ECs thereby activating the innate immune response and eliciting inflammation. We first addressed this supposition by evaluating intra- and extracellular MG infection dynamics with cultured human vaginal, ecto- and endocervical ECs. Next, we elucidated and compared the host cytokine responses to MG infection from reproductive tract ECs and determined whether TLR signaling was involved. To address persistent reproductive tract infection, we determined whether exploitation of intracellular EC survival offered a niche for immune evasion and protection from phagocytic macrophage responses. Finally, using a novel murine model we investigated the capacity of MG to colonize and disseminate to reproductive tract tissues to establish rationale for continued investigation of upper reproductive tract disease in women.

CHAPTER 2: ENHANCED DIAGNOSTIC IDENTIFICATION AND PCR QUANTIFICATION OF *M. GENITALIUM* AND *C. TRACHOMATIS*

OVERVIEW

Sexually transmitted infections (STI) are caused predominately by bacterial pathogens including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum* and the genital mycoplasmas including *Mycoplasma genitalium*. Both *C. trachomatis* and *M. genitalium* are organisms that can establish intracellular infection of reproductive tract EC and thus require special focus for development and testing of diagnostic tools. The need for sensitive and specific clinical and laboratory approaches for quantification of *C. trachomatis* and *M. genitalium* is very high; quantitative real-time PCR assays are an attractive strategy for both pathogens. In this chapter, we describe the development, optimization and validation of two quantitative PCR assays for *C. trachomatis* and *M. genitalium*. Many of the basic approaches were established and validated using the *C. trachomatis* assay and then exported for development of the *M. genitalium* assay that is used throughout this project. Collectively, the findings from this chapter indicate that carefully designed and thoroughly optimized PCR assays for quantification of *C. trachomatis* and *M. genitalium* are a highly sensitive and informative method for clinical and preclinical testing. Portions of this chapter have been published by McGowin et al., *Diagn Microbiol Infect Dis*, January 2009.

INTRODUCTION

The most prevalent sexually transmitted bacterial infections of the genital tract are those caused by *Chlamydia trachomatis* (CT), *Treponema pallidum* (syphilis) and *Neisseria gonorrhoeae* [31]. Other bacterial STI include the genital mycoplasmas, most notably the emerging pathogen, *Mycoplasma genitalium* (MG). With all bacterial STI, early diagnosis is important because long-term infection of reproductive tract tissues can lead to inflammatory syndromes including cervicitis, PID and, in severe cases, tubal infertility and ectopic pregnancy [62]. These consequences have intensified the need for more sensitive and cost-effective tools for clinical diagnostics and pre-clinical evaluation of novel therapeutics for STI.

CT is a gram-negative, obligate intracellular bacterium implicated in ocular and genital tract infections of humans. CT (serovars D-K) is the most prevalent sexually transmitted bacterial pathogen worldwide [2] and is the leading cause of non-gonococcal urethritis (NGU) and mucopurulent cervicitis in males and females, respectively [63]. Although CT infections in women are often subclinical, PID, endometritis and salpingitis can result from ascending infection of the upper genital tract and may have significant deleterious effects on reproductive health [63, 64].

Specific nucleic acid amplification tests (NAATs) including the Gen-Probe APTIMA Combo 2 (Gen-Probe, San Diego, CA), Roche Cobas Amplicor (Roche Diagnostics, Pleasanton, CA) and BD ProbeTec ET (Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ) [65-68] have become the gold standard for clinical diagnosis of CT but are not ideal for high-throughput laboratory use. Therefore, we

developed and tested a PCR assay for clinical and preclinical use that would quantify the most prevalent genital serovars of CT and the *Chlamydia muridarum* Nigg II strain (formerly MoPn). The assay targeted a highly conserved region within the single copy OmpA gene that encodes the major outer membrane protein. By sequence comparisons of the OmpA gene from clinical CT serovars D, E, F, Ia, and *C. muridarum* we identified and targeted a region located at the 3' end of variable domain IV [69] that showed greater than 90% homology among all of the analyzed serovars.

Using similar methodologies, we sought to develop a PCR assay for clinical and preclinical quantification of MG, an emerging sexually transmitted bacterial pathogen implicated in inflammatory reproductive tract syndromes in both men and women. In men, MG has been shown to be a cause of symptomatic urethritis (Reviewed in [70]) and [14]) similar to *Neisseria gonorrhoeae* and CT. In women, MG infection has been strongly associated with PID [71] and cervicitis [21, 23, 72-74] independent of other pathogens including CT. Similar to CT, MG also appears to localize to intracellular spaces of reproductive tract epithelial cells (EC; Refer to Chapter 3) making the approach to diagnosis similar. Despite a considerable amount of research, there currently is no standard approach for diagnosis of genital MG infections nor has any clinical assay been approved by the Food and Drug Administration for clinical use.

Although serological approaches have been somewhat successful (Reviewed in [14]), the most effective diagnostic tools for urogenital MG infections have been PCR-based methods for detection of genomic DNA. The most qualifying characteristic of PCR-based assays is their inherent amplification-mediated sensitivity, an important point

considering the extremely low DNA load in urogenital specimens from male [75] and female [76] MG patients. Jensen and colleagues reported the first PCR assay for MG detection in 1991 [77] followed by more than 15 additional published assays with variable success (Reviewed in [14]).

Importantly, a PCR-based assay with quantitative utility would be beneficial from an experimental and clinical perspective. Yoshida *et al*. reported the first real-time PCR assay for MG detection based upon the 16s rRNA gene [15] but questions have been raised [14] about primer specificity and the strong secondary structure and extensive conservation of 16s rRNA genes among all bacteria that make development of specific PCR assays inherently difficult. In our laboratory, we have developed and validated a TaqMan real-time PCR assay targeting the single-copy MG309 gene. Little is known about the diversity of MG309 among clinical and laboratory MG strains but, to date, we have employed this assay for detection of 5 Danish clinical strains, the type strain G37 and multiple experimental isolates from laboratory animals (See Chapters 6 and 7) with a very low limit of detection and excellent specificity. Quantitative real-time PCR detection systems, although requiring skilled laboratory staff, are the most attractive and cost-effective strategy for high-throughput detection and quantification of bacterial pathogens from diverse clinical and experimental samples. In this chapter, we describe the development and thorough testing of two qPCR assays for detection of MG and CT from clinical and preclinical specimens.

MATERIALS AND METHODS

Bacterial Isolates

Clinical isolates of CT serovars D, E, F and Ia, propagated in HeLa 229 cells, were kindly provided by Dr. Alison Quayle, Louisiana State University-Health Science Center, USA. The *C. muridarum* MoPn biovar strain Nigg II (ATCC VR-123)**,** propagated similarly in McCoy-B cells (ATCC CRL-1696), were maintained in Minimum Essential Medium (MEM; Invitrogen) supplemented with 1x antibiotic-antimycotic, gentamicin (0.1mg/mL) and 10% fetal bovine serum. Infectious CT titers were determined by titration on McCoy-B cells using standard methods and expressed as inclusion forming units (IFU).

MG type strain G37 (MG G37; ATCC 33530) or 5 low-passage Danish strains, denoted M2300, M2341, M2321, M2282 and M2288 (kindly provided by Jorgen Jensen, Staten Serum Institut, Copenhagen, Denmark) were propagated in Friis FB medium [13]. Briefly, frozen MG stocks were inoculated aseptically into tightly sealed tissue culture flasks containing freshly prepared Friis FB medium and incubated at 37°C for 5-8d until harvest for DNA extraction.

Purified PCR-quality DNA from CT, normal and abnormal flora of the human urogenital tract including *Lactobacillus spp.*, *Prevotella bivia*, *Mobiluncus spp.*, group B *β Streptococcus spp.*, *Mycoplasma genitalium* and *Atopobium vaginae* were provided by David H. Martin (Louisiana State University-Health Science Center, USA). Other tested species including *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli K12*, *E. coli* 083, *E. coli* 0157, *E. coli* HS, *Klebsiella pneumoniae* and *Francisella*

tularensis LVS were kindly provided by Tonyia Eaves-Pyles (University of Texas Medical Branch, USA).

DNA Extraction

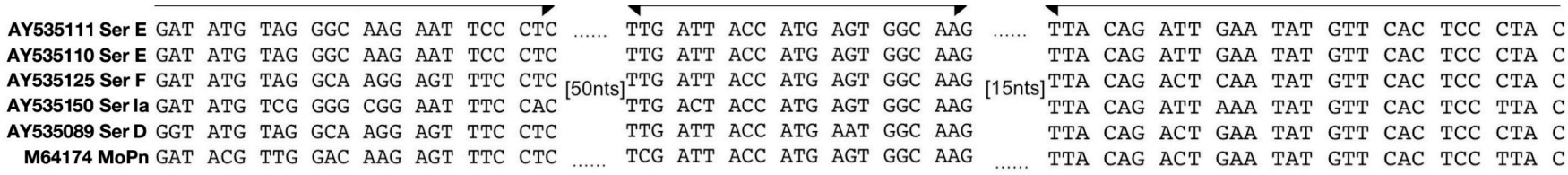
All specimens were processed using the DNeasy 96 Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, eluted into 100 or 200uL of the kit-provided elution buffer and stored at -20° C until PCR reactions were assembled. The selected bacterial species used to test the cross-reactivity of the CT PCR assay were extracted similarly, quantified using spectrophotometry (A_{260}) and then diluted to 10ng of genomic DNA per reaction (~10^6 genomic equivalents).

Real-time quantitative PCR assay development and validation

***C. trachomatis* OmpA assay**

Using Beacon Designer v. 2.04 (Premier Biosoft, Intl., Palo Alto, CA, USA), candidate primer pairs and TaqMan probes (Roche Molecular Systems, Pleasanton, CA, USA) were designed to specifically amplify conserved regions of the major outer membrane protein gene in CT, OmpA. OmpA sequences for clinical CT Serovars D, E (2 isolates), F, Ia and *C. muridarum* strain Nigg II (formerly MoPn) (GenBank Accession no. AY535089, AY535110 and AY535111, AY535125, AY535150 and M64174, respectively) were aligned using the ClustalW algorithm in MacVector v7.0 (Accelrys, San Diego, CA, USA) to select a highly conserved region for primer and fluorogenic probe design (Table 1).

Table 1. ClustalW nucleotide alignment of the primer and TaqMan probe binding positions for *C. trachomatis* serovars D, E, F, Ia, and *C. muridarum* (MoPn) OmpA sequences.



For each PCR reaction, 5uL of DNA template was added to 16.5uL of BioRad iQ Supermix (100mM KCl, 40mM Tris-HCl, 1.6mM dNTPs, 0.825U of iTaq DNA polymerase and 6mM $MgCl_2$), 7.5 pMol of each primer (sense primer, 5'-GATACGTTGGACAAGAATTCCCTC-3' and antisense primer, 5'-GTAAGGAGTGAACATATTCAGTCTGTAA-3') and 11.25pMol of the TaqMan probe [5'-(6-FAM)CTTGCTTGCCACTCATGGTAATCGA($BHQ1_A$)-3'] that was then brought to a final volume of 25uL with sterile RNAse/DNAse-free water. Two-step cycling parameters were as follows: Initial denaturation at 95° C for 3 min followed by 50 cycles of 95° C for 20 sec and 63.5° C for 45 sec. A standard curve was generated using 10-fold serial dilutions of cloned OmpA that had been pre-quantified using spectrophotometry (260nm). The cloned OmpA standard dilutions were used as template in duplicate reactions for each PCR run and used to extrapolate unknown experimental CT DNA loads.

Experimental human clinical or murine specimens were normalized using the single-copy glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene target. Human GAPDH was quantified as described previously [78]. For each murine GAPDH PCR reaction, 5uL of DNA template was added to 16.5uL of BioRad iQ Supermix (100mM KCl, 40mM Tris-HCl, 1.6mM dNTPs, 0.825U of iTaq DNA polymerase, and 6mM $MgCl_2$), 5pMol of each primer (sense primer, 5'-TGCTCCTCCCTGTTCCAGAGA-3' and antisense primer, 5'-AATCCGTTCACACCGACCTTCA-3') and 7.5pMol of the TaqMan probe [5'-(5-TX-RED)TCTTCTTGTGCAGTGCCAGCCTCGTCC($3BHQ2_A$)-

3' in a final volume of 25uL. Real-time PCR was performed using the iQ5 multicolor real-time PCR detection system (BioRad, Hercules, CA). The data was analyzed using the iQ5 Optical System software v1.0 to generate amplification plots and calculate PCR efficiencies. The results presented herein are representative of data generated by multiple operators (n=4) within the laboratory.

***M. genitalium* MG309 assay**

All specimens were processed using the DNeasy 96 or DNeasy Blood and Tissue Spin column kit (Qiagen) as described above. Using Beacon Designer, the G37 genome (GenBank L43967) was used to design specific primers and TaqMan probe for real-time PCR targeting of the single-copy MG309 gene. The sense primer (5'-TTTACACCGAGTAAAATGACCATT -3'), antisense primer (5'-AGATTTTCAATGGTTCTGGAGTTT -3') and TaqMan probe [5'-(6-FAM)ACAGGCAACAGCAACAATTCCCAA(BHQ1A)-3']. The reaction components were assembled similar to the CT OmpA assay described above. Cycling parameters were 95°C for 5 min, followed by 50 cycles of 95°C for 15sec, 50°C for 32sec and 72°C for 15sec. A standard curve of a pre-quantified MG309-containing plasmid was run in duplicate on each 96-well plate and used to extrapolate MG DNA loads. Several types of controls for non-specific amplification were employed throughout this project and included PCRs that lacked template and mock-inoculated animal samples. We also compared the quantitative accuracy of the MG309 assay to a previously published TaqMan-based PCR assay targeting the MG GAPDH gene [79]. The MG309 and MG

GAPDH real time PCR assays were completed using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) and the data presented in this chapter and throughout this project represent multiple users within the laboratory.

RESULTS

C. trachomatis detection and qPCR performance

In order to determine the utility of the CT OmpA qPCR assay we tested whether the assay would identify the most common genital serovars of CT and the widely used *C. muridarum* biovar for animal modeling of CT disease. All tested CT serovars including D, E, F, Ia and *C. muridarum* were reproducibly quantified from infected cell cultures (Table 2).

The linear range of detection spanned from 2log10 to 10log10 copies per reaction (Fig. 2). Although we report a lower limit of linear detection of 100 copies per reaction, 10 copies were detected in 50% of reactions (n=20, data not shown). The interassay reproducibility, completed by 2 technicians, showed that the complete linear range of detection was characterized by coefficient of variation (CV) values of less than 4% among six PCR runs (Fig. 2).

Table 2. Microorganisms (n=20) and relevant human and murine specimens tested

Genus[a]	Species, serovar, biovar[a]	Result
Chlamydia	***C. trachomatis* Serovar D, E, F, Ia**	**Pos**
Chlamydia	***C. muridarum* Nigg II**	**Pos**
Lactobacillus	*Lactobacillus spp.*	Neg
Prevotella	*P. bivia*	Neg
Mobiluncus	*Mobiluncus spp.*	Neg
Streptococci	Group B β *Streptococci spp.*	Neg
Mycoplasma	*M. genitalium*	Neg
Atopobium	*A. vaginae*	Neg
Pseudomonas	*P. aeruginosa*	Neg
Listeria	*L. monocytogenes*	Neg
Escherichia	*E. coli K12, O83, O157, HS*	Neg
Klebsiella	*K. pneumoniae*	Neg
Francisella	*F. tularensis (LVS)*	Neg
uninfected human urine		Neg
human vaginal and cervical flora		Neg
murine vaginal flora		Neg

for specificity of the CT real-time qPCR assay.

[a]Selected organisms represent normal and abnormal flora isolated from the female genital tract and other mucosa.

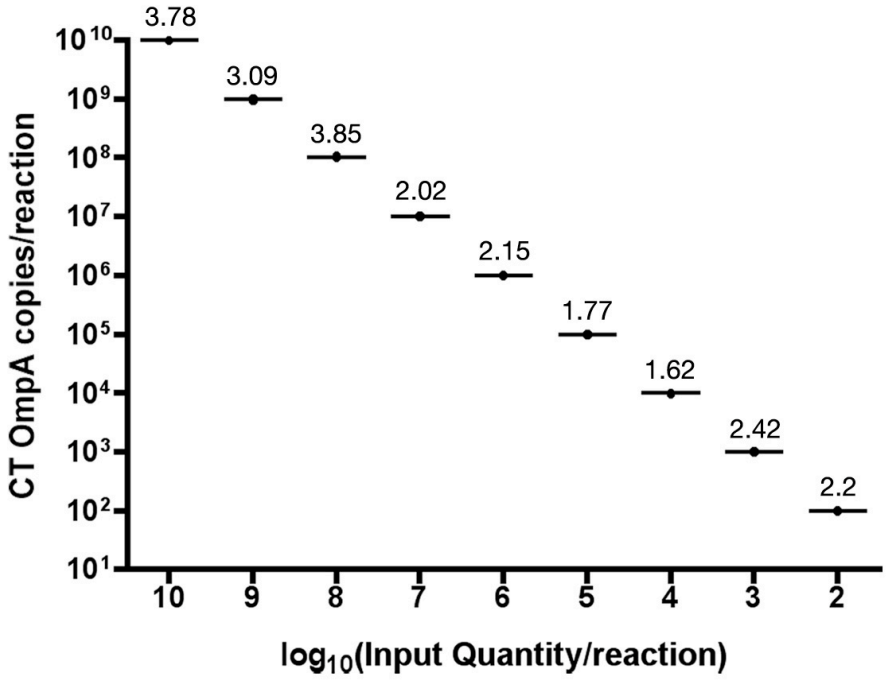


Figure 2. Inter-assay precision of the CT quantitative PCR assay. PCR reproducibility was determined by comparing standard curves of 10-fold serial dilutions of DNA plasmids that encoded CT OmpA from 2log10 to 10log10 copies per reaction among six independent PCR runs. Values above each plot indicate % coefficient of variation.

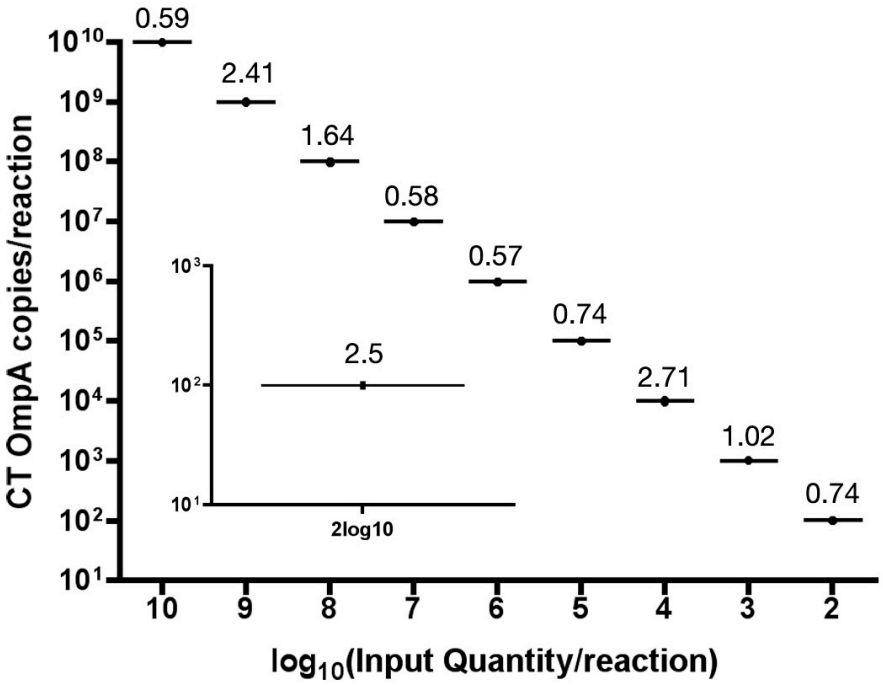


Figure 3. **Intra-assay precision of the CT real-time PCR assay.** Reproducibility within the same PCR run was evaluated by comparing three standard curves of 10-fold serially diluted OmpA DNA plasmids from 2log10 to 10log10 copies per reaction. Inset plot shows reproducibility (n=8 replicates) of the lower detection limit (2log10 copies per reaction). Values above each plot indicate % coefficient of variation.

Within a single reaction plate containing three standard curves of 10-fold OmpA serial dilutions, CV values were reduced to less than 2.7% (Fig. 3). The CV value at the lower limit of detection (2log10 copies per reaction) was 2.5 (n=8; Fig. 3, inset).

PCR specificity was evaluated by testing selected bacterial species common to vaginal or urine samples as well as selected pathogens of other mucosa. Real-time PCR of approximately 6log10 genomic equivalents by SYBR-green detection showed no amplified products from any of the bacterial species tested for cross-reactivity. Importantly, no cross-reactivity was observed from any commensal flora present in samples from the normal human or mouse urogenital tract (Table 2). The lack of amplification was confirmed by agarose gel analyses (data not shown).

Evaluation of CT qPCR for clinical specimens

Patient specimens submitted for CT testing at the UTMB clinical laboratory (n=40) including vaginal, endocervical and male urethral swabs were tested in parallel for CT DNA content using our qPCR assay. The PCR results were compared to those generated using the ProbeTec CT/NG detection system. Twenty ProbeTec-positive and twenty ProbeTec-negative samples were blindly selected and randomized. Of 20 ProbeTec-positive samples the qPCR assay detected CT DNA in 18 samples (90%). The average CT DNA titer in each swab specimen was approximately 5000 genomic copies (100 copies per PCR reaction). Separate PCR amplification of human GAPDH was used to verify the presence of DNA and to normalize CT values based upon the integrity of the collected specimen whereby $>10^8$ GAPDH copies were observed in each sample (data not

shown). Notably, the qPCR assay did identify CT DNA from a single specimen that was reported negative by the ProbeTec assay. The two samples reported positive by the ProbeTec assay that were negative by our qPCR assay were confirmed to be negative by 2 additional independent PCR runs. As a final confirmation of the status, using an independent CT-specific PCR assay [80] the 2 samples again lacked amplifiable CT DNA (data not shown). The human GAPDH levels in both samples were concordant with the rest of the cohort.

The utility of the qPCR assay for detection of CT DNA from human urine specimens was evaluated using human urine spiked with pre-quantified CT Serovar E IFU. Intra-assay CV values for spiked urine samples were less than 2.5% (n=3; Fig. 4) and qPCR copy numbers were similar to the IFU/mL input concentration at a 1:1 ratio of IFU to genomic copies for most dilutions. As expected, the lowest CT concentration deviated from the 1:1 ratio near the lower limit of detection for the PCR assay. The lowest quantity of DNA that was detected from urine using the described methods was approximately 10 copies per reaction (n=3, CV=3.8), a bacterial load equivalent to 500 IFU/mL (data not shown). Importantly, no negative impact on PCR efficiency or reproducibility was observed when processing human urine samples for CT quantification.

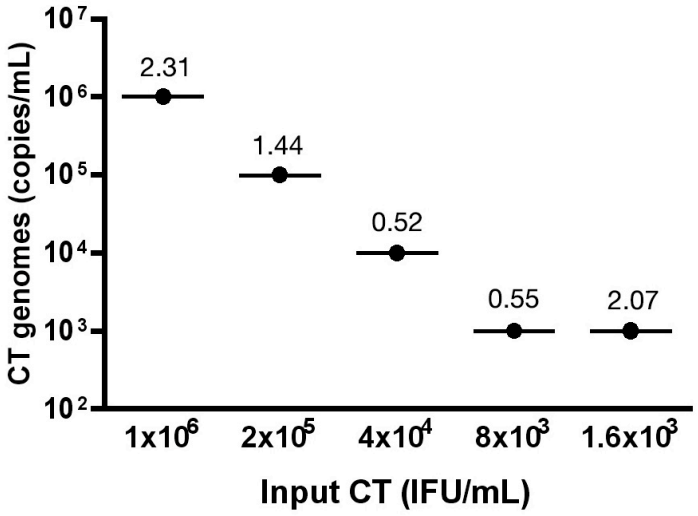


Figure 4. Performance of the qPCR assay for CT detection from urine. Human urine was spiked with CT serovar E elementary bodies. Five-fold serial dilutions were spiked into urine and PCR-amplified in triplicate reactions. Infectious CT titers are shown in IFU/mL and compared to quantitative PCR titers presented as genomic copies per milliliter of urine. Values above each plot indicated % coefficient of variation from 3 replicate PCRs.

Quantification of *C. muridarum* DNA from murine genital specimens

In order to evaluate the utility of this assay for screening CT therapeutics in animals, we quantified bacterial loads in vaginal swabs and upper genital tract tissues from mice experimentally infected with *C. muridarum* after treatment with a candidate CT microbicide or its vehicle. The qPCR results correlated well with IFA data processed in parallel (Table 3). The predicted trends among microbicide-treated and untreated mice also showed strong correlations between the IFA and PCR results. Four samples were identified positive by IFA but negative by PCR. The PCR, however, did detect 3 samples identified as negative by IFA indicating some discordance between IFA testing and PCR quantification. The IFA-positive but PCR-negative samples were at the lowest detection limit (1 area of fluorescence in the undiluted specimen) and were not reproduced in a replicate IFA study suggesting false-positive IFA results. In a separate pre-clinical study using the CT PCR assay, more than 60% of the samples that were positive by PCR but negative by IFA had PCR titers greater than or equal to 3log10 copies per reaction (data not shown). Statistical differences ($p<0.05$; Student's t-test) were observed in the microbicide trial study between the experimental compound and PBS-treated mice at each sampling time (Table 3), a quantitative comparison made possible by the real-time PCR assay. All CT values were normalized to the mean GAPDH value obtained in a separate PCR reaction.

Table 3. Comparison of IFA and qPCR methods for preclinical evaluation of a candidate CT microbicide compound using a murine model of *C. muridarum* genital infection.

Day 3 Swab	Vehicle	Compound
IFA-positive[a]	11/13	1/10
PCR-positive[b]	9/13	2/10
Mean PCR Titer +/- SEM[c]	$2.2x10^3$ +/- 1040	6.97 +/- 4.6
Day 6 Swab		
IFA-positive	11/13	5/10
PCR-positive	11/13	3/10
Mean PCR Titer +/- SEM	$2.4x10^4$ +/- 7723	14.5 +/- 10.8
Oviducts/Ovaries		
IFA-positive	10/13	1/10
PCR-positive	12/13	1/10
Mean PCR Titer +/- SEM	$2.0x10^4$ +/- 16551	4.81

[a]Proportion of mice in treatment group identified positive by IFA (Vehicle n=13; Compound n=10)

[b]Proportion of mice in treatment group identified positive by PCR

[c]Average copies of CT genomes per 5uL reaction in positive samples

M. genitalium detection and qPCR performance

Considering the results from extensive testing of the CT OmpA PCR assay, we next developed and validated a PCR assay for MG using similar approaches. Among 5 separate PCR runs, the interassay reproducibility of cloned MG309 standards was very good with CV values of less than 4.1% (Fig. 5). The MG309 PCR recognized the G37 type strain and 5 Danish clinical strains (M2300, M2321, M2341, M2288 and M2282)

with excellent efficiency and provided quantitative utility to evaluate *in vitro* growth kinetics (Fig. 6). Within a single reaction plate containing 3 standard curves of plasmids encoding MG309, CV values were less than 3% (data not shown). The lower limit of reproducible detection using the MG309 PCR assay was similar to the CT OmpA assay whereby 100 copies were detected in 100% of reactions but lower quantities were routinely detected in experimental specimens (data not shown). Compared to a previously published TaqMan-based qPCR assay [79], quantification of MG *in vitro* growth of the G37 and M2300 strains showed very good correlations (Fig. 7).

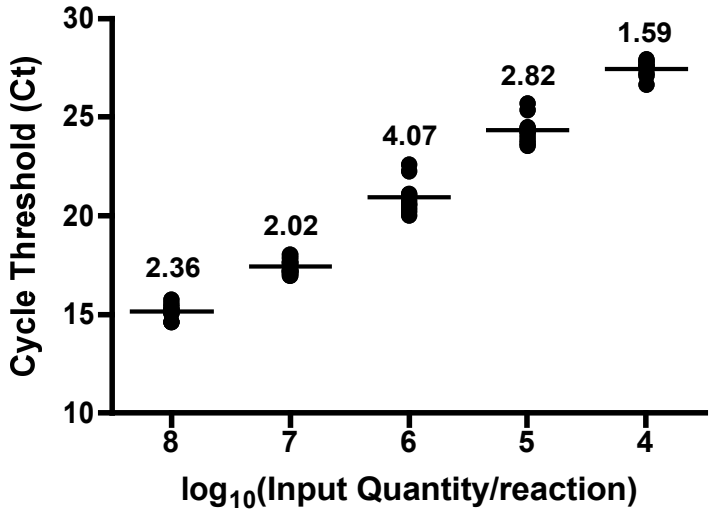


Figure 5. Inter-assay precision of the MG309 quantitative PCR assay. Precision of the MG309 assay among multiple PCR runs was determined by comparing standard curves of 10-fold serial dilutions of DNA plasmids that encoded the MG309 target sequence among 5 independent PCR runs. Values above each plot indicate % coefficient of variation.

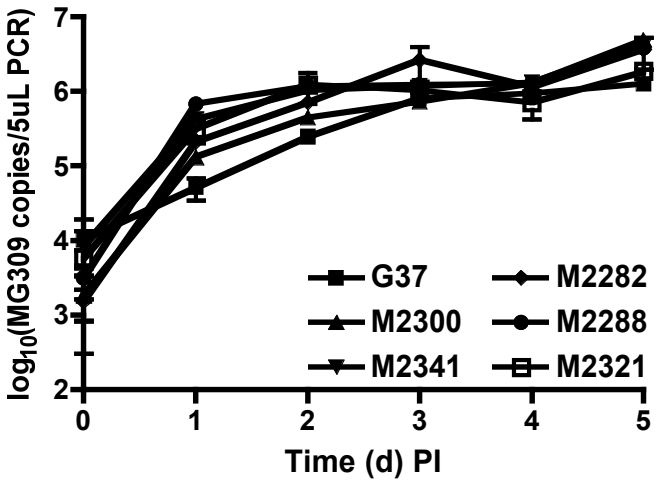


Figure 6. PCR quantification of laboratory and clinical MG strains. The MG309 qPCR assay recognized MG G37 and 5 contemporary Danish strains and provided quantitative utility for kinetic analysis of *in vitro* replication kinetics.

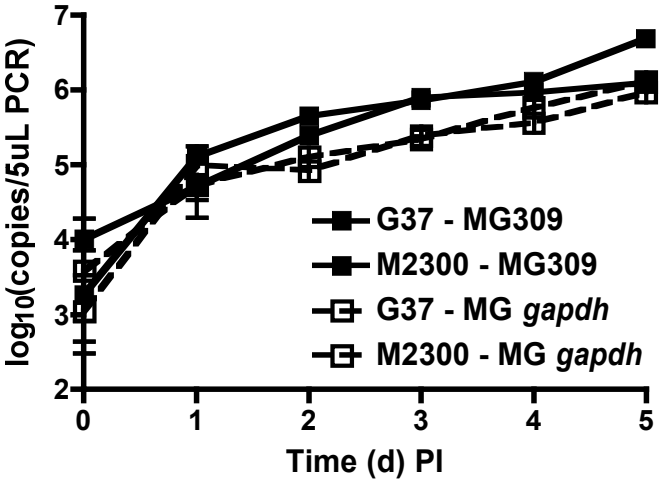


Figure 7. Comparison of the MG309 qPCR assay to the MG *gapdh* assay. *In vitro* replication kinetics for MG strains G37 an M2300 were accomplished using the MG309 qPCR assay and compared to a previously published assay that targeted the single-copy MG *gapdh* gene. Quantitative results were very similar between the two assays.

The MG309 PCR assay was employed extensively throughout this project, most notably for MG quantification from mouse swabs and tissues, with excellent performance (Refer to Chapters 6 and 7). In addition, this assay was indispensable for determining the *in vitro* MG growth kinetics in cell-free conditions and following inoculation of cultured human cells (Refer to Chapters 3 and 5). Importantly, no cross-reactivity was observed in PCR reactions of cultured human and murine cells, samples containing CT or mock-inoculated mouse specimens indicating excellent utility for laboratory use.

DISCUSSION

Genital MG and CT infections in both men and women can result in significant inflammatory sequelae that extend beyond the primary infection. It is now accepted that NAATs including PCR, ligase chain reaction LCR, strand-displacement assays and transcription-mediated amplification of rRNA are more sensitive diagnostic tools for CT than culture, IFA or non-amplified DNA probe assays [81, 82].

For MG, culture of the viable organism from clinical specimens has proven extremely difficult and not a reliable approach to diagnosis [83]. In turn, PCR has been the most attractive strategy and is amendable for high-throughput clinical and experimental use. Fluorescent probe-based PCR assays, such as those utilizing a TaqMan probe, have advantages over other NAATs or SYBR-green qPCR assays because they rely both on complimentarity of the primers and hybridization of a specific oligonucleotide probe. Importantly, real-time PCR can generate quantitative data

extrapolated from a standard curve run in parallel thus permitting a more informative analysis of any specimen type in a high-throughput format.

The ideal PCR detection system should reproducibly detect low levels of bacteria using minimally invasive collection methods and should recognize the most prevalent genital serotypes in circulation. Currently, no data is available for clinical subtypes of MG but the MG309 qPCR assay efficiently amplified the target DNA from the type strain, G37, and 5 Danish clinical strains with excellent efficiency. The genital serovars of CT are denoted D-K but recent analysis of CT genital infections in the United States indicated that serovars D, E, F and Ia currently are the most prevalent cause of urogenital infections [84]. The CT OmpA assay described in this chapter recognized CT serovars D, E, F, Ia and *C. muridarum* for animal modeling of CT disease (Table 2). Based on nucleotide alignment of the primer and probe sequences, the assay should also efficiently recognize serovar G (data not shown) but this was not tested directly. Considering the other genital serovars, some nucleotide divergence was observed within the forward primer binding site of OmpA from Serovar J while OmpA sequence data spanning the target region is not yet available for serovars H and K. Finally, OmpA sequence from the closely related *Chlamydia pneumoniae*, a respiratory tract pathogen, has significant divergence in the target region suggesting it would not be recognized by this assay.

Among the tested CT serovars, quantitative results from the OmpA PCR assay showed highly reproducible inter-assay precision (Fig. 2) and intra-assay reproducibility among replicate samples within the same run (Fig. 3) indicating that the assay generates highly reproducible results within the same PCR run and among multiple technicians

within the laboratory. Similar efficiencies were observed for the MG309 assay (Fig. 5). The lower limit for consistent detection of plasmids containing the OmpA or MG309 target region was 100 copies per reaction. However, the linear range of detection was broad and quantities of less than 100 copies were detected routinely using both PCR assays providing increased utility both clinically and in the laboratory setting.

We next tested the CT OmpA PCR assay for clinical performance using human vaginal, endocervical and penile urethral swabs and compared our results to the BD ProbeTec ET CT detection system. The concordance rate among positive CT samples was very good (90%). The 2 clinical specimens that were identified positive by ProbeTec but not detected by the CT qPCR assay were confirmed negative in 2 additional PCR runs and had normal GAPDH copy numbers indicating sample collection, processing and DNA extraction were successful. Using an independent CT-specific PCR assay [80], we verified the lack of detectable CT DNA in these 2 specimens (data not shown) suggesting false-positive ProbeTec results. However, ProbeTec-positive but PCR-negative results also could have been attributed to testing only a residual portion of the sample after the ProbeTec processing.

Ninety-five percent (19 of 20) of samples that were reported negative by ProbeTec also were reported negative by qPCR. However, CT DNA was detected from a single specimen that was reported negative by ProbeTec. These results were confirmed in a second PCR run with similar quantitative results for both CT and human GAPDH. Collectively, the preliminary evaluation of clinical utility indicated that, the CT PCR assay performance was similar to the ProbeTec system but may afford some enhanced

sensitivity. Although a larger study would be required to fully validate the qPCR assay for clinical application, the concordance rate with the BD ProbeTec system and the quantitative nature of the PCR assay indicate the enhanced utility for quantifying CT loads in reproductive tract specimens and warrants continued investigation.

In this dissertation, small animal modeling of STI pathogens has played a major role in our understanding of the capacity of MG to cause disease. Prior to our work with MG, the laboratory spent considerable time developing more efficient methods for CT detection from mouse reproductive tract specimens. In this chapter, we presented published results showing that the CT PCR assay is a sensitive, specific and reproducible method to quantify CT from mouse lower genital tract swabs and upper-tract tissue specimens (McGowin, et. al, In press.). In a candidate microbicide study, the qPCR assay enhanced the ability to evaluate efficacy with remarkably reproducible PCR titers at each sampling time that mirrored the qualitative IFA results generated in parallel (Table 3). Importantly, these data provided a more informative quantitative measure of CT burden compared to IFA. Many of the findings for CT detection in mouse genital specimens were exported for the development and testing of a novel murine model to evaluate MG reproductive tract disease (See Chapter 6 and 7).

Although MG clinical comparators were not available as few labs routinely screen for this pathogen, both qPCR assays reported in this chapter represent highly specific and sensitive methods for the quantification of CT or MG DNA from various clinical and preclinical specimens. The closed reaction 96-well format provides medium to high-throughput utility for sample screening and greatly reduces the possibility of exogenous

contamination. Specific probe-based real-time PCR assays can reduce the clinical time to diagnosis, provide an accurate and informative monitor of therapy and provide quantitative outcome measures for *in vitro* and animal modeling of human reproductive tract disease.

CHAPTER 3: THE ROLE & INNATE RESPONSES OF VAGINAL AND CERVICAL EPITHELIAL CELLS TO *M. GENITALIUM* EXPOSURE

OVERVIEW

The human female reproductive tract is a compartmentalized organ system that has evolved different levels and specificity of immunological responsiveness among each tissue type. Following sexual transmission, MG and other pathogens make initial contact with epithelial cells (ECs) that play an important role in early activation of the innate response. Studies outlined in this chapter demonstrate that MG strain G37 and M2300 rapidly attach to both vaginal and cervical ECs resulting in intracellular localization. These findings were verified by electron microscopy and by demonstrating protection of MG from extracellular antibiotic treatment. With regard to host response, human vaginal, ecto- and endocervical ECs responded to MG exposure with robust secretion of pro-inflammatory cytokines and chemokines including the potent chemotactic factor, IL-8. Interestingly, long-term MG infection of cervical ECs resulted in a significant down-regulation of IL-8 and concomitant increase in the anti-inflammatory cytokine, IL-10. Using a 3-dimensional cell culture model of polarized vaginal ECs, MG established an intracellular infection and very efficiently transmigrated the entire epithelial multilayer of more than 12 cell layers in thickness. These findings indicated that MG can establish an inflammatory intracellular infection of human vaginal and cervical ECs and provided evidence for dissemination to other reproductive tract tissues. Most importantly, intracellular localization in reproductive tract ECs may provide a survival niche for long-

term evasion of the host immune response and persistent infection. Portions of this chapter have been submitted for publication by McGowin, et al., *BMC Microbiol*, January 2009.

INTRODUCTION

The compartmentalized nature of the female reproductive tract has evolved to tolerate the highly diverse microflora of the vagina and ectocervix while supporting complex and immunologically sensitive biological processes such as conception, fertilization and fetus development [38]. The lower reproductive tract, including the vagina and ectocervix, has adapted to tolerate constant antigenic stimulation by commensal microbial flora. Tissues above the ectocervix include the endocervix, uterus, fallopian tubes and ovaries. These tissues are collectively termed the upper genital tract and are, in general, highly sensitive to microbial contamination via employment of a robust system for recognizing ascending microbes [38]. The cervicovaginal mucosa is a complex multicellular tissue matrix that, similar to other mucosa, simultaneously provides both a portal of entry into the body and protection from potentially pathogenic microbes.

The nature of pathogenicity implies that a microorganism can somehow circumvent the host responses to establish infection and ultimately cause disease. MG has been shown previously to occupy intracellular niches in cultured Vero cells [85] and, more recently, in the EM42 endometrial cell line [86]. It is hypothesized that

intracellular MG localization could provide protection from the host immune responses while simultaneously eliciting inflammatory cytokine secretion from infected cells. However, very little work has addressed the interaction of MG with ECs of female reproductive tract origin. Furthermore, no previous studies have addressed the host immune response to MG and whether or not ECs are capable of initiating innate responses at the reproductive mucosa.

Using electron microscopy and antibiotic protection studies, we found that MG rapidly localized to intracellular sites within vaginal, ecto- and endocervical EC. When vaginal ECs were polarized and inoculated with MG using a novel 3-dimensional culture system, intracellular localization and transmigration through epithelial multilayers was observed. Acute-phase pro-inflammatory cytokine secretion was observed from all tested cell types. However, long-term MG infection of cervical ECs resulted in anti-inflammatory responses suggesting that intracellular survival and an active down-regulation of host innate responses could be occurring. Collectively, the studies presented in this chapter show that MG infection of reproductive ECs results in acute-phase inflammation and highlight the importance of continued investigation of how MG establishes genital tract infection and evades host immune responses.

MATERIALS AND METHODS

Human cell culture

Immortalized human ECs derived from vaginal (n=3 donors; V19I, V12I, V11I), ectocervical and endocervical tissues were maintained as described previously [42]. Keratinocyte serum-free medium (KSFM; Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract (50mg/L), recombinant epidermal growth factor (5ug/L), $CaCl_2$ (44.1mg/L), penicillin-G (100U/mL) and streptomycin sulfate (100ug/mL) was used for culture of ectocervical and endocervical EC at 37°C in a 5% CO_2 humidified incubator [38]. Vaginal ECs were maintained in a 1:1 mixture of KSFM and VEC-100 media (MatTek, Ashland, MA). ME-180 (ATCC HTB-33) cervical carcinoma cells were maintained in RPMI 1640 (MediaTech, Herndon, VA) medium supplemented with 0.1mM non-essential amino acids (Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine, penicillin-G (100U/mL), streptomycin sulfate (100ug/mL) and 10% FBS. Cells were verified to be free of any contaminating mycoplasmas by PCR (Stratagene, Cedar Creek, TX).

Propagation of *M. genitalium* strains G37 and M2300

M. genitalium type strain G37 (MG G37; ATCC 33530) or the lower passage Danish M2300 strain was propagated in Friis FB medium [13]. Briefly, MG stocks were inoculated aseptically into tightly sealed tissue culture flasks containing freshly prepared Friis FB medium and incubated at 37°C for 5-8d. Growth was monitored by the formation of adherent microcolonies and a pH-mediated color change of the medium.

MG was harvested from culture flasks by pouring off the spent medium, extensively washing adherent mycoplasmas with 5 volumes of approximately 5mL each of sterile PBS and then scraping adherent microcolonies into fresh PBS. MG viability was quantified in 96-well plates by serial 10-fold dilution of each sample into fresh Friis FB medium. The last dilution to show a change in color and formation of microcolonies was used to calculate the number of viable organisms in the original sample. UV-inactivation (254nm) of MG was performed using a Stratalinker 2400 (Stratagene, La Jolla, CA) to a total energy of 720,000 microjoules/cm^2. Heat denaturation of MG was accomplished by incubating log-phase cultures at 80°C for 15min and then rapid cooling on ice. Loss of MG viability was verified by an absence of growth in Friis FB medium after 14d incubation at 37°C.

Electron Microscopy

I. Transmission electron microscopy (TEM)

Adherent monolayers or polarized multilayer cultures of MG-inoculated (G37 or M2300; MOI 100) or non-inoculated genital ECs were fixed at indicated times in a mixture of 2.5% formaldehyde and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) containing 0.03% trinitrophenol and 0.03% $CaCl_2$. Cells were washed in 0.1 M cacodylate buffer (pH 7.2) and then postfixed in 1% OsO_4 in the same buffer. Each sample was stained *en bloc* with 1% uranyl acetate in 0.1M maleate buffer, dehydrated in ethanol and embedded in Poly/Bed 812 epoxy resin (Polysciences, Warrington, PA). For polarized ECs grown in transwell culture inserts, prior to embedding into flat molds , the

membrane and cellular multilayer were carefully removed from the culture cup and then cut into small (appx. 1mm wide) strips using a razor blade. Cut strips were then stacked atop each other at one end of the mold and polymerized overnight at 60°C. Semi-thin (1uM) sections were cut from the polymerized stack and then stained with Toluidine Blue. An area with sufficient cells was chosen using a light microscope for ultrathin sectioning. Ultrathin sections were cut using the Ultracut S ultramicrotome (Reichert-Leica) and then stained sequentially in 2% aqueous uranyl acetate and lead citrate. Specimens were examined in a Philips 201 or CM 100 electron microscope at 60 kV.

II. Scanning electron microscopy (SEM)

MG-infected and non-infected control cells were grown in transwell culture inserts and then fixed as described above for TEM for at least 1h at room temperature, postfixed in 1% OsO4 in 0.1 M cacodylate buffer, dehydrated in ethanol, treated with hexamethyldisalazane and then air dried. Next, the samples were mounted on specimen stubs and sputter coated with iridium for 40sec in an Emitech K575X turbo sputter coater (Emitech, Houston, TX). Samples were examined in a Hitachi S4700 field emission scanning electron microscope (Hitachi High Technologies America, Electron Microscope Division, Pleasanton, CA) at 2kV.

Quantification of *M. genitalium* uptake by cervical ECs

To substantiate the electron microscopy data, we next evaluated whether intracellular localization of MG in cervical ECs would protect from extracellular

antibiotic treatment. The sensitivity of MG strains G37 and M2300 to gentamicin was established first by inoculation of log-phase organisms into Friis FB medium with gentamicin concentrations ranging from 100-400ug/mL. No MG growth was observed at 200 or 400ug/mL therefore a working concentration of 200ug/mL was employed in subsequent studies to minimize EC uptake of gentamicin.

As a representative genital EC type, ME-180 cells were seeded into 96-well plates 1d prior to infection at a density of $1x10^5$ cells/well. MG, grown to log-phase, was inoculated onto ME-180 cells (MOI of 100) in triplicate. Following 3h incubation, when mycoplasmas appeared to be attached to and invading genital ECs (see Fig. 9), the supernatant containing residual inoculum was removed and replaced with fresh medium containing gentamicin (200ug/mL). At 15min, 24 and 48h following inoculation, culture supernatants were removed and the infected cells were washed 3x with sterile PBS. The removed supernatants were tested and verified free of any viable MG confirming that gentamicin was killing extracellular MG.

Cells then were removed from the well by scraping into Friis FB medium followed by plating serial 10-fold dilutions prepared in Friis FB medium into a 96-well plate. Outgrowth of MG from infected ME-180 cells was observed for 14d. Viable MG was determined by a dramatic color change of the Friis FB medium and formation of adherent microcolonies in the culture well. The load of viable MG from each sample was calculated using a color changing unit (CCU) assay as described above.

In parallel cultures, following MG inoculation and subsequent removal of the inoculum, we also investigated egress of MG from infected cells in the absence of

gentamicin. This allowed us to measure the titer of viable MG at extracellular sites after infection and whether or not MG was capable of re-emerging from infected cells. Extracellular MG viability was quantified from supernatants of infected cells processed in the same manner and quantified similarly using the CCU assay.

3-Dimensional cell culture model

Immortalized human vaginal ECs (n=3 donors; V19I, V12I, V11I) were cultured separately in a 1:1 mixture of KSFM and VEC-100 media KSFM medium exactly as described for non-polarized cells. Approximately $1x10^6$ cells were seeded into transwell cell culture insert cups (24-well, 0.4 or 3.0μM pore size; Becton Dickinson, Franklin Lakes, NJ) in a 0.5mL volume and cultured for 9d. After 2d incubation, the medium atop the cells (apical side) was removed and, for the remainder of the study, the cells received fresh medium every other day from the basolateral side only (termed 'dry interface'; See Fig. 8). The apical-most cells, representing the lumenal ECs, are elongated and often lack a nucleus (Fig. 9). Polarization of the cell multilayer was evaluated by measuring Trans-Epithelial Electrical Resistance (TEER) and electron microscopic visualization of tight junction formation. TEER across reproductive EC multilayers was measured by adding 500uL

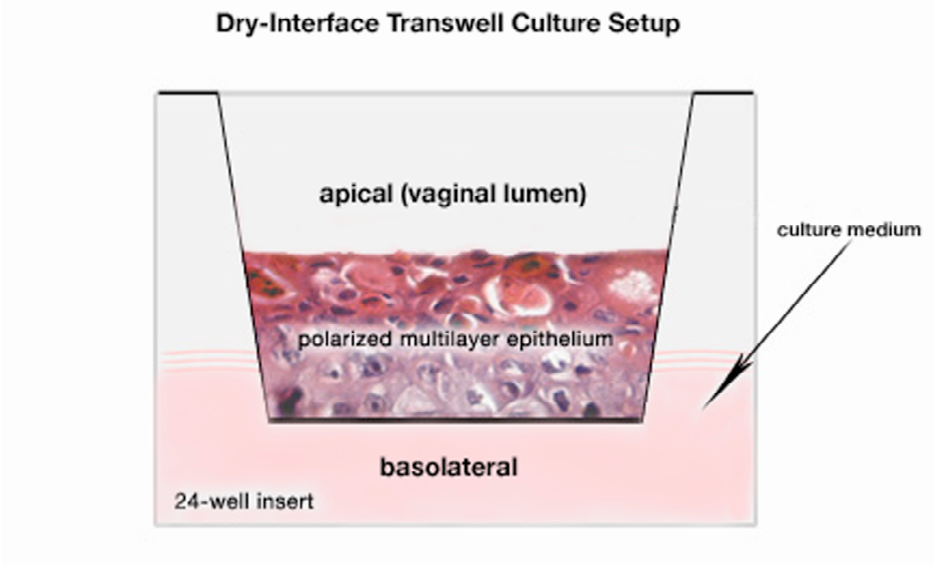


Figure 8. Polarized human vaginal EC transwells. Immortalized human vaginal EC were polarized by culturing in 24-well transwell inserts for 9d with medium provided exclusively from the basolateral surface (A). These 'dry interface' cultures were used for investigation of MG replication and dissemination dynamics.

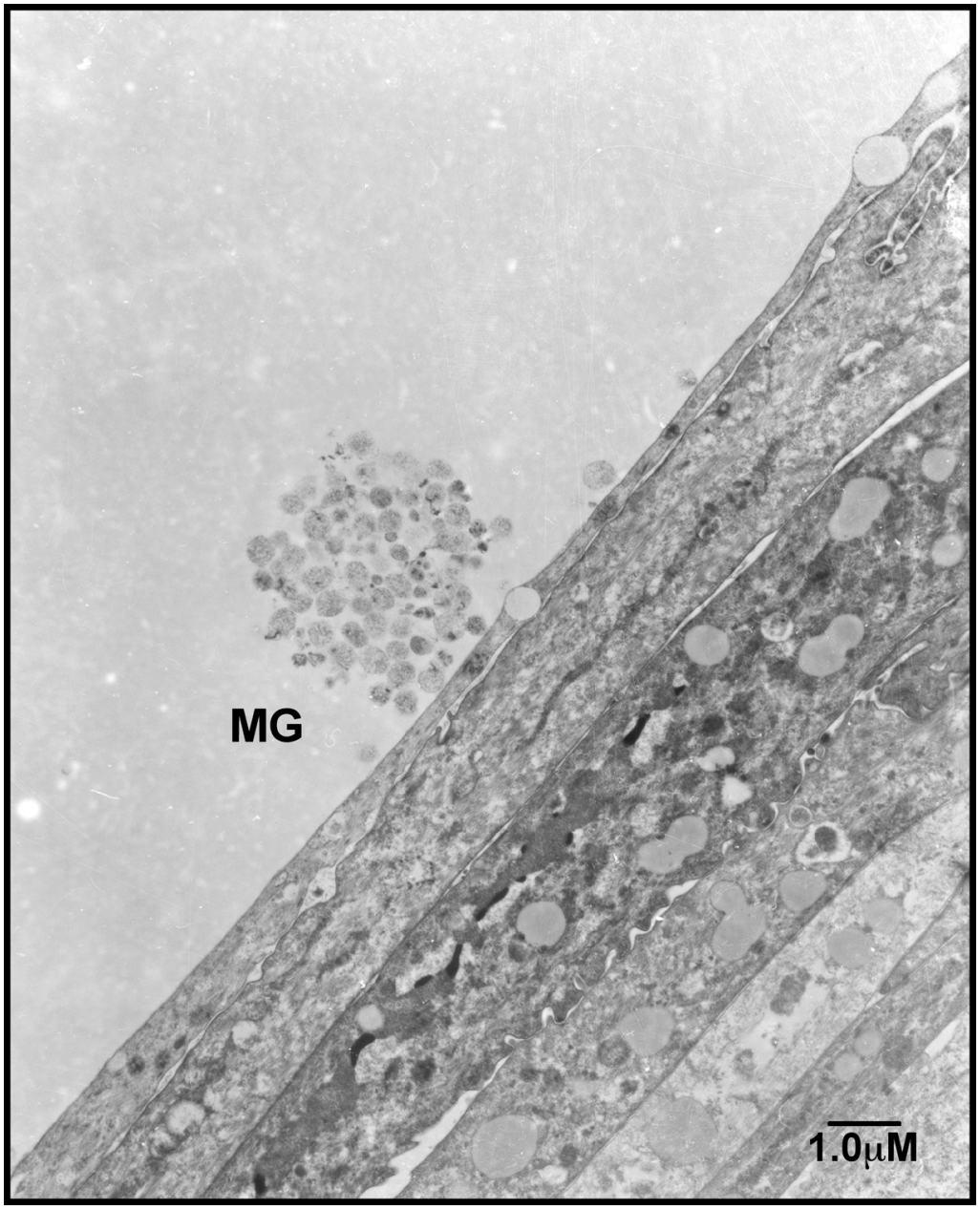


Figure 9. MG inoculum on polarized vaginal ECs. Immortalized human vaginal EC were polarized in 24-well transwell inserts for 9d with medium provided only from the basolateral surface. These 'dry interface' cultures were used for investigation of MG replication and dissemination dynamics. The apical-most EC, corresponding to the vaginal lumen, were elongated (pictured above) while larger, stratified squamous EC were abundant near the transwell membrane (not pictured).

PBS to the apical surface, collecting the TEER measurement and then promptly removing the PBS to maintain the 'dry' interface format. Following harvest and inoculation of MG G37 or M2300 (MOI 100), cell multilayers were fixed and processed for TEM and SEM at indicated times as described above.

Stimulation and quantification of cytokines from genital ECs

Non-polarized human vaginal, ectocervical or endocervical ECs were seeded into 96-well plates at a density of $1x10^5$ cells/well. Following overnight incubation at 37°C, culture supernatants were removed and replaced with fresh medium to remove any constitutively secreted cytokines. Polarized vaginal ECs were seeded into transwell culture insert cups at $1x10^6$ cells/well and cultured as described above. Log-phase MG G37 or M2300 was harvested as described above, re-suspended in fresh PBS and then inoculated onto each cell type (MOI of 10). Controls for innate immune stimulation included the *M. salivarium*-derived TLR2/6 agonist, FSL-1 (0.1ug/well) or an equal volume of the PBS vehicle added to triplicate wells and processed in parallel. Secreted cytokines were quantified from culture supernatants from non-polarized cells 6 or 48h PI. Supernatants from both the apical and basolateral sides were collected from polarized vaginal EC cultures at indicated times following inoculation. Cytokines were quantified via a cytometric bead array (CBA) assay using the human 27-Plex panel of cytokine targets (BioRad) that included the following targets: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12(p70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF,

GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF.

Persistent cervical EC infection

To evaluate the immunologic effect of long-term MG infection of reproductive tract ECs, ME-180 cervical carcinoma ECs were inoculated with MG G37 and M2300 (MOI 0.1). Inoculated cells were maintained and passaged approximately every 4d to establish a persistent infection. At each passage time, following centrifugation and resuspension in fresh medium, approximately $1x10^6$ cells were collected for DNA extraction and PCR quantification of MG as described in Chapter 2. After 33d infection, basal cytokine secretion was measured by plating $1x10^5$ cells into triplicate wells of a 96-well plate and the measuring secreted cytokines from culture supernatants collected 24h after plating. The profile of secreted cytokines from MG-infected cells was compared to non-infected cells maintained in parallel.

Statistical Analyses

The Student's t test was used to calculate significant differences in intra- and extracellular MG titers and when comparing secretion of individual cytokines from a single cell type to basal (PBS-treated) levels. The one-way ANOVA followed by Dunnett's post-test (Prism v. 4.0, GraphPad, San Diego, CA) was used to calculate significant differences in cytokine secretion levels when more than 2 conditions were compared. Significance was indicated when $p<0.05$.

RESULTS

M. genitalium attachment and invasion of non-polarized human genital ECs

MG has been shown previously to occupy intracellular spaces in cultured cells of non-reproductive origin [85, 87, 88] and cells obtained clinically from vaginal swabs of MG-positive women [76] but the invasion kinetics for genital cell types are not well understood. Inoculation of non-polarized genital ECs with MG strains G37 or M2300 (MOI 100) resulted in attachment and early entry of vaginal (V19I) and cervical (ME-180) EC (V19I and MG G37 shown in Fig. 10A-C). By 6h PI, MG was seen in intracellular vacuoles (Fig. 10C) that were most often adjacent to the nucleus (ME-180 shown in Fig. 10D). At this time point, some extracellular mycoplasmas were observed but were often in aggregates and showed no evidence of attachment or invasion because cellular invaginations were absent. Morphologically, the intracellular mycoplasmas were highly pleomorphic and appeared to have normal ultrastructure indicated by a dense content of ribosomes and few degraded bacterial membranes. A previously described tip structure [85] was observed (Fig. 10C; starred arrow) but was not always visible on attached mycoplasmas in each stained section. No similar organisms or structures were observed in non-infected cells processed in parallel.

We next quantified MG G37 and M2300 viability from intra- and extracellular fractions of non-polarized ME-180 cells using a gentamicin protection assay as described in the *Materials and methods*. Briefly, in order to quantify intracellular titers, the MG inoculum was incubated for 3h to allow attachment to and entry of host cells followed by

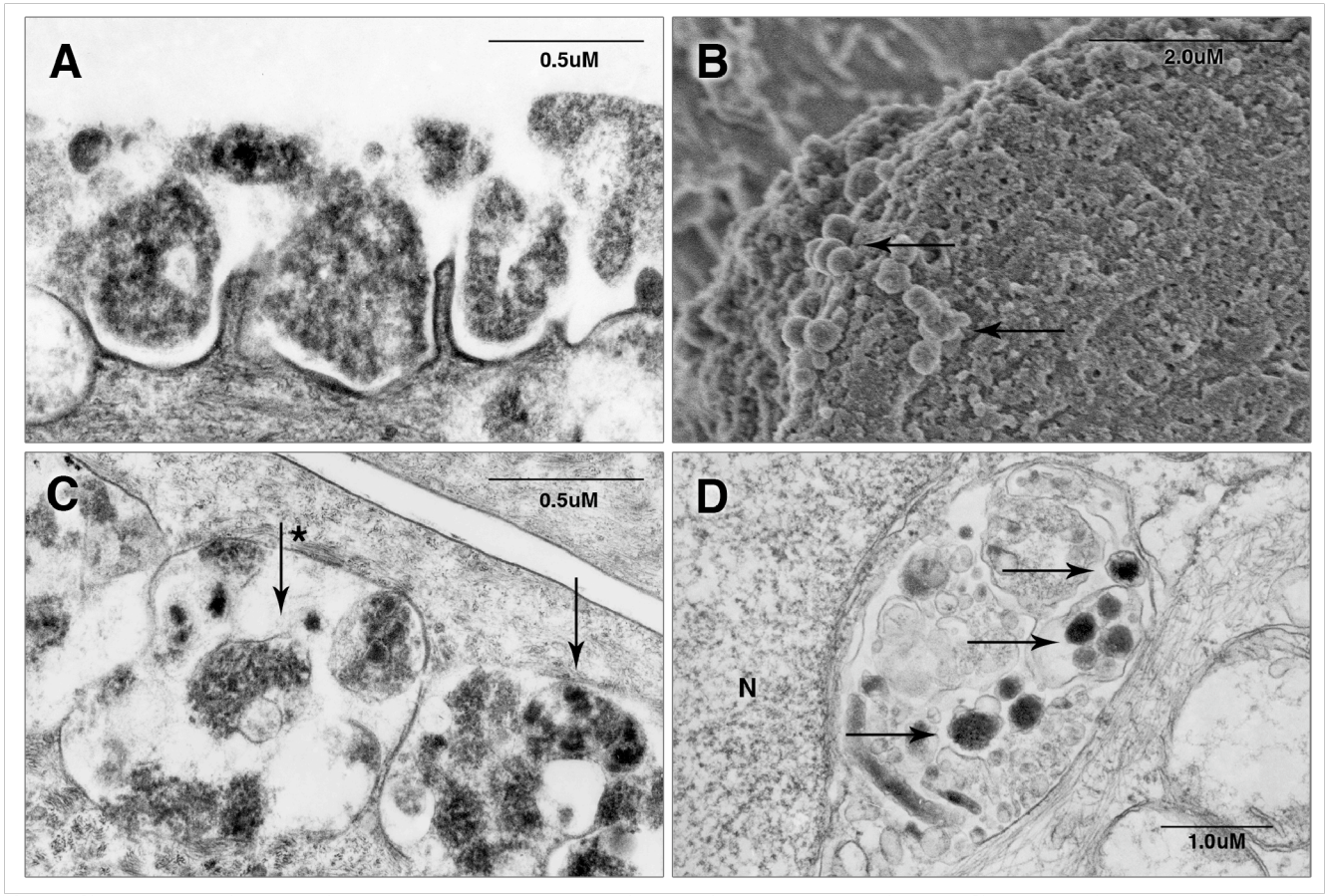


Figure 10. MG was localized to intracellular vacuoles in vaginal and cervical ECs. Log-phase cultures of MG G37 were harvested and inoculated onto vaginal (V19I; panels A, B, C) or cervical (ME-180; panel D) EC and collected kinetically for transmission (A, C, D) or scanning electron microscopy (B). By 2h PI, MG G37 had attached to vaginal EC with evidence of early invasion of the host cell indicated by pit formation surrounding attached mycoplasmas (A, B). MG was observed in membrane-bound intracellular vacuoles by 2h PI (C) that were often adjacent to the nucleus following extended culture (Panel D; N denotes nucleus). Arrows indicate MG. Starred arrow denotes a tip structure.

removal of the inoculum and replacement of fresh culture medium containing a bactericidal concentration of gentamicin (200ug/mL). Fifteen minutes following inoculation with either G37 (Fig. 11A) or M2300 (Fig. 11B), no viable MG were detected in the cells (solid line).

Using a color changing unit assay (CCU), high titers of viable intracellular MG were detected at both 24 and 48h PI. No MG viability was detected in supernatants containing gentamicin at any time point indicating that the observed titers were due exclusively to intracellular mycoplasmas that were protected from gentamicin exposure.

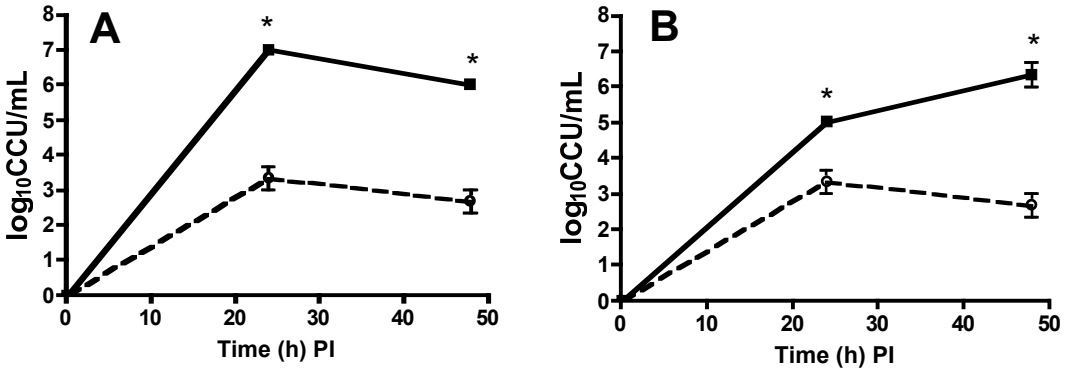


Figure 11. Intra- and extracellular localization of MG in ME-180 cervical EC. Non-polarized ME-180 cells were inoculated with log-phase cultures of MG strain G37 (A) or M2300 (B) to determine whether MG can invade human reproductive tract EC. To quantify intracellular MG loads (solid line), the inoculum was removed following 3h incubation for attachment and entry and replaced with medium containing gentamicin (200ug/mL). Extracellular MG viability (dashed line) was quantified from parallel culture supernatants (separate wells) processed the same way except, following the 3h incubation, the inoculum was removed and replaced with fresh medium without gentamicin. Cell fractions or culture supernatants were collected at 0, 24 and 48h following removal of the inoculum for quantification of intracellular or extracellular bacterial loads, respectively, using a color changing unit assay (CCU). * indicates a significant difference between intracellular and extracellular MG titers at the indicated time point ($p<0.05$; Student's t-test).

Extracellular MG titers were quantified in parallel wells from supernatants of infected cells that were inoculated in the same fashion but did not receive gentamicin. Extracellular titers from culture supernatants (dashed line) were significantly less than intracellular titers ($p<0.05$) indicating preferential localization to intracellular sites.

It is also important to note that, although streptomycin was a component of the ME-180 culture medium during these assays, MG was found to be resistant to streptomycin. In addition, streptomycin was also employed in Friis FB medium for axenic culture of MG. Together there should be no antibiotic-mediated pressure on MG to avoid the extracellular environment. The findings of intracellular MG infection also were consistent with intracellular localization observed by electron microscopy (Fig. 10).

M. genitalium traverses 3-D multilayers of vaginal ECs

Following inoculation of polarized, 3-dimensional cultures of human vaginal ECs with MG G37 or M2300, rapid attachment was observed (Fig. 12). The apical-most cells of these polarized cultures, representing the luminal surface of the vagina, appeared very elongated and, similar to the human vagina (See Fig. 13), were atop many cells with typical stratified squamous morphology (See Fig. 9). Heavy colonization of the apical-most cells by MG was commonly observed (Fig. 13) followed by rapid invasion (as early as 30min PI, data not shown) and localization to intracellular vacuoles (Fig. 14).

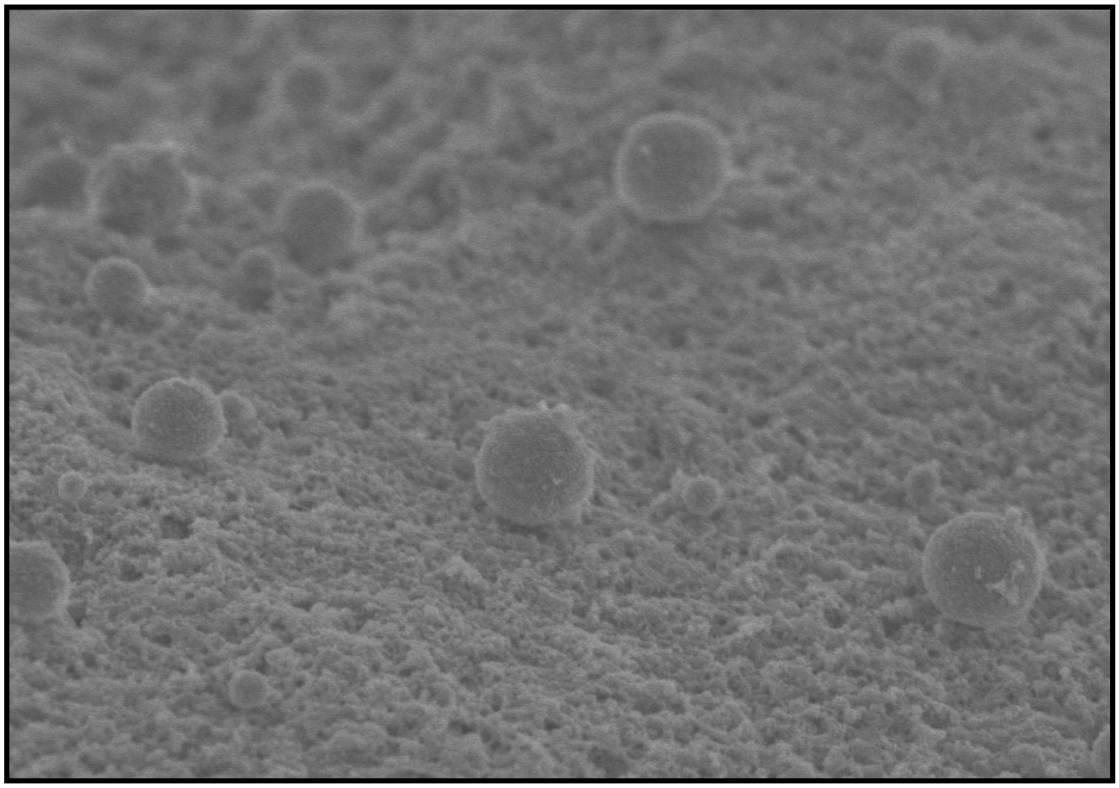


Figure 12. Scanning electron micrograph of MG G37 attached to polarized vaginal ECs. Human vaginal EC were cultured in transwell inserts and then inoculated with MG G37. MG was observed to be attached to polarized vaginal EC 2h PI.

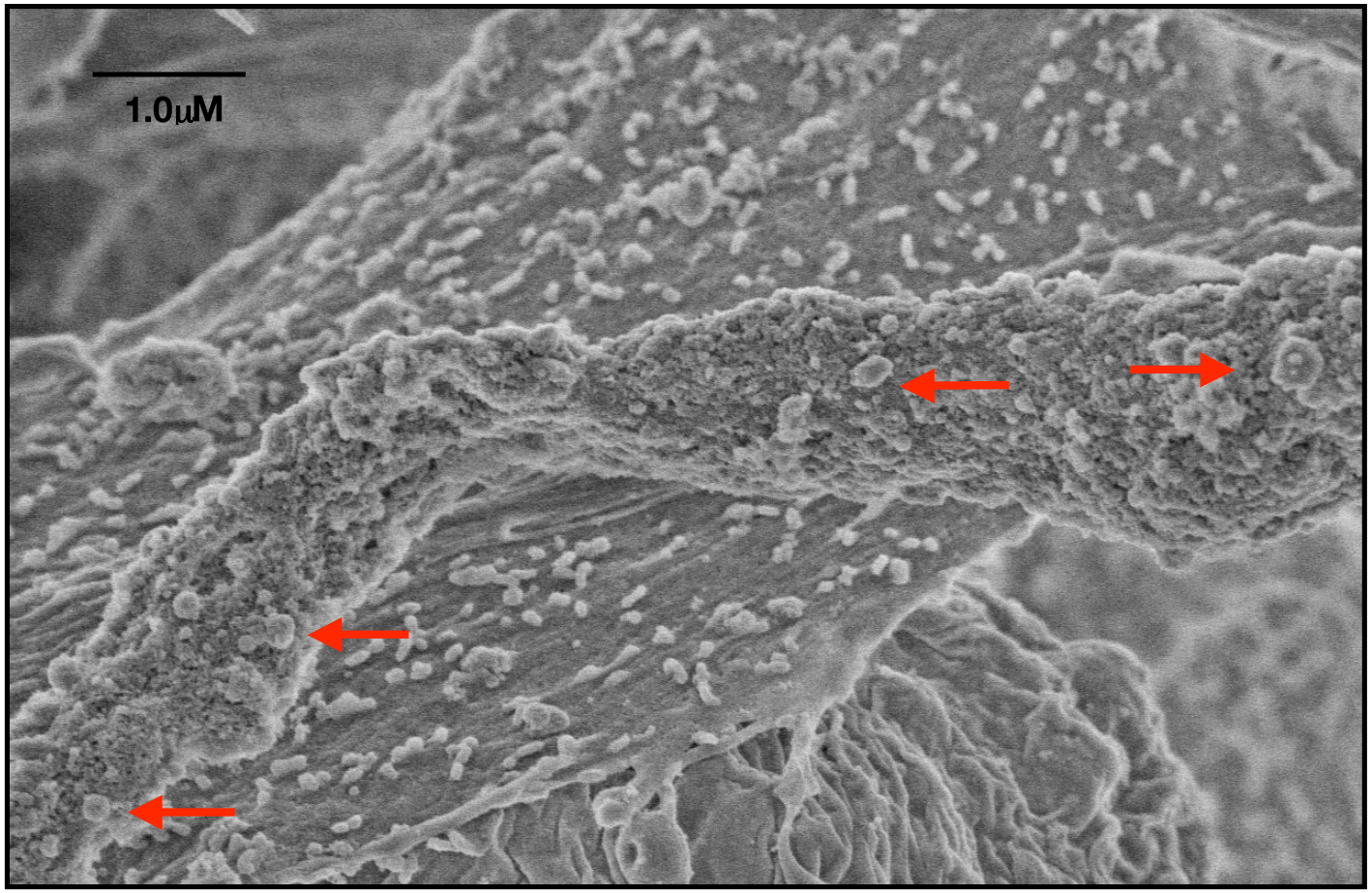


Figure 13. Attachment of MG to apical-most vaginal ECs. Human vaginal EC were cultured in transwell inserts and then inoculated with MG G37 or M2300. Images collected from infected cells harvested for SEM 2h PI showed heavy attachment of MG to apical-most EC. The apical-most cells represent the superficial cells at the luminal surface of the vagina. Several MG are attached to each cell (red arrows).

Interestingly, microscopic examination of infected cultures showed transmigration of MG from the apical surface towards the basolateral membrane at 6, 12 and 24h PI (Fig. 14A, B and C, respectively).

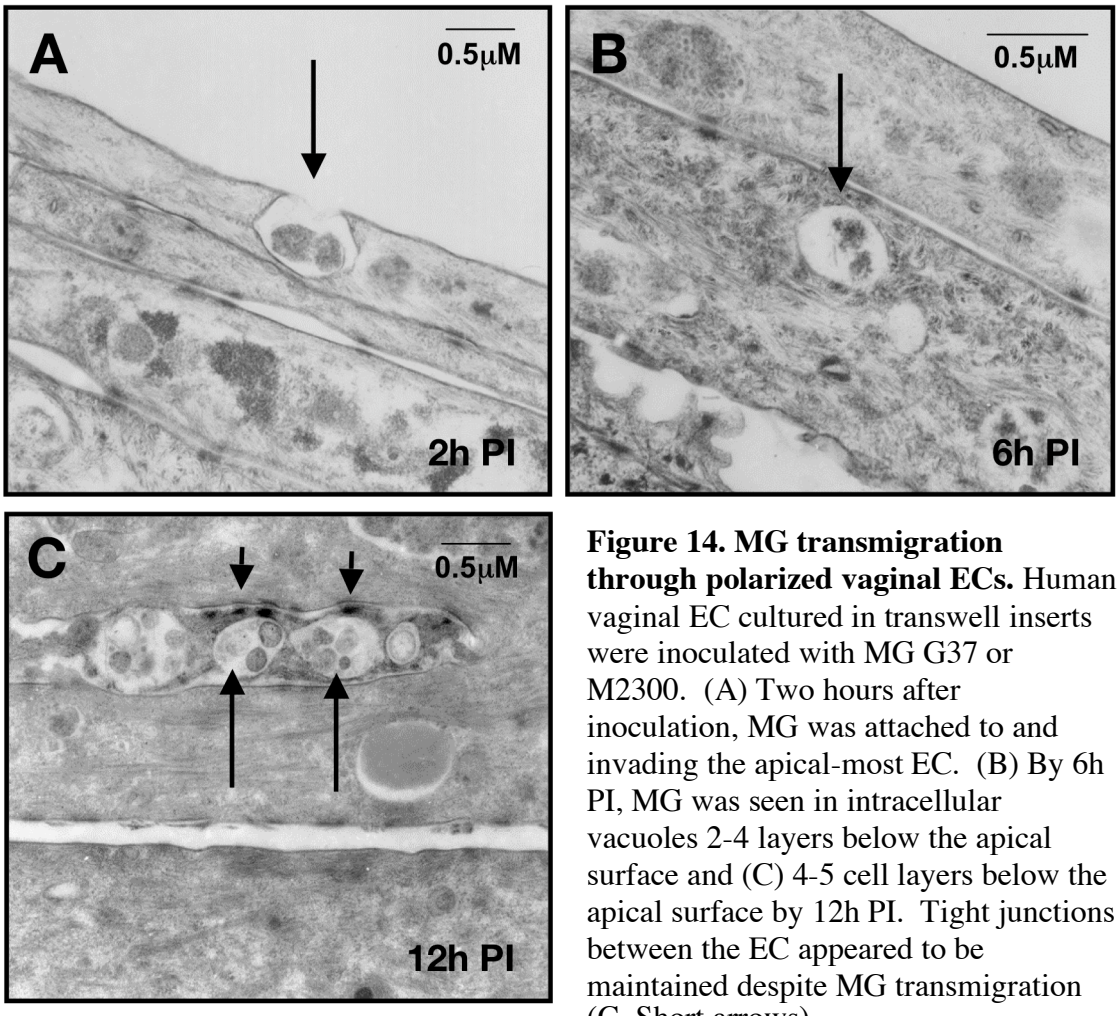


Figure 14. MG transmigration through polarized vaginal ECs. Human vaginal EC cultured in transwell inserts were inoculated with MG G37 or M2300. (A) Two hours after inoculation, MG was attached to and invading the apical-most EC. (B) By 6h PI, MG was seen in intracellular vacuoles 2-4 layers below the apical surface and (C) 4-5 cell layers below the apical surface by 12h PI. Tight junctions between the EC appeared to be maintained despite MG transmigration (C, Short arrows).

These findings were confirmed by titration of viable MG from the apical surface, the cellular multilayer or the basolateral compartment throughout the infection process. Apical titers were measured from 15min to 24h PI whereby, following 6-8h incubation, a significant decrease in MG viability was observed (Fig. 15A) suggesting penetration of the multilayer culture. Titration of viability from the cells showed the presence of MG and subsequent bacterial replication from 0-72h PI in all 3 vaginal EC types (Fig. 15B). Importantly, complete transmigration of the polarized multilayer was observed by a subset of MG organisms from 24-72h PI (Fig. 15C). These studies quantified the kinetics of MG infection in polarized vaginal ECs and confirm the visualization of invasion and transmigration that was observed using electron microscopy.

Throughout the infection process (0-72h PI), no significant decrease in TEER was observed indicating that MG infection and replication did not dramatically affect the integrity of the EC multilayer (Fig. 16).

M. genitalium elicited pro-inflammatory cytokines from genital ECs

Following demonstration of intracellular localization within reproductive tract ECs, we first evaluated the host cytokine response from 3 human non-polarized vaginal (V11I, V12I, and V19I) and 2 cervical EC lines (sA2EN and 3ECI) [42] in 2-dimensional cultures. Of the tested time points, peak cytokine values were obtained 48h PI and are presented in Table 4. Vaginal ECs exposed to viable MG G37 or M2300 (MOI 10) responded with significant secretion of interleukin-6 (IL-6), IL-8 and GM-CSF ($p<0.05$ vs. PBS control).

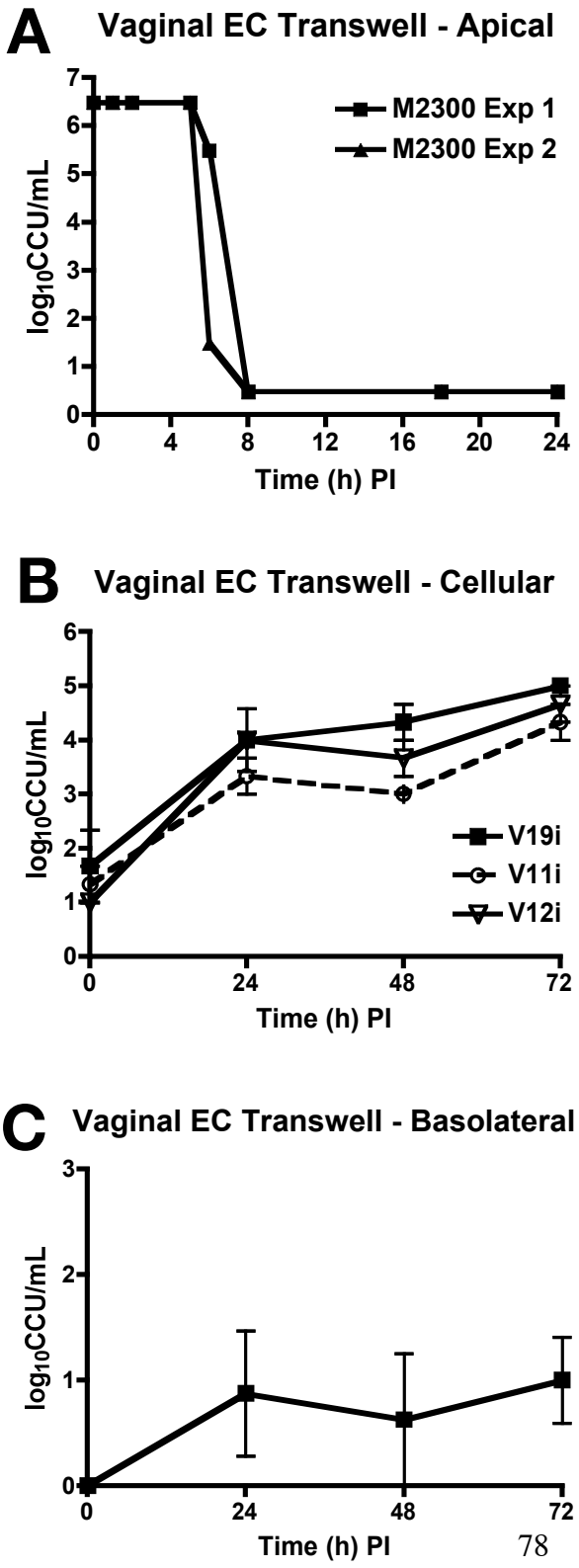


Figure 15. MG replication in polarized human vaginal ECs. Human vaginal EC were cultured in transwell inserts and then inoculated with MG strain M2300. Viable MG was titrated from the apical, cellular and basolateral fractions using a color changing unit (CCU) assay. (A) Six to 8h following inoculation, apical titers were dramatically reduced. MG replication was observed up to 72h PI indicating localization from the apical surface to the multilayer. (C) Complete transmigration of MG was observed from 24-72h PI.

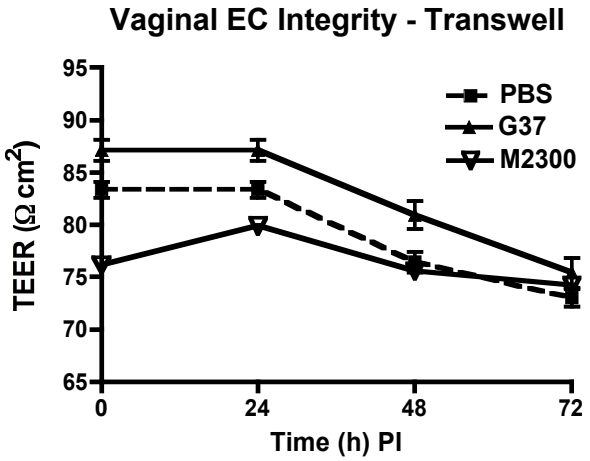


Figure 16. Epithelial integrity through MG infection. Polarized human vaginal ECs were inoculated with MG strain G37 or M2300. Transepithelial electrical resistance (TEER) was measured 0, 24, 48 and 72h PI to determine whether MG infection was significantly impacting the integrity of the epithelial multilayer. No significant reductions were observed.

Following MG exposure, ectocervical ECs secreted significant levels of IL-6 and IL-8 ($p<0.05$ vs. PBS control). Endocervical ECs responded to MG with the most number of secreted cytokines that included IL-8, G-CSF, GM-CSF and MCP-1.

Using IL-8 as a representative comparator for all cell types, endocervical ECs were more responsive than vaginal or ectocervical cells when the fold increase of cytokine secretion by infected cells was calculated and compared to cells that received only PBS (ANOVA; $p<0.01$, See Table 4). A similar pattern of cytokine elaboration was observed following inoculation of MG at a MOI of 1 (data not shown). Cytokines that were not significantly induced by MG G37 or M2300 in any genital EC type included IL1-β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, MIP1-α, MIP1-β, Basic FGF, Eotaxin, IP-10, PDGF-BB and VEG-F.

Table 4. Cytokine elaboration from human genital ECs after MG G37 exposure[a].

	Vaginal (V19I, V12I, V11I)		Ectocervical (3ECI)		Endocervical (sA2EN)	
	MOI 10	**PBS**	**MOI 10**	**PBS**	**MOI 10**	**PBS**
IL-6	**127±13.1***	**69±1.7**	**63.7±1.8***	**21.3±2.4**	**348±13***	**196±15**
IL-8	**1458±117***	**785±11.3**	**3304±300***	**722±98**	**5e7±1347***	**6e4±367**
G-CSF	**261±46**	**227±37**	**548±143**	**779±122**	**155±6.2***	**93±21**
GM-CSF	**24±1.8***	**8±3.1**	**16±2.6**	**10±1.0**	**160±9.4***	**45±12**
MCP-1	**5.8±1.4**	**7±2.1**	**11.4±1.3**	**10±3.1**	**7.2±1.1***	**0.46±0.02**

[a]Human vaginal (n=3 donors), ectocervical or endocervical ECs were inoculated with MG G37 (MOI 10). An equal volume of the PBS vehicle was added and processed in parallel as a control. Culture supernatants were collected 48h PI. Values are expressed as the mean ± SEM pg/mL from triplicate wells. Cytokine elaboration pattern and magnitudes induced following exposure to strain M2300 were not significantly different than G37. PBS values are presented to indicate basal cytokine elaboration from each cell type. ND, not detected. *, $p<0.05$ vs. PBS control using Student's t-test. ($p<0.05$ vs. PBS control).

In polarized, 3-dimensional cultures of human vaginal ECs (V11I, V12I, and V19I) MG inoculation resulted in pro-inflammatory cytokine elaboration in a pattern similar to 2-dimensional cultures measured 6h PI. The single notable exception was IL-8 whereby a significant increase in acute phase secretion was observed 6h PI but at 12, 24 48 and 72h PI a significant decrease was observed compared to non-infected cells (Fig. 17).

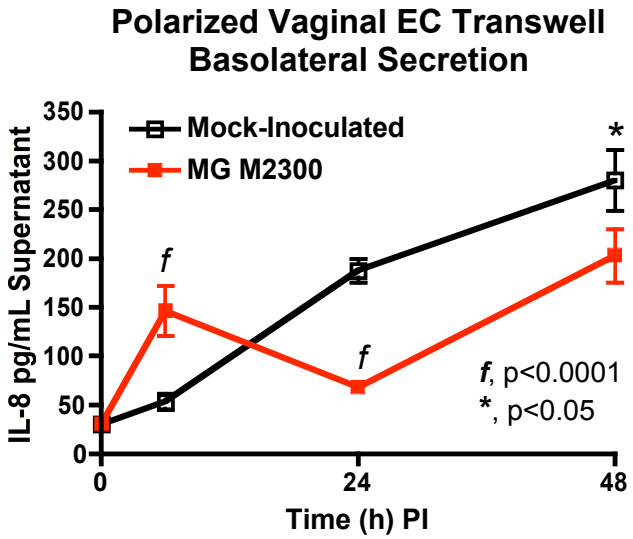


Figure 17. Dynamics of basolateral IL-8 secretion. Polarized Human vaginal ECs were inoculated with MG strain G37 or M2300. Basolateral supernatants were collected for cytokine quantification at indicated times after inoculation. Statistical significance was determined using the Student's t test.

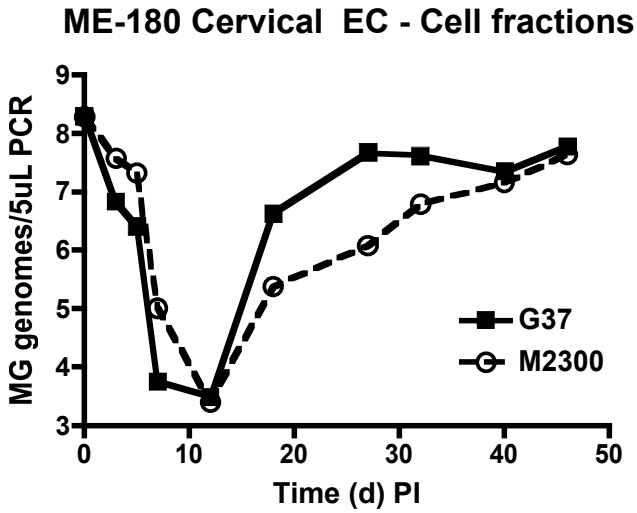


Figure 18. Dynamics of persistent MG infection of cervical ECs. ME-180 cells were inoculated with MG strains G37 or M2300 and then passaged for 48d after inoculation. Cells were collected at indicated times for PCR quantification of MG load.

Anti-inflammatory cytokine profiles from persistently infected cervical ECs

Considering that IL-8 secretion in polarized vaginal ECs was reduced following extended infection, we tested the hypothesis that prolonged exposure of MG to genital ECs results in a shift to anti-inflammatory cytokine secretion. Following inoculation of ME-180 cervical ECs with MG G37 or M2300, cells were passaged approximately every 4d for up to 48d PI to establish a persistent infection.

PCR titers of both the G37 and M2300 strains were reduced markedly during the first 10d of infection but were followed by a robust increase 15-25d PI. Subsequently, MG titers remained at roughly 1-5 MG/cell (Fig. 18). It is important to note that the presented values in figure 18 are from the cellular fraction of the ME-180 cultures

whereby cells were rinsed 3 times with a total of 25mL of PBS prior to collection of the cells for PCR quantification of MG. Therefore, the values represent either intracellular MG (observed by TEM; data not shown) or tightly attached extracellular MG not removed by the washing. Taken together with the observed protection from extracellular antibiotic treatment (Fig. 11), these data support strongly the hypothesis that MG preferentially targeted intracellular sites in persistent cultures of ME-180 cervical ECs.

Basal cytokine secretion was measured from supernatants of infected and mock-infected cells 33d PI and showed a significant down-regulation of IL-8 and significant increase in IL-10 secretion (Fig. 19). No additional cytokines were consistently down or up-regulated compared to non-infected cells among 3 independent trials of persistently infected cervical ECs.

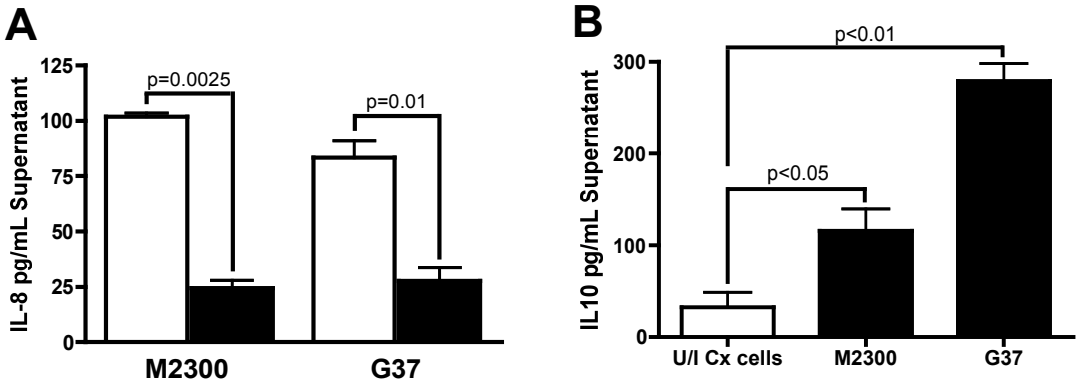


Figure 19. Anti-inflammatory cytokine profile in persistently infected cervical ECs. Following 33d of persistent MG infection of ME-180 cells, (A) IL-8 secretion was significantly reduced compared to non-infected cells. (B) Secretion of IL-10, an anti-inflammatory cytokine, was significantly increased relative to non-infected cells. This experiment is representative of 3 independent trials of persistently infected ME-180 cells. Significance was calculated using ANOVA.

DISCUSSION

Considering that MG reproductive tract infections in humans [89, 90] and non-human primates [91] can often become persistent, it seems likely that MG employs some tactic(s) to elude the host response to establish infection. Consistent with this supposition, attachment and invasion of MG strains G37 and M2300 to non-polarized vaginal and cervical ECs was rapid and observed as early as 2h PI (Fig. 10) suggesting that intracellular localization could provide a survival niche. Following inoculation, MG was found in peri-nuclear vacuoles similar in appearance to those reported in cultured Vero [85] and EM42 cells [86]. Using ME-180 cells, we next showed that MG was protected from extracellular gentamicin treatment by localization to intracellular sites within the cells (Fig. 11). In these studies, we also investigated egress of MG from infected ME-180 cells in cultures without gentamicin. We found that egress of MG from infected ECs resulted in MG titers in the culture supernatants that were 100 to 1000-fold lower than intracellular titers. These data indicated that MG preferred intracellular sites but that some egress was occurring. Importantly, these findings are the first report of intracellular localization in cultured human ECs from the vagina, ecto- and endocervix.

Knowing that the human vaginal mucosa is not simply a single monolayer of ECs, we next investigated the dynamics of MG infection using polarized, 3-dimensional multilayer EC cultures. Using electron microscopy, MG was shown to rapidly attach to the apical-most ECs (Fig. 12 and 13) followed by intracellular localization and transmigration of the cells within the multilayer (Fig. 14). These findings were confirmed using quantitative viability assays whereby, following inoculation of the apical

surface, MG viability decreased at the apical surface 6-8h PI but significantly increased in the multilayer from 0-72h PI ultimately leading to complete transmigration of the basolateral membrane by 24-72h PI (Fig. 15). It is evident that a loss of MG viability occurs after inoculation of the apical surface because cellular titers measured 24-72h PI do not reach the level of the inoculum. This likely was due to a subset of MG that did not invade the EC multilayer and was, in turn, removed during the washes of the apical surface during collection of the cellular multilayer. Alternatively, MG that only invade the apical most cells but do not completely transmigrate the multilayer also might be removed during these washes. Indeed, in viable titration assays of the apical surface, ECs were observed in the lowest dilution indicating some cells were collected in the washes. Collectively, this might be an important indicator that MG at the luminal surface could be shed readily via infected EC, specifically during sloughing of these cells during sexual intercourse. To this end, we did observe attachment to the elongated apical-most cells of the polarized vaginal EC multilayer (See Fig. 12 and 13).

The findings of intracellular localization in 2- and 3-dimensional EC cultures are consistent with the clinical observation of heavy intracellular MG loads in PCR-positive vaginal specimens [76] and earlier reports of intracellular localization in cells of non-reproductive tract origin [85, 87, 88]. Importantly, in both 2- and 3-dimensional cultures, MG also was found in extracellular fractions of infected cells (Fig. 11). In 3-dimensional cultures, MG were observed to move throughout the multilayer, presumably both on a vertical and horizontal plane. This provides an explanation for why only a subset or organisms completely transmigrate the multilayer by 72h PI. In addition, some

organisms also may have genetic characteristics that facilitate dissemination throughout reproductive tract tissues. To this end, additional studies using clonally derived G37 or low-pass clinical strains are warranted to evaluate the potential genotypic and phenotypic differences due to culture adaptation. However, the presented results firmly indicate MG's capacity for invasion and prolonged intracellular survival and also suggest that the MG life cycle in reproductive tract tissues may include an extracellular component.

Because ECs likely serve as the first responders to STI we next investigated the acute-phase cytokine response to MG from non-polarized human vaginal and cervical ECs. We found that MG elicited minimal innate responses from human vaginal ECs from 3 donors but ecto- and endocervical EC were highly responsive and secreted cytokines consistent with recruitment of immune cells including IL-8, G-CSF, GM-CSF and MCP-1 (Table 4). The increased responsiveness of endocervical ECs may have biological relevance, as the normally sterile upper tract tissues likely are more sensitive to microbial contamination than the lower genital tract. Using polarized, 3-dimensional vaginal ECs, the cytokine response to MG infection was similar to non-polarized cells with the exception of IL-8. IL-8 is a potent pro-inflammatory chemokine secreted by mucosal ECs for the recruitment of immune cells to inflamed tissues and was found to be suppressed following 24, 48 and 72h of infection (Fig. 17). The clinical implications for this finding currently are unclear but, in limited studies, we have also observed a trend of IL-8 suppression by MG in long-term cervical EC cultures (Fig. 19). Although MG has been associated with inflammation in several clinical investigations, such as of cervicitis, many cases of MG infection of the reproductive tract do not seem to be associated with

inflammatory sequelae (personal communication, David H. Martin). These outcomes could be attributable to different stages of the infection process, differences in the host immune response for controlling the infection or MG strain heterogeneity among the population and within the individual.

Determining the mechanisms by which MG both activates and evades the host immune response likely will be a topic of considerable research in the future. In Chapter 5, the susceptibility of MG to macrophage responses will be addressed and whether intracellular EC localization provides protection from phagocytosis. However, the findings of immune suppression suggest that MG might actively suppress EC responsiveness during persistent reproductive tract infections and may impair recruitment of immune cells to the reproductive mucosa. Although not observed in our studies using non-polarized vaginal ECs, this notion warrants continued investigation. Immune suppression by MG could enhance the susceptibility to other STI and provide a putative mechanism for the clinically observed associations of MG severity with HIV-1 shedding [92, 93].

Establishment of primary infection and persistence of MG in host tissues is not well understood. Our findings suggest that MG rapidly invades host ECs thereby exploiting an intracellular survival niche. Importantly, MG infection resulted in inflammatory cytokine responses from vaginal and cervical ECs in a pattern most consistent with recruitment of monocytes and macrophages but intracellular localization may provide protection from host immune responses (See Chapter 5). Considering that the upper tract tissues are where inflammation due to microbial infection likely has the

most severe consequences, it seems possible that persistent infection of female reproductive tract tissues may indeed result in inflammatory outcomes that could affect reproductive health but continued research is necessary to fully elucidate the mechanisms of MG-induced urogenital disease.

CHAPTER 4: TOLL-LIKE RECEPTOR RECOGNITION OF *M. GENITALIUM* AND THE IMMUNOSTIMULATORY ROLE OF MEMBRANE PROTEINS

OVERVIEW

MG has been shown to elicit pro-inflammatory cytokine secretion from human vaginal, ecto- and endocervical ECs (See Chapter 3). ECs of the vagina and cervix have been shown to express robust levels of TLR2, 3, 5, 6 and CD14 with low levels of TLR1, 4 and 7-9. We sought to determine which TLR were involved in recognizing MG and identify specific components capable of activating highly expressed TLR to better understand how MG initiates inflammation. The results showed that human TLR2/6 heterodimers recognized the viable MG organism and three specific recombinant antigenic proteins encoded by MG307, MG309 and MG338. In addition, TLR9 was also shown to recognize viable MG and MG DNA supporting the hypothesis that intracellular localization also leads to inflammation. Importantly, these findings provided seminal evidence for the role of extra- and intracellular TLR for initiation of acute-phase inflammation at reproductive tract mucosa. Portions of this chapter have been published by McGowin, et al., *Infect Immun*, December 2008.

INTRODUCTION

Despite the significant association of MG with inflammatory reproductive tract disease syndromes, including pelvic inflammatory disease[71] and cervicitis [21, 23, 72-74], the mechanisms for initiation of the innate immune response in these tissues have yet to be elucidated. The female reproductive tract includes the normally sterile upper genital tract composed of the endocervix, uterus, fallopian tubes and ovaries. The lower genital tract includes the polymicrobial environment of the vaginal and the transitional ectocervical tissues. ECs of the reproductive tract provide a physical barrier against invading pathogens and are differentially equipped as non-traditional immune cells capable of activating early innate immune responses [38, 39]. These cells recognize several classes of conserved pathogen-associated molecular patterns (PAMPs) via the TLR of transmembrane receptors. Ligation of TLR results in activation of the innate response through secretion of pro-inflammatory cytokines but no studies, to our knowledge, have addressed the genital EC response to MG infection.

ECs of the vagina and cervix express robust levels of TLR2, 3, 5, 6 and CD14 with low levels of TLR1, 4 and 7-9 [42] and are the first cells to encounter MG following transmission. Among TLR implicated in recognition of bacteria, the TLR2/6 heterodimer is hypothesized to be important for recognition of bacterial ligands by reproductive ECs because their expression level and immunologic responsiveness is increased relative to other TLR [42]. It is well documented that lipoproteins from select pathogenic *Mycoplasma spp*. can interact with TLR leading to inflammatory cytokine production. To this end, vaginal and cervical ECs respond to specific TLR2/6 agonists with robust

pro-inflammatory cytokine elaboration including the *Mycoplasma fermentans* lipopeptide (MALP-2) [94] and the fibroblast-stimulating lipopeptide-1 of *Mycoplasma salivarium* (FSL-1) [42].

Like other MLO, MG contains a significant proportion of lipoproteins in the cell membrane [49] consistent with the identification of 21 putative lipoprotein genes in the 580-kb genome [43]. It has been shown recently that Triton X-114 preparations of detergent-soluble components from MG upregulate transcription of TNF-α, interleukin 1-β (IL1-β) and IL-6 mRNA in the human THP-1 monocytic cell line [48, 50] suggesting that lipoproteins may, at least in part, be responsible for immune activation. Of the 21 putative lipoprotein genes, we selected MG307, MG309 and MG338 for inflammatory characterization because each is expressed during MG infection and is recognized by sera of MG patients suggesting immune recognition by the host (L. Ma and D.H. Martin, unpublished findings). Interestingly, a specific region within the MG309 coding sequence has been shown previously to have significant variability in the number of short tandem repeats (STR) among and within clinical isolates [95]. This suggests that surface-exposed membrane lipoproteins could be under immune-mediated selective pressure and, combined with MG's capacity for invasion of genital ECs (See Chapter 3), provides rationale for investigation of intracellular TLR.

TLR9 was first identified as a receptor for bacterial DNA and unmethylated CpG motifs in 2000 [96] and is localized to the intracellular membrane compartment including the endoplasmic reticulum ER, endosomes and lysosomes (Reviewed in [97]). Bacterial DNA is known to be a potent PAMP capable of eliciting robust activation of the innate

immune response (Reviewed in [98]). However, TLR9 does not recognize mammalian DNA because most CpG motifs are methylated in mammals [98] making it a specific detector of DNA from bacterial pathogens. Considering that MG has been shown to occupy the intracellular spaces of ECs from patient samples and cultured cells *in vitro* we sought to evaluate the capacity for MG G37 to activate pro-inflammatory responses through TLR9

Collectively, our results established that the intact MG organism and 3 immunogenic MG proteins activate NF-κB via TLR2/6 heterodimers ultimately leading to pro-inflammatory cytokine elaboration from human vaginal, ecto- and endocervical ECs. In addition, intracellular MG infection and purified MG genomic DNA also led to activation of NF-κB via TLR9. These findings establish two putative mechanisms for induction of inflammatory reproductive tract disease in MG patients and elucidate a specific immunogenic protein capable of activating the innate immune response.

MATERIALS AND METHODS

Cloning and purification of recombinant *M. genitalium* proteins

Mycoplasmas utilize the UGA codon to encode tryptophan rather than a termination codon making it difficult to express mycoplasma genes in *E. coli* hosts. We investigated 3 antigenic proteins that have been identified as putative membrane proteins: MG309, MG307 and MG338. The MG309 gene from MG G37 (bp 382732-386409; GenBank L43967) contains 7 UGA codons while MG307 (bp 377975-381508) contains 5

and MG338 (bp 423100-426912) contains 14. The largest fragment of MG309 free of UGA stop codons (bp 2755-3678) was amplified from MG G37 genomic DNA by PCR using primers 5'- GGATCCACCAACAACAAAACCCCAACAG-3' and 5'-GTCGACGCTGTGAACTGATTGCTGA-3', that contained *Bam*HI and *Sal*I restriction sites added to the 5' ends (underlined), respectively. The largest region free of UGA codons was PCR-amplified similarly from MG307 (bp 826-2463) and MG338 (bp 1294-1668). Each PCR product was cloned into the pROEX HtB expression plasmid (Invitrogen) that directs expression of the recombinant protein with a 6-histidine (6-his) tag allowing purification using a nickel affinity column (See Fig. 20).

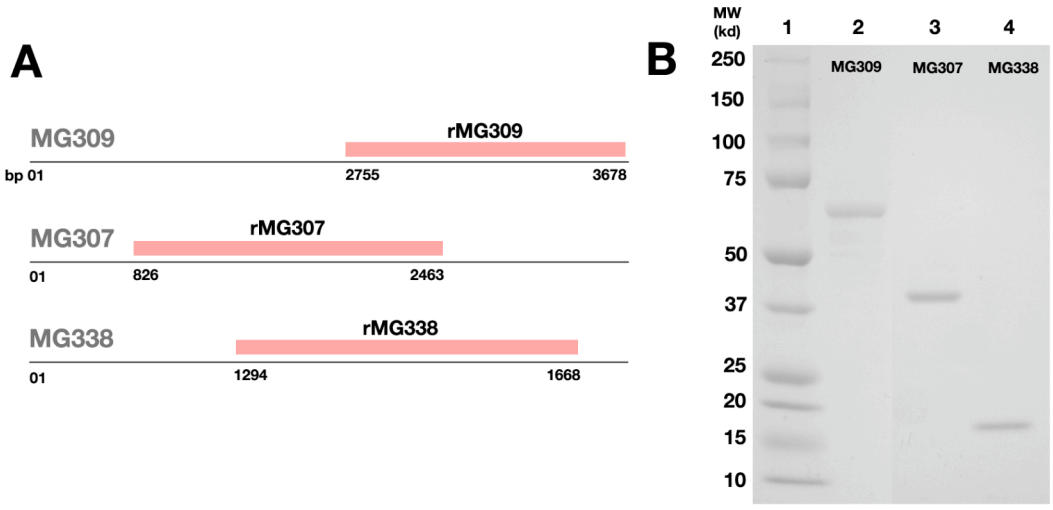


Figure 20. Purification of recombinant MG proteins. The largest fragment of MG309, MG307 and MG338 free of any UGA codons (A; red region) was cloned into the pROEX-HtB 6-his expression plasmid and then purified on nickel resins. (B) The correct size and purity were verified by coomassie staining on SDS-PAGE gels.

The 'empty' pROEX vector and the *Salmonella dublin* flagellin (FLAG) fliC gene [99] in pROEX were used as controls. All plasmid constructs were confirmed by restriction digest and DNA sequencing and then expressed in *E. coli* BL21 DE3 (Protein Express, Cincinnati, OH). Expression of recombinant MG proteins or FLAG was induced using Isopropyl β-D-1-thiogalactopyranoside (0.1M). The optimum duration of expression was determined by kinetic evaluation of induced *E. coli* lysates using SDS-PAGE.

Purification of 6-his fusion proteins was accomplished first by lysozyme-mediated lysis and sonication of induced *E. coli* cell pellets. Cell lysates then were applied to Ni^{2+}-charged resins and purified under denaturing conditions with 6M urea (His-Bind Purification; Novagen, Darmstadt, Germany). Purified proteins were dialyzed overnight at 4°C in 3L of PBS to remove urea and any free LPS. Correct size and purity of rMG309 preparations were verified using SDS-PAGE with coomassie staining and reactivity with a polyclonal rabbit serum against a MG G37 whole cell lysate (See Fig. 20). The yield of recombinant protein was measured using the Protein Quantification Assay (BioRad). Contaminating *E. coli* endotoxin was quantified using the Pyrotell Limulus amebocyte lysate assay (Associates of Cape Cod; East Falmouth, MA). Heat denaturation was performed by incubating purified rMG309c preparations (0.5μg/mL) at 95°C for 15min and then cooling on ice. Proteinase-K digestion was done by mixing 3μL Proteinase-K solution (Qiagen) with 20uL of the stock recombinant protein preparation and incubation at 56°C for 1h. Protein digestion was verified by SDS-PAGE.

Following proteinase-K treatment, the digested sample was heat-inactivated as described above to ensure no protease activity was carried forward into the TLR or cytokine assays.

Propagation of *M. genitalium* strains G37 and M2300

M. genitalium type strain G37 (MG G37; ATCC 33530) or the lower passage Danish M2300 strain were propagated in Friis FB medium [13]. MG grown to mid-log phase were harvested from culture flasks by pouring off the spent medium, extensively washing adherent mycoplasmas with 5 volumes of approximately 5mL each of sterile PBS and then scraping into fresh PBS.

Cell culture and TLR stimulating compounds

Human embryonic kidney cells stably transfected with selected human TLR (HEK293; InvivoGen) were maintained in DMEM supplemented with 10% FBS, penicillin-G (100U/mL), streptomycin sulfate (100ug/mL), 0.1mM non-essential amino acids (Sigma-Aldrich, St. Louis, MO) and 2mM L-glutamine (Invitrogen). Human vaginal, ectocervical or endocervical EC were maintained in KSFM medium as described previously [42]. The synthetic lipopeptide Pam2CGDPKHPKSF (FSL-1) derived from *Mycoplasma salivarium* (InvivoGen, San Diego, CA) was used as a TLR2 and TLR2/6 agonist for the HEK293 cell studies and for quantification of cytokine secretion from genital EC (0.1ug/96well). Recombinant endotoxin-free *S. dublin* flagellin (FLAG; 1ng/96well) [99], provided by Dr. T. Eaves-Pyles, was used as a positive control for TLR5-dependent NF-κB activation. *E. coli* LPS (Sigma-Aldrich) was applied to

HEK293 cells expressing human TLR4/CD14 as a positive control for NF-κB activation. The active CpG-containing oligonucleotide (1018 ISS, Dynavax Technologies) was used as a positive control for TLR9 stimulation.

NF-κB reporter system for TLR-mediated responsiveness

HEK293 cells stably expressing TLR2, TLR2/6, TLR9, TLR4/CD14 or TLR5 (InvivoGen) [100] were seeded into T-75 culture flasks and incubated overnight at 37°C in an atmosphere containing 5% CO_2. Cells then were transfected with a NF-κB-responsive secreted alkaline phosphatase (SEAP) reporter plasmid (Clontech, Mountain View, CA) using Lipofectamine Plus (Invitrogen) per the manufacturer's instructions. Transfected cells were seeded into 96-well plates ($5x10^4$/well) and incubated overnight. Purified MG proteins (10ng-1ug/well), FSL-1 (0.1ug/well), *S. dublin* FLAG (1ng/well) or viable MG G37 (MOI 1-100) were applied to triplicate wells of HEK293-hTLR cells and incubated for 24h. Supernatants were removed and heat-inactivated for 30min at 65°C to destroy endogenous alkaline phosphatases. SEAP activity, directly representing NF-κB activation, was quantified using the Phospha-Light Chemiluminescence Reporter Assay (Tropix, Bedford, MA) and a TR717 microplate luminometer with WinGlow software (Tropix/PE Applied Biosystems, Bedford, MA). Relative fluorescent unit values were normalized by calculating the mean SEAP secretion from cells expressing TLR2/6 treated with the PBS vehicle and then adjusting the data set from the other cell types to facilitate a comparison of relative responsiveness.

Quantification of TLR-mediated cytokine secretion

Human vaginal, ectocervical or endocervical ECs were seeded at $1x10^5$ cells/well. Following overnight incubation at 37°C, culture supernatants were removed and replaced with fresh medium containing purified MG proteins (0.5ug/well). Each experimental condition was tested in triplicate wells. Positive controls for TLR-mediated stimulation included FSL-1 (0.1ug/well) and *E. coli* LPS (100ng-1pg/well). As a negative control for experimental manipulation, an equal volume of the PBS vehicle was added to triplicate wells and processed in parallel to establish baseline cytokine levels. Following 6h incubation at 37°C, secreted cytokines were quantified from culture supernatants via cytometric bead array (CBA) assay using the human 27-Plex panel of cytokine targets (BioRad).

Determination of the TLR-activating domain of MG309

The membrane lipoprotein encoded by MG309 has been hypothesized to be under immune-mediated pressure [95] and therefore we investigated specific MG309 sub-fragments for TLR specificity. To determine the specific pro-inflammatory regions, three sub-fragments of rMG309 corresponding to the N-terminal one third (bp 383382-383655; (5'- GGATCCACCAACAACAAAACC-3' and 5'-CGTCGACCCACCACTTGATTGAGTAGA-3'), the middle one third (bp 383067-383381; 5'-GGATCCTCTACTCAATCAAGTGG-3' and 5'-CGTCGACCCATTTGTACCTATATACCC-3') and the C-terminal one third (bp 382746-383067; 5'-GGATCCGGGTATATAGGTACAAAT-3' and 5'-

CGTCGACCCGAATTTACGCACC-3') were amplified with primers containing *BamHI* and *Sal I* restriction sites (See Fig. 20). PCR products were purified and cloned into the pROEX HtB expression plasmid as described above. IPTG-induced expression, Ni^{2+} purification and LPS testing of the recombinant sub-fragments were done as described above for the full-length rMG309.

Statistical Analyses

The one-way ANOVA followed by Dunnett's post-test (Prism v. 4.0, GraphPad, San Diego, CA) was used to calculate significant differences in SEAP activity or cytokine elaboration when more than two conditions were compared. The Student's t-test was used for comparison of differences in magnitude for individual cytokines. Significance was indicated when $p<0.05$.

RESULTS

***M. genitalium* G37 activated NF-κB via TLR2/6**

Activation of NF-κB is a key step in TLR-mediated responses and is necessary to induce the innate immune response to pathogens ultimately resulting in inflammatory cytokine production [101]. Mechanistically, MG was predicted to ligate TLR that are expressed at high levels in genital ECs via antigenic proteins such as MG309. Consistent with this hypothesis, viable MG elicited significant NF-κB activation in HEK293 cells expressing TLR2/6 at a MOI of 100 ($p<0.01$; Fig. 21A). In cells expressing TLR2 alone,

viable MG elicited low but insignificant ($p>0.05$) NF-κB activation only at a MOI of 100 that was significantly less than HEK293 cells expressing TLR2/6 heterodimers (*f*, $p<0.01$; Fig. 21A). Result from experiments using strain M2300 were indistinguishable (data not shown). In HEK293 cells expressing hTLR5, no significant NF-κB activation was observed in response to MG G37 but these cells were highly responsive to purified recombinant FLAG ($p<0.01$ vs. PBS vehicle control; Fig. 21B).

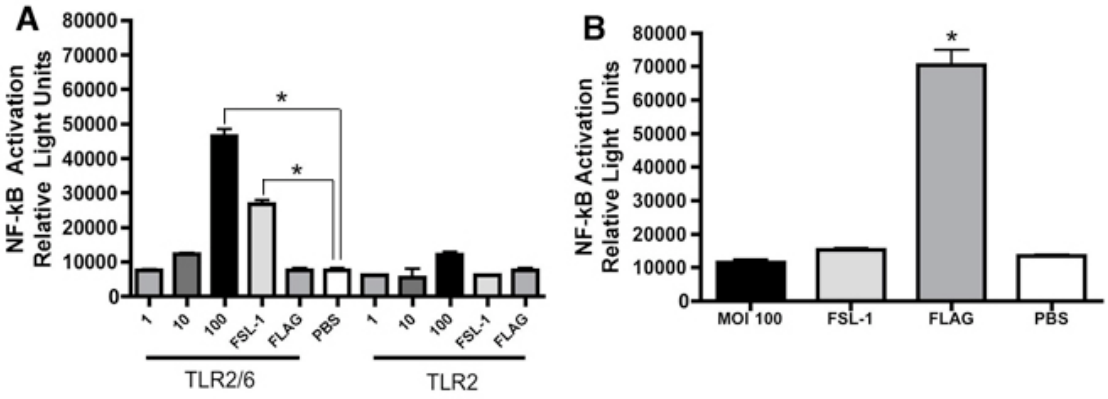


Figure 21. MG G37 induced TLR2/6-mediated NF-κB activation. HEK293 cells, stably expressing human TLR2/6, TLR2 (A) or TLR5 (B) were transfected with a NF-κB responsive SEAP reporter plasmid to quantify NF-κB activation. Extensively washed viable MG (MOI 1-100) was applied to HEK293 cells and then culture supernatants were collected 24h PI for SEAP quantification. Purified recombinant *S. dublin* FLAG or FSL-1 from *M. salivarium* were used as TLR specificity controls and processed in parallel on each cell type. Data shown are the mean ± SEM of SEAP induction collected in two independent experiments performed in triplicate wells. Statistical comparisons were made using one-way ANOVA followed by Dunnett's post-test; * indicates $p<0.01$ vs. PBS vehicle control.

Purification of endotoxin-free recombinant *M. genitalium* proteins

We cloned the largest portion of MG307, MG309 and MG338 (rMG307, rMG309, rMG338) free of UGA stop codons and expressed the resulting proteins to high levels in *E. coli*. Each clone was verified by DNA sequencing. Purified MG307, MG309 and MG338 proteins were each present as a single band on SDS-PAGE gels at approximately 60, 40, and 20 kd, respectively (Fig. 22B, lane 1). These sizes were consistent with the predicted molecular mass calculated from the cloned DNA sequence. Stock preparations of purified MG proteins were recognized by a rabbit polyclonal antiserum raised to MG lysates (MG309 shown in Fig. 22) and by MG-infected patient sera (Liang Ma and David H. Martin, unpublished findings). These proteins were not recognized by pre-immune serum from the same rabbit (Fig. 22B, lane 3).

The protein encoded by MG309 was investigated further to determine the TLR-activating and pro-inflammatory domain. The three sub-fragments of rMG309 corresponding to the N-terminus, middle and C-terminus were expressed and purified to similar concentrations and purity (Fig. 22C). The amount of LPS endotoxin in undiluted recombinant protein preparations was quantified using the Limulus amebocyte assay and was consistently less than 10pg/mL. This concentration was below the minimum stimulatory concentration for HEK293 cells expressing TLR4/CD14 or genital ECs. Furthermore, HEK293 cells expressing TLR4 and CD14 were not responsive to rMG309c (data not shown) indicating a lack of LPS contamination.

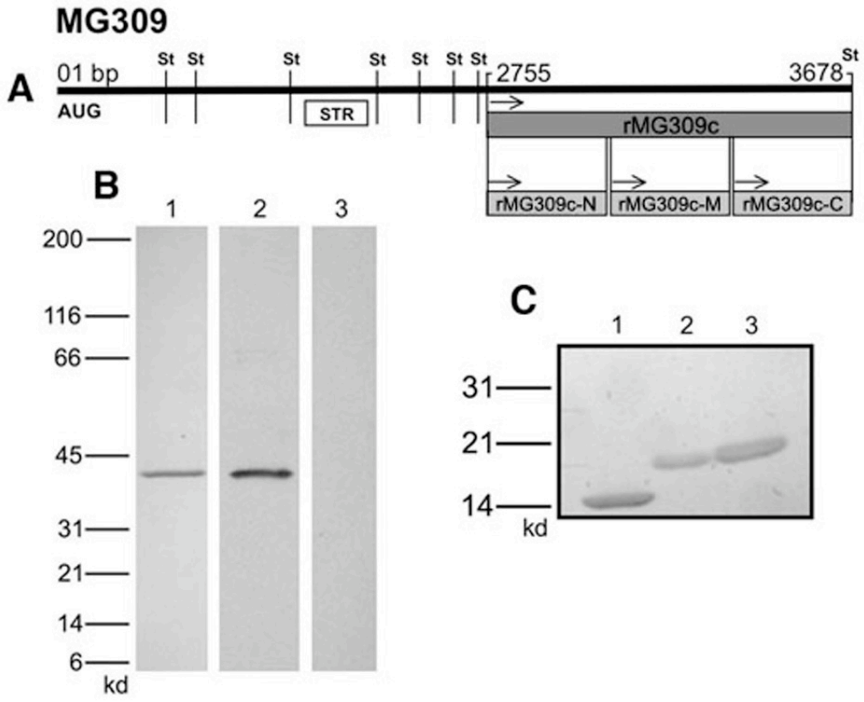


Figure 22. Purification of recombinant MG309 (rMG309c) and subfragments. (A) The largest coding fragment of MG309 (designated rMG309c) free of UGA codons (stop signal (St) in *E. coli*) was PCR amplified and cloned into pPROEX HTb for expression of a 6his-tagged recombinant protein in BL21 *E. coli*. The chosen coding region did not include the highly variable short tandem repeat (STR) stretch that varies significantly in length within *in vitro* and clinical specimens. (B) Nickel-purified rMG309c ran at approximately 40kd using SDS-PAGE (25ug load; coomassie stain, lane 1) and was recognized by a rabbit polyclonal antibody generated to MG G37 lysates (lane 2) but not by pre-immune serum (lane 3). (C) Three sub-fragments of rMG309c representing the N-terminus (rMG309c-N; appx. 15kd), middle (rMG309c-M; appx. 18kd), and carboxyl (rMG309c-C; appx. 18kd) terminus were purified similarly and ran on SDS-PAGE gels (lanes 1, 2 and 3, respectively; coomassie stain).

Purified *M. genitalium* proteins activated NF-κB via TLR2/6

Purified rMG307, MG309c and rMG338 (1ug and 0.1ug/well) elicited significant NF-κB activation in HEK293 cells expressing human TLR2/6 compared to PBS ($p<0.01$; Fig. 23A, D and E, respectively). Using rMG309 as a representative membrane protein, HEK293 cells expressing TLR2 without TLR6 were not responsive indicating TLR2/6 heterodimers were necessary for efficient recognition (Fig. 23A). Heat denaturation and proteinase-K digestion of purified rMG309c reduced the SEAP production to baseline levels in cells expressing TLR2/6 (Fig. 23B). An equimolar quantity of FSL-1 (0.1ug/well) induced significant SEAP production in cells expressing TLR2/6 (Fig. 23B; $p<0.01$). As expected, cells expressing TLR2/6 were not responsive to the purified recombinant TLR5 agonist, FLAG from *S. dublin* among 3 independent experiments (1ng/well; Fig. 23B, D, and E). HEK293 cells expressing human TLR5 (Fig. 23C and F) or TLR4/CD14 (data not shown) did not respond to rMG307, rMG309, rMG338 (1ug/well) or FSL-1 and served as a control for TLR specificity.

***M. genitalium* proteins elicited cytokine secretion from human genital ECs**

ECs of the female reproductive mucosa express TLR1-9 [102-104] but express the highest levels of TLR2, 3, 5 and 6 [42]. To determine whether rMG309 was immunostimulatory to female genital tract ECs, purified rMG309c was applied to immortalized human vaginal, ecto- and endocervical ECs to quantify induced cytokine responses.

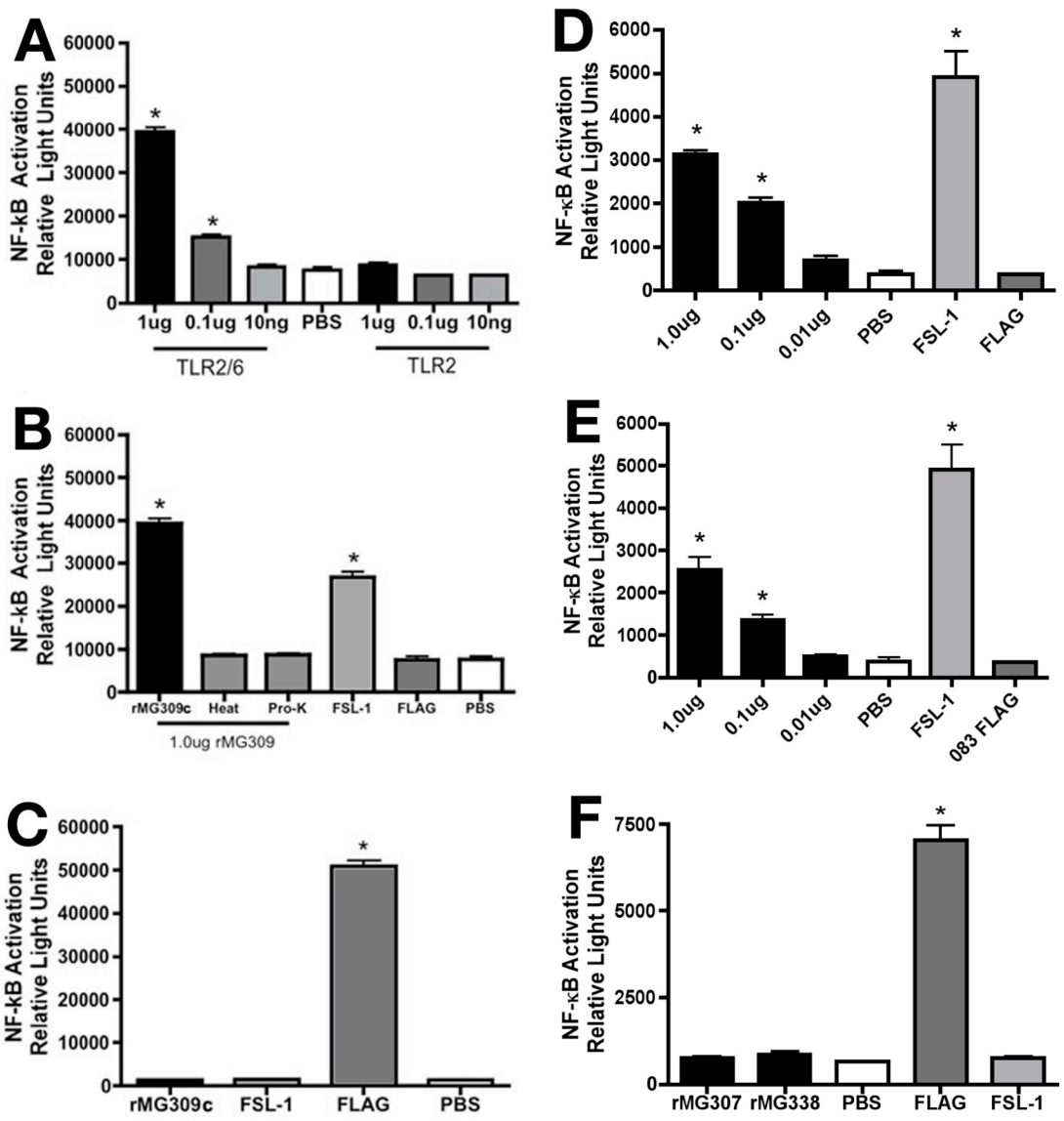


Figure 23. rMG307, rMG309 and rMG338 induced TLR2/6-mediated NF-κB activation. HEK293 cells, stably expressing TLR2/6 (A, B, D, E), TLR2 (A) or TLR5 (C, F) were transfected with a NF-κB responsive SEAP reporter plasmid to quantify NF-κB activation. Purified MG proteins were applied to each HEK293 cell type (1ug-0.01ug/well) and then culture supernatants were collected 24h later for quantification of SEAP. An equivalent molar amount of purified recombinant *S. dublin* FLAG or FSL-1 was processed in parallel on TLR2/6- or TLR5-expressing cells as a control for TLR specificity. Data shown are the normalized mean ± SEM of SEAP induction collected in two independent experiments performed in triplicate wells. Statistical comparisons were made using one-way ANOVA followed by Dunnett's post-test. *, $p<0.01$ vs. PBS vehicle control.

rMG309 elicited significant levels of IL-8 secretion that peaked 6h following application to each EC type ($p<0.05$; Table 5). Secretion of G-CSF and GM-CSF was significantly increased compared to PBS controls from both ecto- and endocervical ECs but not vaginal ECs. Finally, endocervical ECs, but not vaginal or ectocervical ECs, exposed to rMG309 also secreted significant levels of IL-6 ($p<0.05$; Table 5).

The patterns and magnitudes of cytokines induced by rMG307 and rMG338 were not distinguishable from rMG309 (data not shown) indicating a conserved response following TLR2/6-specific activation. As observed with the HEK293 cells, heat-denaturation or proteinase-K digestion significantly reduced the inflammatory capacity of rMG307, rMG309 and rMG338 to levels equivalent to the PBS vehicle (data not shown).

The inflammatory capacity of rMG309 was localized to a 91 amino acid region

Because these recombinant proteins activated NF-□B via TLR2/6 without the native N-terminus and traditional lipoylation, we tested the hypothesis that specific peptides could be responsible for TLR2/6 activation. Using rMG309 as a representative membrane protein, 3 sub-fragments (See Fig. 22A and C) were cloned, purified and then tested for TLR2/6 activation. Application of equimolar amounts of each sub-fragment showed that the TLR2/6 stimulating capacity was localized to amino acids 919-1009 composing the N-terminus of the rMG309 protein (rMG309c-N; Fig. 24A).

Table 5. Cytokine elaboration from human vaginal, endo- and ectocervical ECs following exposure to purified rMG309[a].

	Vaginal EC		Ectocervical EC		Endocervical EC	
	rMG309c	**PBS**	**rMG309c**	**PBS**	**rMG309c**	**PBS**
IL-6	34.8±5.1	27±2.6	27.1±3.2	14.2±0.63	114±3.1*	26.6±2.9
IL-8	186±0.6*	132±11	1792±38*	167±17	62997±6667*	3705±1440
G-CSF	17±0.5	12±0.1	368±7.5*	181±3.2	200±5.1*	24±2.4
GM-CSF	<LL	<LL	10.8±0.9*	4.9±0.4	32±4.0*	6±1.9

[a] Vaginal EC, ecto- and endocervical ECa were stimulated with rMG309 (0.5ug/well) or an equal volume of the PBS vehicle as a control. Culture supernatants were collected 6h following stimulation to quantify secreted cytokines. Values are expressed as mean ± SEM pg/mL supernatant. <LL (less than the lower limit), values too low to be calculated from the standard curve. *, significantly increased compared to PBS vehicle control ($p<0.05$; Student's t-test).

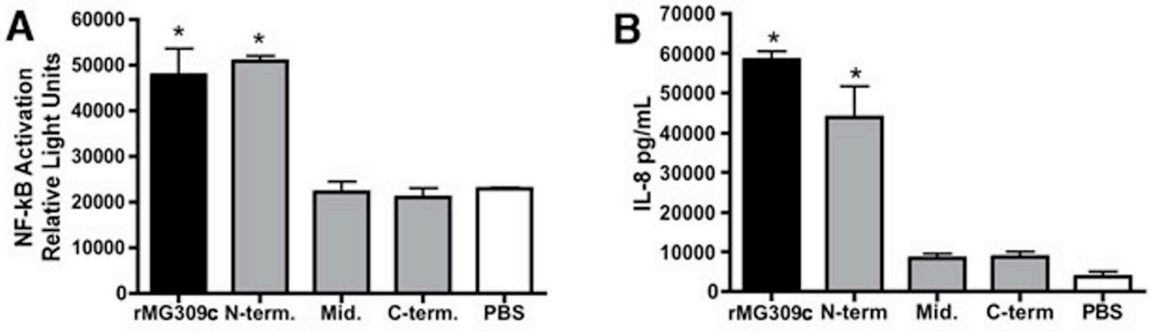


Figure 24. TLR2/6 binding and inflammatory capacity of rMG309c were localized to a 91 amino acid region. (A) HEK293 cells, stably expressing TLR2/6, were transfected with a NF-κB responsive SEAP reporter plasmid to quantify NF-κB activation. Three sub-fragments of rMG309c were purified and tested for their ability to activate NF-κB via TLR2/6. (B) To quantify the cytokine response to the rMG309c sub-fragments, equimolar amounts of purified rMG309c or the three sub-fragments were applied to human vaginal, ecto- and endocervical ECs. Cytokines were quantified from culture supernatants 6h PI. Data shown are the mean ± SEM from a representative study that was replicated in two independent experiments performed in triplicate wells. Statistical comparisons were made using one-way ANOVA followed by Dunnett's post-test. *, $p<0.01$ vs. PBS vehicle control.

This 91 amino acid region also elicited significant pro-inflammatory IL-8 elaboration from endocervical ECs (Fig. 24B), the most sensitive genital EC type in our studies. The IL-8 levels were similar in magnitude to the complete rMG309 peptide (Table 5).

The pattern of tested cytokines induced by the N-terminal sub-fragment was identical to the full-length rMG309 in vaginal, ecto- and endocervical EC cultures (data not shown). NF-κB activation and induction of inflammatory cytokine secretion by the N-terminal region of rMG309 was equivalent to the induction from the complete rMG309 protein indicating the TLR2/6 activating capacity was specifically localized to this region. No TLR2/6-mediated NF-κB activation or pro-inflammatory cytokine secretion from vaginal or cervical ECs were observed from the middle or C-terminal rMG309 sub-fragments (Fig. 24) thereby confirming the specific activity of the N-terminal region within the C-terminus of MG309.

Live *M. genitalium* and purified *M. genitalium* DNA activated NF-κB via TLR9

Following exposure to HEK293 cells expressing TLR9, viable MG G37 elicited significant NF-□B activation in at a MOI 10 and MOI 100 ($p<0.05$; Fig. 25A). UV-inactivated MG G37 elicited significant NF-□B activation at MOI 100 ($p<0.05$; Fig. 25A) but not at MOI 10 or MOI 1 ($p<0.05$ vs. PBS vehicle control). However, UV-inactivation significantly reduced the TLR9-activating capacity relative to viable MG ($p<0.05$; MOI 100). Purified DNA from MG G37 also activated NF-□B in cells expressing human TLR9 at concentrations ranging from 1.0-2.0μg/mL (Fig. 25B). Purified CpG-containing oligonucleotides (1018 ISS; 50μg/mL) were used as a positive control and also elicited significant NF-□B activation via TLR9. Similar to the studies using TLR2/6-expressing cells, HEK293 cells expressing hTLR5 were not responsive to MG G37 or purified MG DNA but were highly responsive to purified FLAG resulting in

significant NF-□B activation (See Fig. 23, p<0.05 vs. PBS vehicle control). Experiments using MG strain M2300 and purified M2300 DNA were similar (data not shown).

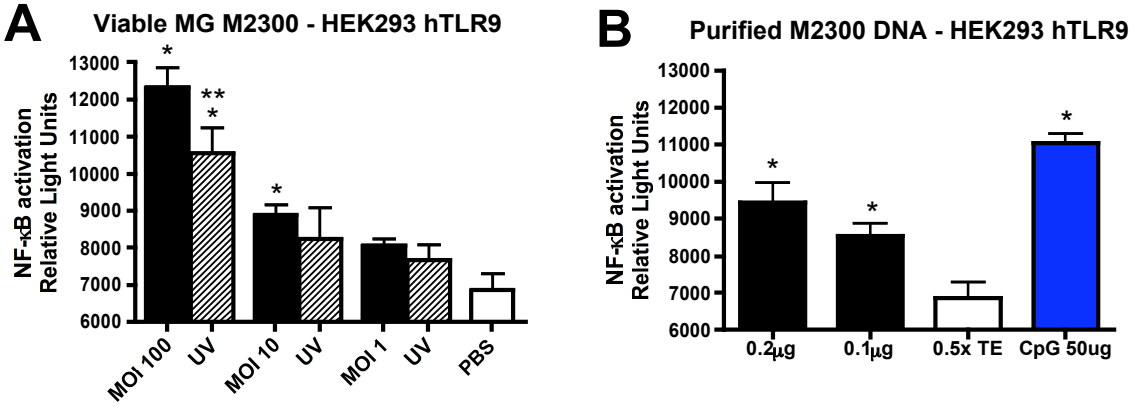


Figure 25. TLR9 activation by MG. HEK293 cells expressing human TLR9 were transfected with a NF-κB responsive SEAP reporter plasmid to quantify TLR9-specific NF-κB activation. (A) Viable MG M2300 (MOI 100-1) or UV-killed MG was inoculated onto HEK293 cells and then culture supernatants were collected 24h later for quantification of SEAP. (B) Purified MG DNA (0.2-0.1μg) was also evaluated for its TLR9-specific immunogenicity. Purified CpG-containing oligonucleotides were used as a positive control from TLR9 stimulation. Significance was determined using ANOVA. *, p<0.05 compared to PBS or TE baseline control. **, p<0.05 compared to viable MG (MOI 100).

DISCUSSION

MG infection of the reproductive tissues in women is associated with cervicitis [21, 23, 72-74], endometritis [24], PID [71] and, with less conclusive evidence, tubal

infertility [28, 29]. The mechanisms and cell types involved in the early recognition of MG infection and activation of the innate immune response remain unclear. We investigated whether MG G37 was recognized by highly expressed TLR throughout the female reproductive tract and verified these findings using 3 recombinant antigenic proteins encoded by MG307, MG309 and MG338.

We observed significant activation of NF-κB in response to viable MG via TLR2/6 heterodimers expressed in HEK293 cells (Fig. 21A). NF-κB activation was observed via TLR2 alone but at a significantly reduced level and only at the highest concentration of MG suggesting that TLR2 homodimers likely are not sufficient for robust recognition of MG. Interaction of MG with cell-surface TLR, such as TLR2 and 6, is a plausible mechanism for innate immune response activation as clinically isolated MG strains have been shown directly to interact with the host cell surface during the process of adhesion and entry into host cells (See Chapter 3) [85, 88]. Activation of TLR2/6 by the intact MG organism suggested that the membrane-associated proteins likely mediate a significant proportion of the innate immune activation from reproductive tract tissues as TLR2 is involved commonly in recognition of bacterial proteins [105].

Mycoplasmas have no cell wall but contain a single plasma membrane system [106, 107] comprised heavily of proteins [5, 49] that are associated with the ability to adhere to the epithelium [108, 109]. Considering the observed antigenic characteristics of membrane proteins, it was hypothesized that MG307, MG309 and MG338 might also activate innate immune responses via TLR2/6. These proteins were targeted for investigation because they are expressed during genital tract infection and axenic culture

([95] and L. Ma, personal communication). A homolog of MG309 exists in *M. pneumoniae* (MPN444) but no studies to our knowledge have addressed antigenicity or a role in inflammation. Similar to the intact MG organism, significant activation of NF-κB was observed via TLR2/6 heterodimers but not TLR2 alone (Fig. 23). A recent report showing that MG149 activates NF-κB via TLR1/2 [51] further supports the hypothesis that MG is capable of eliciting innate immune responses via cell surface TLR. However, TLR1 expression in vaginal and cervical EC is very low [42] so we did not evaluate whether these membrane proteins were capable of activating TLR1/2 heterodimers. Importantly, MG307, MG309 and MG338 are likely among several MG proteins capable of inducing pro-inflammatory cytokines following MG exposure. In fact, other highly expressed antigenic lipoproteins including MgPa are putative agonists for cellular TLR and currently are under investigation for their role in immune stimulation and antigenic variation.

We further investigated the ability of MG307, MG309 and MG338 to elicit pro-inflammatory innate responses from vaginal and cervical ECs. Immortalized human vaginal, ectocervical and endocervical ECs responded to rMG307, rMG309 and rMG338 with low, but significant elaboration of pro-inflammatory cytokines (rMG309 presented in Table 5). The pattern of acute-phase cytokines induced by rMG309 in these cells was largely similar to that induced by FSL-1, a *M. salivarium*-derived lipopeptide known to specifically ligate TLR2/6 [42]. Importantly, the pattern was consistent with immune cell recruitment [110, 111] and was very similar to EC responses to the intact MG organism (C.L. McGowin, V.L. Popov, and R.B. Pyles, submitted for publication; See Chapter 3).

The robust secretion of IL-8, IL-6, G-CSF and GM-CSF, most notably from endocervical ECs (Table 5), strongly suggested that monocytes and macrophages might be recruited to infected reproductive tract tissues. These findings are important for predicting the capacity of MG to elicit inflammation in upper reproductive tract tissues and warrant continued investigation at these sites. Collectively, these findings suggest that TLR2/6 ligation by membrane proteins likely is an important mechanism for activation of the innate response to MG in the reproductive tract.

The focus of this work was to elucidate a putative mechanism for the observed inflammatory responses using the intact MG organism and 3 representative antigenic proteins. More focus was given to the protein encoded by MG309 because there is preliminary evidence to suggest this gene is under immune-mediated pressure [95]. The full-length MG309 gene encodes a putative lipoprotein with a predicted N-terminal transmembrane domain. An extensive analysis of the lipoylation state of the recombinant MG309 used in our studies has not been completed. However, the native N-terminus of MG309 was not included in the regions we investigated and thus it was speculated that the TLR2/6 activating capacity was independent of lipoylation and likely amino acid based. The conclusion that rMG309 activated TLR2/6 independent of N-terminal lipoylation was supported strongly by the ablation of intact MG and rMG309's TLR2/6-binding capacity following proteinase-K digestion and heat denaturation (Fig. 23B). In contrast, the macrophage stimulating capacity of enriched lipoproteins from cultured *M. fermentans* [112], *M. hyorhinis* [113], *M. salivarium* [114] and *M. arthritidis* [115] was not significantly affected by proteinase-K digestion. However, enriched detergent phase

proteins from *M. hominis* [116] and *U. urealyticum* [117] were sensitive to proteinase-K digestion suggesting that some genital mycoplasmas may encode proteins capable of activating inflammatory cytokine elaboration independent of lipidation.

It has been demonstrated previously that human TLR2/6 specificity of mycoplasmal lipoproteins is not solely dependent upon N-terminal lipoylation but also the amino acid composition of the C- and N-termini and 3-dimensional protein folding [118, 119]. To determine which region was activating TLR2/6, we cloned and purified 3 rMG309c sub-fragments to test the TLR2/6 stimulating capacity of each fragment independently. It was discovered that the 91 amino acids composing the N-terminal region of rMG309c were solely responsible for the TLR2/6 activating capacity (Fig. 24). Furthermore, only the N-terminal sub-fragment was capable of eliciting pro-inflammatory cytokine elaboration from endocervical ECs with levels and patterns equivalent to the full-length rMG309. It is likely that the specific region responsible for TLR ligation is a smaller polypeptide sequence but these studies are not yet complete. It is important to note that, although these data are quite convincing, we analyzed roughly half of the full-length MG309-encoded protein and therefore some inhibitory or additional stimulatory activity may be associated with the region we did not investigate. Therefore, although the TLR2/6 mechanism for immune stimulation is supported both by rMG309 and the viable organism, the magnitude of the observed responses may not accurately represent the inflammatory capacity of the native protein.

Collectively, our results indicate that MG is capable of eliciting early innate immune responses including NF-κB activation via TLR2/6. Similarly, the antigenic

proteins encoded by MG307, MG309 and MG338 were shown to activate TLR2/6 ultimately resulting in immune activation and pro-inflammatory cytokine elaboration from relevant EC types of the vagina and cervix. Considering the high expression levels of TLR2 and 6 in EC of the reproductive tract, and because MG is capable of attachment to and invasion of genital ECs used in our studies (Refer to Chapter 3), it seems possible that acute and persistent colonization of genital tissues by MG may indeed result in prolonged inflammation of infected tissues. Therapeutically, we have also investigated the use of purified recombinant MG proteins as topical immunomodulating microbicides (Refer to Chapter 8). Continued investigation into the specific mechanisms of TLR-mediated activation of the innate immune response is ongoing and will be important to better understand the capacity of MG to cause urogenital disease and to establish targeted antimicrobial therapies.

CHAPTER 5: SUSCEPTIBILITY OF *M. GENITALIUM* TO MACROPHAGE KILLING AND EVASION OF PHAGOCYTOSIS BY INTRACELLULAR EPITHELIAL CELL SURVIVAL

OVERVIEW

Earlier studies in our laboratory and others have firmly shown the capacity of MG to invade, replicate and survive within reproductive tract ECs. As a microbe also capable of eliciting potent TLR-mediated inflammatory responses from these cells, it is implied that intracellular localization must afford MG some evolutionary advantage for survival. Other intracellular bacterial pathogens, including the Chlamydiae, are hypothesized to prefer intracellular localization to parasitize necessary metabolic requirements and to evade host immune responses. We hypothesized that intracellular localization of MG could provide protection from cellular immune responses thereby providing a survival niche for persistent infection of reproductive tract tissues. We found that, when exposed directly, MG was highly sensitive to human and murine macrophage phagocytosis coincident with a rapid loss of viability and a potent cytokine response by exposed macrophages. The macrophage response included significant secretion of several inflammatory cytokines known to enhance HIV-1 replication. However, intracellular MG infection of vaginal ECs provided significant protection from macrophage phagocytosis thereby highlighting a putative mechanism for evasion of the host immune response. Importantly, these results also provide evidence that long-term intracellular survival within reproductive tract ECs could lead to persistent infection and inflammation of the genital mucosa.

INTRODUCTION

MG has been associated with long-term clinical infection of both the male and female reproductive tract (Reviewed in [14]). The mechanisms for evasion of the host immune responses have yet to be elucidated but, considering clinical infections are associated with inflammatory sequelae, it is evident that MG employs effective means for persistence despite a robust immune response. We have shown that MG can rapidly attach to and invade cultured human vaginal and cervical ECs resulting in pro-inflammatory cytokine secretion (Refer to Chapter 3). In addition, vaginal swabs obtained from MG-positive patients contained ECs colonized heavily by intracellular MG [76] collectively suggesting that intracellular survival could be an efficient means for evading the host immune response.

The female reproductive tract is a complex multicellular and polymicrobial environment that is capable of both sustaining normal endogenous flora and responding to pathogenic organisms. Physiologically, the vaginal and cervical mucosa are highly susceptible to pathogen exposure and capable of generating robust inflammatory responses (Reviewed in [38]). Macrophages are versatile cells of the innate immune system and play a central role in mucosal immune responses through recognition of a pathogen, phagocytic killing of the organism and potent secretion of pro-inflammatory cytokines throughout the infected tissue [120]. Although macrophages are not always resident in the vaginal lumen, they are distributed throughout the epithelial and sub-epithelial mucosa of the vagina and cervix and make up a significant proportion of the total immune cell population of the reproductive tract [121]. Importantly, studies were

needed to address directly the interaction of MG with human macrophages and whether infection of reproductive tract ECs elicits chemokine secretion for recruitment of phagocytic cells infected tissues.

The role of macrophages during MG infection of female reproductive tract tissues may be of particular interest considering the severity of cervical MG infection is strongly associated with HIV-1 shedding [92]. Previous studies from our lab have shown that the pattern of cytokine secretion by human vaginal and cervical ECs is most consistent with recruitment and stimulation of monocytes and macrophages (Refer to Chapter 3) suggesting they likely have a role in MG infection. These cytokines included IL-6, IL-8, GM-CSF and MCP-1. IL-6 secretion may be of particular importance considering that IL-6 from vaginal secretions is positively correlated with HIV-1 burden [122] and known to up-regulate HIV-1 replication [123]. Furthermore, macrophages are one of two major cellular reservoirs for latent HIV-1 infection and contribute to early-stage virus transmission and dissemination throughout the host (Reviewed in [124]). Together, these findings provided important rationale for investigating the putative role of macrophages for the mucosal response to MG infection of the female reproductive tract.

Considering the potential role of macrophages for recognition of MG in infected genital tissues, we next investigated the capacity of MG to elicit pro-inflammatory cytokine secretion from macrophages. Lipoprotein-enriched detergent phase preparations from MG strain G37 have been reported to activate inflammatory cytokine secretion from a transformed monocytic cell line [48, 50] but these fractions, nor the viable organism, have yet to be tested using primary human macrophages. Published results presented in

Chapter 4 showed that viable MG activates TLR2/6 resulting in inflammatory cytokine secretion [125] suggesting that human macrophages would also be highly responsive to MG exposure. In our studies, we found that human monocyte-derived macrophages (MDM) rapidly phagocytosed and killed MG resulting in a robust secretion of pro-inflammatory cytokines. These data provide the first characterization of the human innate immune response to viable MG from relevant cell types of the female reproductive tract and provide insight into the dynamic interaction of MG with the reproductive mucosa.

MATERIALS AND METHODS

Isolation of human monocyte-derived macrophages (MDM)

Human macrophages were generated as described previously [126] from peripheral blood mononuclear cells (PBMCs) collected from healthy volunteers with UTMB IRB approval. Briefly, PBMCs were isolated using Hypaque-Ficoll (Amersham Biosciences, Piscataway, NJ) density-gradient separation. Selection was performed using the magnetic column separation system (StemCell Technologies, Vancouver, Canada). Purified monocytes were differentiated into macrophages by culturing in RPMI 1640 medium supplemented with 10% FBS, L-glutamine, HEPES, sodium pyruvate and GM-CSF (100ng/mL). Following 7d of differentiation, MDM were removed from the culture plastic using a non-enzymatic cell dissociation solution (Sigma-Aldrich) and then resuspended in fresh RPMI 1640 medium. Macrophage differentiation was verified by

flow cytometric confirmation of CD11b, CD80 and CD86 expression showing typical purities of >95% (data not shown). Macrophages were differentiated from PBMCs collected from 3 different blood donors and used in 3 independent experiments.

Propagation of *M. genitalium* strains G37 and M2300

M. genitalium type strain G37 (MG G37; ATCC 33530) and M2300 were propagated and quantified in Friis FB medium [13] as described in earlier chapters. MG was harvested from culture flasks by pouring off the spent medium, extensively washing adherent mycoplasmas with sterile PBS and then scraping adherent microcolonies into fresh PBS. Loss of MG viability following macrophage exposure was verified by an absence of growth in Friis FB medium after 14d incubation at 37°C.

Transmission electron microscopy (TEM)

Adherent monolayers of MG-inoculated (G37 or M2300; MOI 100) or non-inoculated human MDM (MOI 100) were fixed at indicated times in a mixture of 2.5% formaldehyde and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) containing 0.03% trinitrophenol and 0.03% $CaCl_2$. Following fixation, cells were scraped, centrifuged briefly at 1,000 x g, washed in 0.1 M cacodylate buffer (pH 7.2) and then postfixed in 1% OsO_4 in the same buffer. Each sample was stained *en bloc* with 1% uranyl acetate in 0.1M maleate buffer, dehydrated in ethanol and embedded in Poly/Bed 812 epoxy resin (Polysciences, Warrington, PA). Ultrathin sections were cut using the Ultracut S ultramicrotome (Reichert-Leica, Depey, NY). Sections were stained

sequentially in 2% aqueous uranyl acetate and lead citrate and then examined in a Philips 201 or CM 100 electron microscope at 60 kV.

Stimulation of human MDM

Primary human monocytes were isolated and differentiated into macrophages as described above and then seeded into 96-well plates ($5x10^4$/well). Following overnight incubation at 37°C, culture supernatants were removed and replaced with fresh medium to remove any constitutively secreted cytokines. Log-phase MG G37 or M2300 was harvested as described above and then inoculated onto each cell type (MOI of 10). Controls for innate immune stimulation included the *M. salivarium*-derived TLR2/6 agonist, FSL-1 (0.1ug/well) or an equal volume of the PBS vehicle added to triplicate wells and processed in parallel. Secreted cytokines were quantified from culture supernatants 6h PI via a cytometric bead array (CBA) assay using the human 27-Plex panel of cytokine targets (BioRad). For testing of MG viability following macrophage exposure, infected macrophages were inoculated into Friis FB medium 30min, 2, 6 and 12h PI and observed for MG outgrowth indicated by a pH-mediated color change and adherent microcolony formation.

Protection of *M. genitalium* from macrophage phagocytosis

Considering the observed susceptibility of MG to macrophage phagocytosis, we next tested whether intracellular EC infection offered MG protection from macrophage exposure. Polarized human vaginal ECs (V19I, V12I and V11I) were cultured in

transwell inserts in the 'dry-interface' format as described in Chapter 3. Following 9d culture, ECs were inoculated with MG strain M2300 (approximately $5x10^8$ CCU/well) and incubated for 6h to allow MG attachment and invasion of the ECs (See electron micrographs in Chapter 3). Primary human MDM were then added to the apical surface and co-cultured with infected ECs for 24h prior to harvest of the EC multilayer for MG titration. MG viability was quantified from infected EC using a color changing unit (CCU) assay as described in earlier chapters. As a comparator for EC protection, MG was exposed directly to human MDM and then titrated in parallel.

Statistical Analyses

The Student's t test was used to calculate significant differences in cytokine secretion levels following MG exposure compared to basal (PBS-treated) levels. The one-way ANOVA followed by Dunnett's post-test (Prism v. 4.0, GraphPad, San Diego, CA) was used to calculate significant differences in cytokine secretion levels when more than 2 conditions were compared. Significance was indicated when $p<0.05$.

RESULTS

Phagocytosis of *M. genitalium* by human MDM

To determine the susceptibility of MG to macrophage phagocytosis, human MDM were exposed to log-phase MG strains G37 or M2300 (MOI 100) and processed at selected time points for transmission electron microscopy (TEM). Within 5min of

inoculation, MG appeared dark staining with a dense content of ribosomes and no signs of membrane degeneration (Fig. 26A). As early as 30min PI, MG uptake by MDM was observed that was associated with morphologic changes of the bacterium including a loss of ribosome density and a hollow appearance (Fig. 26B) consistent with a loss of viability. Internalized mycoplasmas were prevalent and localized within membrane-bound phagolysosomes. Similar morphological changes were observed 2h PI (data not shown). By 6h PI, the macrophages appeared to have many phagocytic vesicles but no intracellular MG could be located (Fig. 26C). MG viability following macrophage exposure was evaluated by seeding infected MDM (6h PI) into Friis FB medium at 37°C. These cultures were observed for MG outgrowth by a pH-mediated color change and microcolony formation. No MG growth was detected over a 14d period from any of these cultures collectively indicating that MG was susceptible to rapid phagocytosis and killing.

M. genitalium elicited pro-inflammatory cytokines from human MDM

Because MG was phagocytosed rapidly by human MDM with no evidence of bacterial viability by 6h PI, we sought to determine whether MG exposure to human MDM would elicit acute-phase cytokine responses. Viable MG G37 and M2300 inoculated at MOI 10 or MOI 1 elicited significant cytokine elaboration from macrophage cultures measured from supernatants collected 6h PI (G37 [MOI 10] results presented in Table 6). No significant differences were observed between G37 and M2300 (data not shown).

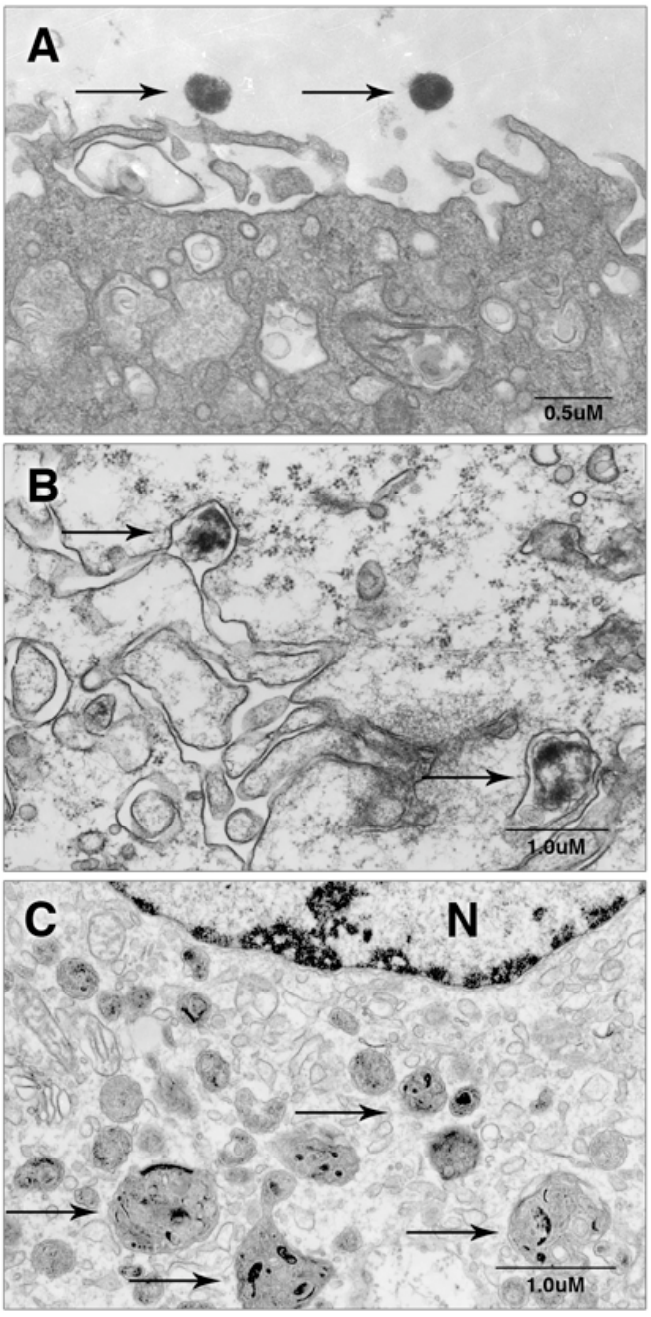


Figure 26. MG G37 was phagocytosed rapidly by human MDM resulting in a loss of bacterial viability. Primary human MDM were inoculated with log-phase MG G37 or M2300 (MOI 100) and collected at 30min, 2h and 6h PI for TEM. Viable extracellular MG with dense intracellular ribosomes and an intact outer membrane were observed at the time of inoculation (A). Thirty minutes later, phagocytosis of MG was observed with localization within phagolysosomes (arrow) and morphological changes of the bacterium (B). By 6h PI, macrophages contained many phagocytic vacuoles (arrows) and no identifiable intracellular mycoplasmas could be located (C). Similar findings were observed for strain M2300. N denotes nucleus.

The profile of induced cytokine responses from human macrophages was composed predominately of early pro-inflammatory markers including significant secretion of IL-1β, TNF-α, IL-6, IL-8, G-CSF, IFN-γ, MCP-1, MIP-1β, MIP-1α and RANTES ($p<0.05$; Table 6). These findings were consistent with results from 2 additional blood donors (data not shown). Following UV inactivation, MG elicited a similar profile and magnitude of cytokine secretion (Table 6) indicating that the immunostimulatory capacity of MG was not dependent upon bacterial viability. Immune markers that were not induced by MG in human MDM included IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, IP-10, PDGF-BB and VEG-F. Considering IL-6, a representative acute-phase cytokine with a central role in innate responses to bacterial pathogens [127], heat denaturation or proteinase-K digestion of MG significantly reduced the inflammatory capacity from human macrophages (Fig. 27) suggesting that a significant proportion of the inflammatory capacity was mediated by MG-derived protein components.

Protection from macrophage phagocytosis via intracellular EC survival

Studies presented in Chapter 4 showed that MG G37 and M2300 rapidly attach to and invade vaginal and cervical EC ultimately resulting in intracellular localization and long-term infection. Clinical MG cases in both men and women are known to be persistent (reviewed in [14]) indicating that MG must employ some strategy to evade the host immune response.

Table 6. Cytokine elaboration from human MDM following exposure to MG G37[a].

	Human MDM		
	Viable	**UV-Inactivated**	**PBS**
IL-1β	31±6.1*	33±1.4*	0.7±0.04
IL-6	385±13.8*	439±4.0*	3.2±0.1
IL-8	5784±149*	5368±564*	116±7.8
G-CSF	63.1±5.5*	72±2.4*	6.2±0.1
IFN-γ	270±24*	339±3.9*	9±3.6
MCP-1	298±9.3*	318±8.3*	36±3.9
MIP-1α	1056±16*	1068±4.0*	176±10.9
MIP-1β	2514±57*	2403±19*	810±47
RANTES	66±1.5*	74±9.9*	11.4±0.4
TNF-α	7456±334*	8616±697*	20±2.0

[a] Human MDM were inoculated with MG G37 (MOI 10) or an equal volume of the PBS vehicle as a control into triplicate wells. Culture supernatants were collected 6h PI to quantify secreted cytokines as described in *Materials and methods*. Values are expressed as the mean ± SEM pg/mL supernatant. PBS values are presented to indicate basal cytokine elaboration. Data collected following exposure to MG strain M2300 were similar in pattern and magnitude. ND, not detected. *, $p<0.01$ using ANOVA.

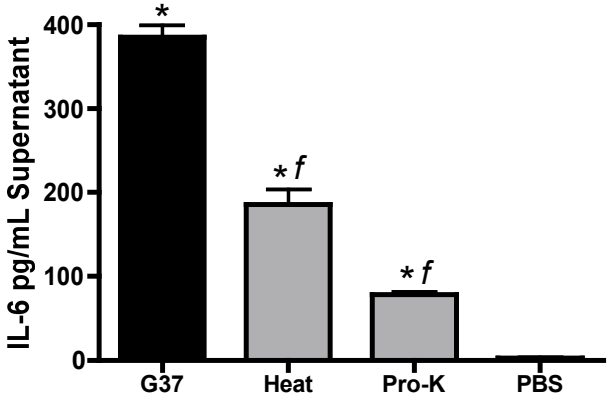


Figure 27. The MG-induced inflammatory cytokine secretion from human MDM was mediated predominately by proteins. Human MDM were exposed to viable MG G37 (MOI 10) or MG that had been denatured by heat or digested with proteinase-K (MOI 10). Cytokine secretion was quantified from culture supernatants collected 6h PI. Data shown are the mean ± SEM of IL-6 induction from a representative experiment using MG G37 performed in triplicate wells. Data collected for each experiment using strain G37 or M2300 were similar in pattern and magnitude between 2 additional blood donors. *, $p<0.01$ vs. PBS control using ANOVA. *f*, $p<0.01$ vs. viable MG G37.

Intracellular localization in host cells would be an efficient means to circumvent phagocytic cellular immune responses, such as those by macrophages, simply through physical protection. In Chapter 3, we showed that acute MG infection of reproductive tract ECs results in secretion of pro-inflammatory cytokines in a pattern most consistent with recruitment of monocytes and macrophages to the infected epithelia. Earlier in this chapter, we demonstrated the susceptibility of MG to human macrophage phagocytosis suggesting evasion of these cells would be necessary for survival. To address directly

whether intracellular localization within reproductive tract ECs afforded MG protection from macrophage phagocytosis, we employed the polarized, 3-dimensional transwell culture model of human vaginal ECs. Following inoculation, MG M2300 established a predominately intracellular infection (Refer to Chapter 3) throughout the EC multilayer. Six hours after infection, primary human MDM were added to the apical surface to evaluate whether co-culture of macrophages with MG-infected vaginal ECs resulted in MG killing. Titration of viable MG was done 24h after addition of macrophages to allow sufficient time for co-culture. MG viability was observed in polarized vaginal ECs from each donor (V19I, V12I and V11I) at a significantly higher titer than when macrophages were mixed with MG directly (Fig. 28). Importantly, among three independent experiments macrophages were observed to transmigrate the EC multilayer (mean= $8x10^3$ migrated cells; data not shown) indicating that intracellular localization was providing protection from macrophage exposure.

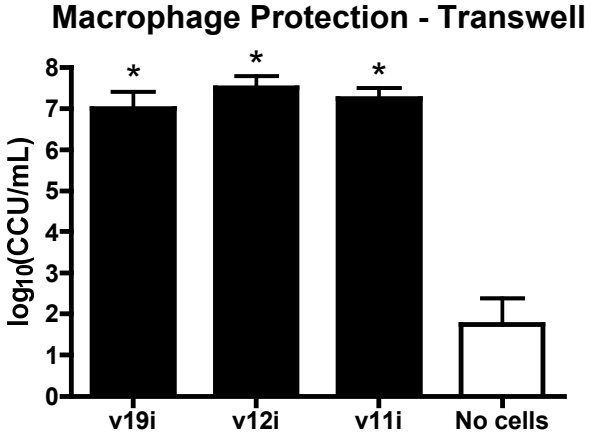


Figure 28. Protection of MG from macrophage phagocytosis. Polarized human vaginal EC from 3 donors (V19I, V12I and V11I) were inoculated with MG M2300 and incubated for 6h to allow attachment and cellular invasion. Following addition of primary human MDM and co-culture for 24h, significant MG viability was observed compared to MG exposed directly to MDM. *, $p<0.05$ vs. no cell control.

DISCUSSION

Considering that MG can establish intracellular infection of vaginal and cervical ECs resulting in inflammatory cytokine secretion consistent with recruitment of monocytes and macrophages, we investigated the putative role of macrophages and their cytokine response to MG infection. Following exposure to human MDM, phagocytosis of MG occurred rapidly (Fig. 25) resulting in complete ablation of MG viability by 6h PI. Because of the morphological changes of MG following macrophage exposure, it seems likely that MG death was due to an active macrophage killing mechanisms. Susceptibility to macrophage phagocytosis is a novel finding and indicates the importance for evading exposure to phagocytic immune cells for long-term survival in reproductive tract tissues.

Importantly, several key pro-inflammatory cytokines were secreted by human macrophages in response to MG exposure (See Table 6). Among these included IL-6 secretion that may be of particular importance because vaginal IL-6 has been shown to be positively correlated with HIV-1 burden [122]. Importantly, IL-6 also has been shown to up-regulate HIV-1 replication [123] suggesting the inflammatory response to MG from macrophages could lead to enhanced HIV-1 susceptibility or disease severity. Indeed, the microbial burden of MG in clinically-obtained cervical specimens also is strongly associated with HIV-1 shedding [92]. Furthermore, macrophages are one of two major cellular reservoirs for latent HIV-1 infection (Reviewed in [124]). To this end, we observed significant secretion of 4 potent chemokines responsible for granulocyte recruitment, MIP1-a, MIP1-b [128], MCP-1 and RANTES [129] (Table 6) indicating that

macrophage exposure to MG in reproductive tissues likely would result in significant inflammation consistent with enhanced HIV-1 replication. Considering these results, and the findings in Chapter 3, the data suggest that both infected genital ECs and recruited immune cells are responsible for secretion of IL-6 and other cytokines that may contribute to HIV-1 pathogenesis. Although these putative mechanisms are intriguing, continued research is necessary to formally dissect the cellular dynamics of HIV-1 and MG co-infections in reproductive tissues.

Only suggestive evidence exists for the mechanisms of MG-induced activation of pro-inflammatory responses. All Mycoplasmas lack a cell wall but contain a single plasma membrane system [106, 107] containing membrane-associated proteins [5, 49] of which some have been associated with the ability to adhere to the epithelium [108, 109]. In our studies, the macrophage-stimulatory capacity of MG was not dependent upon bacterial viability. This outcome likely is due to the highly sensitive nature of macrophages. However, both heat denaturation and proteinase-K digestion significantly reduced the cytokine response (Fig. 27) suggesting that a large proportion of MG's inflammatory capacity was indeed mediated by protein components. This hypothesis is supported by published results presented in Chapter 3 whereby viable MG and 3 immunogenic membrane proteins were shown to induce TLR2/6-dependent NF-□B activation and inflammatory cytokine secretion [125]. Collectively, these findings indicate that macrophages are highly sensitive to MG exposure and highlight the putative pressure on MG to evade cellular immune responses.

Despite MG's ability to activate TLR and elicit pro-inflammatory cytokine secretion from reproductive tract ECs and macrophages, persistent clinical infections are well documented and indicate that immune evasion is occurring. Our studies showed that, following vaginal EC infection, intracellular localization of MG provided significant protection against macrophage phagocytosis and killing (Fig. 28). These results suggest that a significant advantage is created through intracellular EC localization and provides a mechanism for long-term survival in reproductive tract tissues. Most importantly, intracellular MG localization in genital EC resulted in macrophage-stimulatory cytokine secretion (Refer to Chapter 3) but protected from recruited macrophages. However, because MG exploits an intracellular survival niche it is hypothesized that long-term inflammation is possible through inflammatory cytokine secretion both from infected ECs and recruited immune cells.

CHAPTER 6: A NOVEL MURINE MODEL FOR *M. GENITALIUM* FEMALE REPRODUCTIVE TRACT DISEASE

OVERVIEW

MG has been intensively investigated as an independent cause of inflammatory reproductive tract syndromes in both men and women. However, despite significant clinical associations of MG with urethritis, PID, cervicitis and endometritis in women, no experimental evidence existed previously for the ability of MG to establish reproductive tract infection. These impediments were due largely to the lack of a reproducible small animal model for investigation of MG infection and pathogenesis. We set out to develop a murine model of female reproductive tract infection to better understand how MG establishes the primary infection and to investigate the host immune response. Using several strains of both outbred and inbred mice, we established that inoculation of MG strain G37 results in long-term infection of the lower genital tract. Because hormones are known to greatly impact the susceptibility and pathogenesis of other STI, we tested and determined that conditioning with either progesterone or estradiol facilitated MG colonization. Outlining the necessary parameters for successful establishment of MG infection in mice was essential for investigation of disease pathogenesis and the potential of MG to disseminate to the upper genital tract and peripheral tissues (See Chapter 7). Findings presented in this chapter, combined with a portion of Chapter 7, have been submitted for publication by McGowin, et al, *PLoS Pathogens*, March 2009.

INTRODUCTION

MG has been established as a sexually transmitted pathogen [4, 90] associated with several inflammatory syndromes in women including cervicitis [21, 23, 72-74], PID [71] and tubal-factor infertility [28, 29]. In studies outlined in the previous chapters of this dissertation, we have established that MG is capable of long-term intracellular survival within reproductive tract EC resulting in TLR-mediated inflammation. It is important to note that the female reproductive tract mucosa are not simply comprised of ECs but are multicellular, polymicrobial tissues. Further investigation into the dynamic characteristics of the genital tract and whether or not MG can cause disease have been largely impeded by the lack of a cost-effective animal model of reproductive tract disease. We first sought to develop a mouse model of MG reproductive tract infection for investigation of the early events following sexual transmission and how MG.

Five years after the initial isolation of MG from men with urethritis [8], several large animal species were found susceptible to experimental urogenital MG infection including male cynomolgus monkeys (*Macaca fascicularis*), male chimpanzees (*Pan troglodytes*), female squirrel monkeys (*Saimiri sciureus*), female tamarins (*Saguinus mystar*) and female marmosets (*Callithrix jacchus*; [60]). These studies established only preliminary evidence that MG was pathogen of the human genital tract. With regard to the capacity to cause disease, female marmosets and grivet monkeys developed salpingitis only after direct inoculation of MG to the oviduct [60]. Additional studies by David Taylor-Robinson and colleagues provided preliminary evidence that the type strain of MG (G37) could establish lower reproductive tract infection in female balb/c mice

[61]. Importantly, these studies provided essential evidence that MG could colonize the reproductive tract of female mice but no evidence as to whether or not MG could ascend to the upper genital tract.

All of the currently available STI murine models employ systemic pre-conditioning with either progesterone or estrogen prior to intravaginal pathogen inoculation. Regulation of sex hormones affords a number of important advantages for experimental manipulation including rendering otherwise resistant animals susceptible to infection and synchronizing the estrus cycle of animals within a study. With regard to mycoplasmas, it is unclear why some species, such as *M. pneumoniae* and *M. pulmonis*, require progesterone treatment while others including the genital pathogen, *M. fermentans*, only colonize the genital tract following estrogen treatment [61]. The balb/c model proposed by Taylor-Robinson's group showed that balb/c mice were susceptible to vaginal MG infection only following progesterone but not after estradiol benzoate treatment [130]. In the course of our studies, we addressed the effects of progesterone and estrogen for establishment of vaginal MG infection in order to design a reproducible platform for investigation of upper genital tract infection and other aspects of pathogenesis (Refer to Chapter 7).

The model developed in our laboratory allowed for reproductive tract colonization of outbred Swiss Webster mice following either estradiol or progesterone treatment. Following hormone treatment 7 and 1d prior to inoculation, mice were inoculated intravaginally with approximately $1x10^8$ viable MG organisms. Animals were then assessed daily for hair loss, erythema, genital ulceration and survival for up to 8 weeks

following inoculation. Vaginal swabs were collected at selected times following inoculation for real-time PCR quantification of vaginal MG titers as presented in Chapter 2. The parameters outlined for establishment of reproductive tract infection presented in this chapter provided valuable insight into how MG establishes primary infection and elucidated several immunodominant proteins important in the mouse immune response.

MATERIALS AND METHODS

Propagation of *M. genitalium*

M. genitalium type strain G37 (MG G37; ATCC 33530) was propagated in modified Friis FB medium as described previously [13]. Immediately following a pH-mediated color change of the culture medium, adherent microcolonies were washed extensively with sterile PBS before being scraped into fresh PBS for immediate inoculation. MG was cultured minimally prior to inoculation of animals to reduce the probability of culture adaptation. Following receipt from the ATCC, MG G37 was passaged once to establish frozen stocks and then a second time to generate the log-phase MG mouse inocula. For studies addressing active vaginal colonization, MG was inactivated by UV irradiation (254nm) using a Stratalinker 2400 (Stratagene, La Jolla, CA) to a total energy of 720,000 microjoules/cm^2. Loss of MG viability was verified by an absence of growth in Friis FB medium after 14d incubation at 37°C.

Hormone treatment and vaginal inoculation paradigm

Five-week-old female Swiss Webster, balb/c or ICR mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved quarters and provided with unlimited access to food and water. All procedures were performed humanely in concordance with a UTMB Animal Care and Use Committee-approved protocol. A subcutaneous injection of either estradiol cypionate (0.25mg; Depo-estradiol, Pfizer, New York, NY) or medroxyprogesterone acetate (3mg; Sicor, Irvine, CA) was administered 7d and 1d prior to MG inoculation. Mice were swabbed once with a PBS-soaked calcium alginate swab and then once with a 'dry' swab to remove vaginal debris. Without anesthesia, animals were inoculated intravaginally with $2x10^7$ CCU/20uL MG G37 in sterile PBS. Included in each independent study, mock-infected mice were hormone treated but received only the PBS vehicle to control for non-specific PCR detection and false-positives in viability testing.

Establishment of vaginal infection and PCR quantification of *M. genitalium*

Vaginal swabs were collected at selected times after inoculation into 0.5mL sterile PBS for DNA purification. Swab specimens were processed using the DNeasy 96 or DNeasy Blood and Tissue Spin column kit (Qiagen) and eluted into a 0.1mL volume. To quantify the MG burden, real-time PCR targeting the single-copy MG309 gene was performed as outlined in Chapter 2. A standard curve of a pre-quantified MG309-containing plasmid was run in duplicate on each 96-well plate and used to extrapolate

MG DNA loads. Specimens from mock-inoculated animals and PCRs that lacked template served as controls for contamination and false-positive MG detection. Real time PCR assays were completed using the 7500 Real Time PCR System (Applied Biosystems.

Mouse cytokine and antibody response to *M. genitalium* G37

Because MG reproductive tract infection is associated with inflammatory sequelae, we investigated the vaginal cytokine response to MG G37 inoculation of outbred mice. Following progesterone treatment, Swiss Webster mice (n=5/group) were inoculated with MG G37 as described above. Control mice were delivered PBS only. Following 6, 12 and 24h infection, vaginal lavages were collected from separate groups of mice for quantification of cytokine secretion into the vaginal lumen. Cytokine quantification was accomplished via the BioPlex CBA assay using the Mouse 23-Plex panel of cytokine targets (BioRad). Cytokine secretion from MG-inoculated mice was compared to mice receiving PBS.

To investigate the host antibody response to MG infection, serum was collected from MG G37-infected or mock-infected Swiss Webster mice (77d PI) for immunoblot against MG G37 lysates using standard methods. Each of the immunogenic MG proteins that were recognized by infected mouse sera were identified by MALDI-TOF/TOF mass spectrometry using a 4700 Proteomics Analyzer (Applied Biosystems).

RESULTS

Long-term detection of *M. genitalium* in the vagina of estradiol- or progesterone-treated mice

Maintenance of vaginal colonization would be an important factor for MG transmission as well as the potential for ascending infection of the upper reproductive tract. We first evaluated the early dynamics of vaginal infection and long-term shedding. MG G37 was first delivered intravaginally to progesterone- or estrogen-treated mice (n=5/group). MG titers were detected in vaginal swabs from both estradiol- (Fig. 29A) and progesterone-treated (Fig. 29B) mice whereby the MG burden 1d PI was similar for each genotype of mouse. In Swiss Webster mice conditioned with estradiol (Fig. 29C) or progesterone (Fig. 29D), vaginal MG titers were highest on day 1 PI and then decreased on day 2 and 3 PI indicating most of the inoculum was cleared from the vaginal lumen

Next, in two independent studies using estradiol-conditioned animals, MG G37 was delivered intravaginally to Swiss Webster mice (n=5, experiment 1; n=6, experiment 2) to evaluate long-term shedding. A large proportion of MG was cleared from the vagina or moved to sites not adequately sampled by a vaginal swab from 1-3d PI in both experiment 1 and 2. These data confirmed the findings from the previous experiment (data not shown). Importantly, delivery of an equivalent titer of UV-killed MG G37 did not yield detectable MG 24h PI (data not shown). Similarly, instillation of an equivalent amount of purified MG DNA was not detectable 24h PI indicating that the observed PCR titers from inoculation of viable MG were due to active colonization of the vaginal tissues.

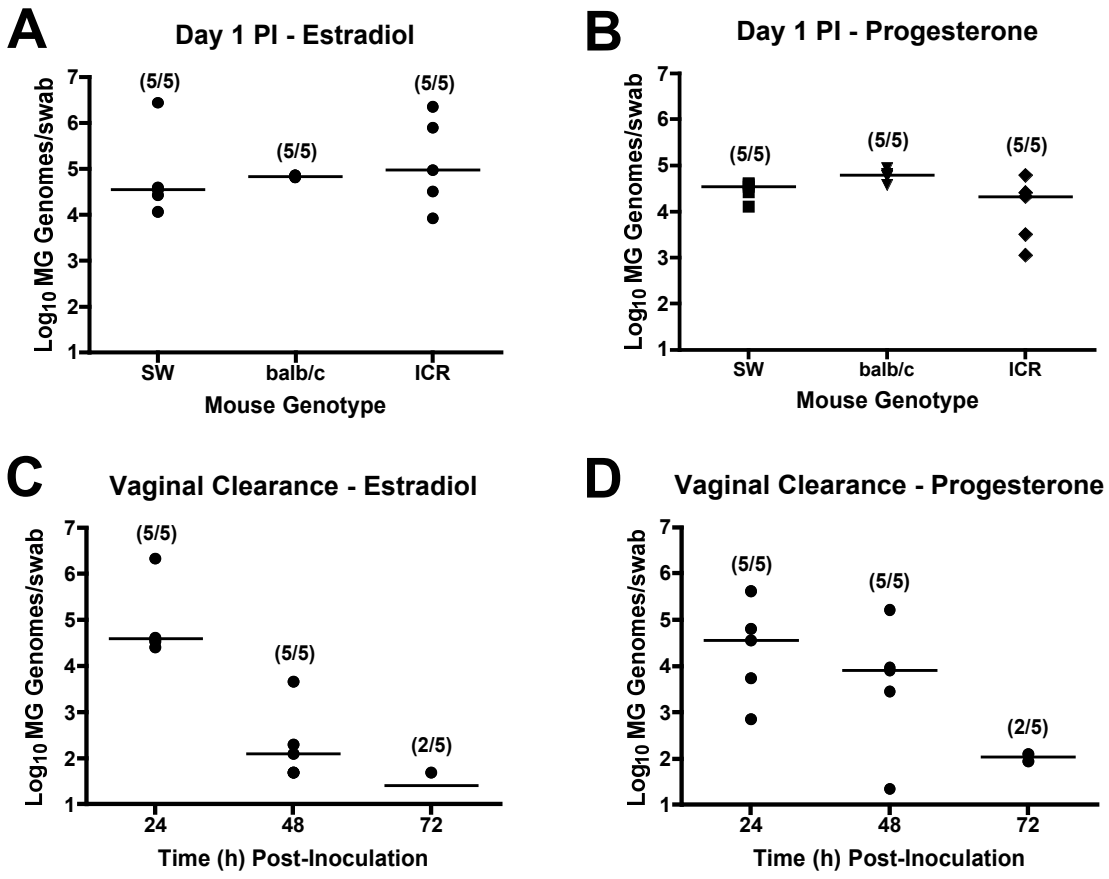


Figure 29. Colonization and clearance of MG from the murine vagina. Swiss Webster (SW), balb/c or ICR mice were treated with estradiol (A) or progesterone (B), inoculated intravaginally with MG G37 and then swabbed 1d PI. Clearance of MG from the vaginal lumen was evaluated using SW mice following estradiol (C) or progesterone (D) pre-treatment. Values on the Y-axis are expressed as genomes/swab (line indicates median value) and were obtained by PCR quantification of vaginal swabs collected at the indicated time points after inoculation. Values in parentheses indicate the proportion of mice PCR-positive at each time point.

Vaginal titers were maintained at very low levels (mean of positive samples = $5.0x10^1$ genomes/swab) for up to 5d PI in experiment 1 and up to 77d PI in experiment 2 (Table 7). Although every mouse from experiment 2 was PCR positive at least once from 12-77d PI, on average, approximately 40% of the collected swabs were PCR-positive at each sampling time. MG DNA was not detected in swabs from mock-infected mice (n=10). In a single experiment using progesterone-conditioned mice, vaginal swabs were collected on d7, d21 and d35 PI whereby a similar rate of PCR-positive samples and MG titers were observed (data not shown). Collectively, these data indicate that, despite the significant reduction of vaginal titers 1-3d PI, both estradiol- and progesterone-treated animals maintained detectable MG loads in the vaginal lumen for extended time periods.

Table 7. Long-term PCR detection of MG G37 in vaginal swabs of estradiol-treated mice.

Days PI	1	2	3	4	5	12	24	31	55	77
Exp #1	5/5	5/5	2/5	2/5	2/5	--	--	--	--	--
Exp #2	6/6	6/6	2/6	3/6	2/6	2/6	2/6	3/6	3/6	3/6

[a]Indicated proportions are # of PCR-positive mice/group at each sampling time.
--indicates that vaginal swabs were not measured at this time point in Experiment 1.

Vaginal cytokine response to *M. genitalium*

Following vaginal inoculation of MG G37 of Swiss Webster mice, vaginal lavages were collected 6, 12 and 24h PI for quantification of cytokine secretion. Vaginal secretion of several cytokines were significantly increased 24h PI compared to PBS-treated animals including IL-12(p40), KC (mouse IL-8), MCP-1, IFN-γ (Fig. 30), IL-12(p70), TNF-α and IL-10. For most cytokines, no significant increase was observed until 24h PI.

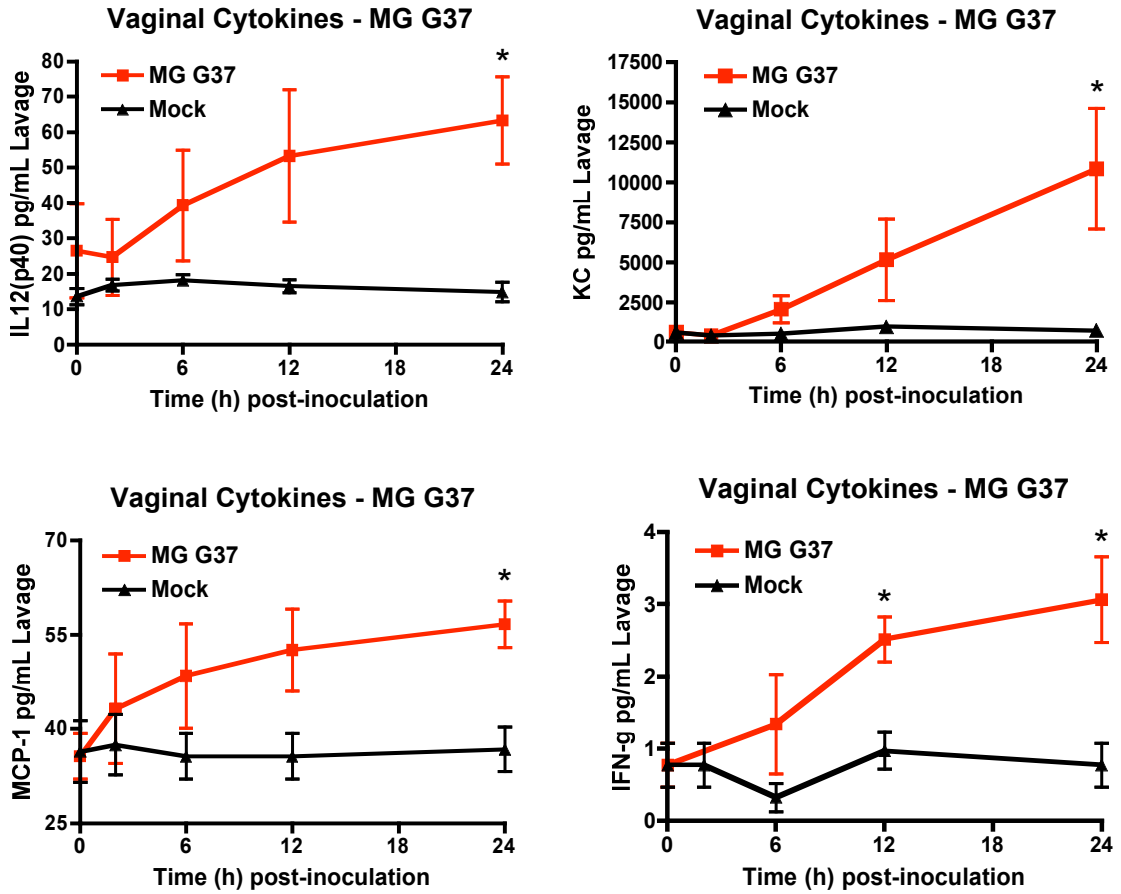


Figure 30. Vaginal cytokines in response to MG inoculation. Swiss Webster mice were inoculated intravaginally with MG G37 and then lavages were collected 6, 12 and 24h PI for quantification of secreted cytokines. * indicates a significant increase at the indicated time point relative to mock-inoculated mice that received only the PBS vehicle.

Murine antibody responses to *M. genitalium* infection

Urogenital MG infection of women[28] and men [47] is associated with the development of antibodies to the major outer membrane protein, MgPa. Estradiol-conditioned Swiss Webster mice (n=6/group) were inoculated intravaginally with MG G37 or PBS as a control. Pooled sera from MG-inoculated mice (77d PI) showed antibody responses to a number of MG proteins (Fig. 31, lane 3) at a 1:100 dilution but the strongest responses were observed to 3 proteins of approximately 40, 45 and 150kD that were recognized by high titer antibodies (dilutions of 1:1000; lane 4, and 1:5000; lane 5). Using MALDI-TOF/TOF mass spectrometry we identified these MG proteins as pyruvate dehydrogenase component E1α, elongation factor Tu, and MgPa, respectively (100% identity confidence for each protein). A weaker response to the highly expressed MG DnaK (Hsp70) protein also was observed. Mock-infected mice showed only a very weak antibody response to EF-Tu (Fig. 31, lane 6) but this response also was observed when *E. coli* lysates were probed (lane 7) indicating the response was not MG-specific.

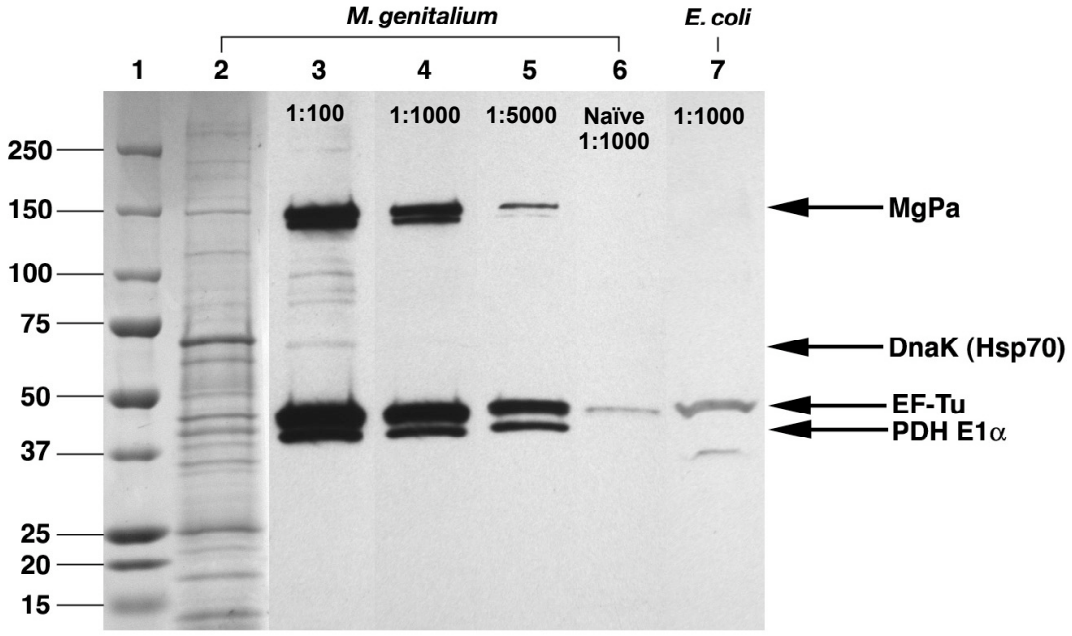


Figure 31. Host antibody response to MG G37 infection. Estradiol-conditioned Swiss Webster mice were inoculated with MG G37 or the PBS vehicle (n=6/group). Pooled sera from each group (77d PI) were used for immunoblot of purified MG lysates (coomassie, lane 2). Sera from MG-infected mice were used to probe MG lysates at indicated dilutions. Incubation of sera from mock-infected animals on MG G37 (lane 6) or E. coli (lane 7) lysates was performed in parallel to determine the specificity of the antibody response. The indicated immunogenic MG proteins were identified via MALDI-TOF/TOF mass spectrometry.

DISCUSSION

These studies served primarily to develop a reproducible animal model to better understand how MG establishes genital tract infection and to investigate reproductive tract disease. Following inoculation, Swiss Webster, balb/c and ICR mice had detectable MG in vaginal swabs but a decrease in titer was observed from 1-3d PI. This suggests either a large proportion of the inoculum was cleared by the mucosal immune system or MG disseminated to other tissue sites. The small variability among vaginal swab titer from 1-3d PI indicated that the inoculum was not simply lost in the cage bedding whereby significant variation among mice would be expected. Importantly, the observation of long-term shedding in the vaginal lumen indicated that MG was capable of establishing a persistent reproductive tract infection and that immune evasion was occurring.

The observation of persistence is in concordance with several reports of long-term clinical MG infections in women [55, 56, 89] and is similar to other pathogenic *Mycoplasma spp*. [5, 131, 132]. In our studies, persistent vaginal titers were maintained up to 77d PI and could be attributed to shedding from upper genital tract tissues into the vagina or active vaginal infection. Maintenance of vaginal MG titers would be an important factor for sexual transmission whereby we observed intermittent detection by PCR (approximately 40%). Although all animals had detectable vaginal titers throughout the study, the intermittent detection could be due to the sensitivity of the PCR assay or simply the variable presence of MG in the vaginal lumen. From our previous studies in cultured human vaginal EC, it is evident that intracellular infection is preferred and that

dissemination of MG through the mucosal matrix likely occurs. This could significantly impact the efficiency of swab collection for MG detection as luminal titers may not expected, especially if the vaginal tissues are not the primary reservoir. Nonetheless, the long-term presence of MG in the vagina is an important finding that provides rationale for evaluating the capacity of MG to establish upper genital tract infection (Refer to Chapter 7).

An important factor in development of an animal model is to accurately parallel most aspects of the human infection. Because the overarching theme of this project is to understand the host-pathogen relationship and relevant aspects of the host immune response, characterization of the vaginal cytokine response was important. We observed significant secretion of several pro-inflammatory cytokines that peaked 24h PI (Fig. 30). Included in the profile of secreted cytokines was a robust secretion of IL-8, a chemokine responsible for recruitment of immune cells. Importantly, acute-phase secretion of IL-8 and other cytokines is similar to the observed responses from MG-infected human vaginal and cervical ECs (Refer to Chapter 3). This finding supports the hypothesis that MG infection results in acute inflammation but studies evaluating the long-term cytokine responses are warranted.

We next evaluated the host antibody response following MG infection to determine the immunodominant MG proteins that elicit antigenic responses. Long-term infection of mice in our studies ultimately resulted in the development of specific antibodies to several highly expressed MG proteins including the major adhesin MgPa (Fig. 31). Clinically, urogenital infection of both women and men by MG is associated

with the development of antibodies to MgPa [28, 47] indicating the mouse response is similar to the human. Additional immunogenic MG proteins discovered during our studies included elongation factor Tu, DnaK (Hsp70) and the E1 α subunit of pyruvate dehydrogenase. All of these proteins have been shown to be putative components of the cytoskeleton of *M. pneumoniae* [133] suggesting that these proteins could also be targets of the human host response to MG.

Together, these findings indicated that MG can establish a long-term infection of the mouse reproductive tract that results in relevant antibody responses similar to human patients. In addition, acute-phase inflammatory cytokine responses were observed in vaginal lavages indicating a robust innate response to MG exposure. The cytokine responses observed from vaginal lavages of MG-inoculated mice were similar to those observed from human vaginal and cervical ECs in vitro (See Chapter 3). Collectively, these results highlighted the relevance and utility of the mouse for modeling human infection dynamics. Continued investigation using this described model was and continues to be important for testing more contemporary MG isolates and for comparing viable infection to the PCR outcomes described in this chapter. An important next step in evaluating the capacity of MG to cause reproductive tract disease will be to determine whether vaginal inoculation results in dissemination to upper genital tract sites (Refer to Chapter 7).

CHAPTER 7: VAGINAL EXPOSURE TO *M. GENITALIUM* RESULTS IN RAPID DISSEMINATION TO UPPER REPRODUCTIVE TRACT AND JOINT TISSUES IN MICE

OVERVIEW

Since it was first discovered in 1980, MG has been intensively investigated as an independent cause of several reproductive tract syndromes in both men and women. Despite significant clinical associations of MG with pelvic inflammatory disease, cervicitis and endometritis, no direct experimental evidence existed previously for the ability of MG to disseminate to upper reproductive tract tissues. Following the observation that MG can establish long-term vaginal infection (Refer to Chapter 6), we next demonstrated in this chapter that MG can rapidly establish long-term *viable* infection of lower and upper reproductive tract tissues with dissemination to peripheral joints following vaginal exposure. These studies combine more than 3 years of experimentation and development of parameters for evaluation of MG infection dynamics in mice. Sex hormones such as estrogen and progesterone have profound affects on regulation of susceptibility and pathogenesis for other STI including HSV-2 and *Neisseria gonorrhoeae*. In turn, we found that both progesterone and estrogen pre-treatment induced susceptibility and allowed for a persistent infection at reproductive tract and synovial sites. These findings provide the first experimental evidence that MG can indeed establish long-term infection of the upper reproductive tract and joint tissues following vaginal exposure and should heighten awareness to this emerging sexually transmitted pathogen.

INTRODUCTION

MG is an emerging sexually transmitted pathogen identified as a cause of inflammatory urogenital disease in men (Reviewed in [70, 134]). Convincing associations with inflammatory syndromes in women including cervicitis [21, 23, 72-74] and PID [71] have led to an increased awareness of MG as a pathogen that can adversely affect reproductive health. It also may be of considerable importance that MG is positively associated with HIV shedding from cervical tissues [92] suggesting that reproductive tract infection may increase the likelihood of acquiring or transmitting other genital pathogens.

Although the preferred site of colonization seems to be the genital tract, MG also has been a suspected cause of reactive arthritis since DNA was detected in the knee joints of arthritic patients [135-137] and in synovial fluid from temporomandibular joints [138, 139]. Localization of sexually transmitted pathogens to synovial sites can result in an arthritic syndrome termed sexually acquired reactive arthritis (SARA). SARA patients typically present with joint pain in up to 6 joints simultaneously but most often in the lower extremities including the knees and ankles (Reviewed in [140]). The most probable pathogens of the genitourinary tract that have been implicated in SARA are *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma fermentans*, MG and *Ureaplasma ureulyticum* [140]. However, only indirect evidence exists for MG's role in any arthritic condition. Furthermore, no experimental evidence has been presented that indicate whether MG has the ability to disseminate from the vagina to colonize upper genital tract or peripheral joint tissues.

As epidemiological data continue to implicate MG as a cause of reproductive tract disease in women, relevant models to understand pathogenesis and evaluate therapeutic interventions are of utmost importance. A number of small and large animal species, including non-human primates, have been shown susceptible to MG infection. In female marmosets and grivet monkeys, MG elicited inflammation when delivered directly to the upper genital tract [60] suggesting that ascending infection could result in upper genital tract disease. In this chapter, we describe more than 3 years of experimentation for successful establishment of a murine model to investigate MG reproductive tract disease. Our findings provide strong evidence that, following vaginal exposure, the lower and upper reproductive tract tissues become persistently colonized resulting in long-term shedding from the vagina. Importantly, colonization of the knee joints also was observed providing evidence that MG can disseminate from the vagina to colonize synovial sites as well. A reproducible small animal model to study MG pathobiology will be indispensable for continued investigation into the mechanisms of disease, co-infection with other STI pathogens and to evaluate novel therapeutics, preventatives and vaccines.

MATERIALS AND METHODS

Vaginal inoculation paradigm

M. genitalium type strain G37 (MG G37; ATCC 33530) or the low passage Danish M2300 strain were propagated in modified Friis FB medium [13] and harvested as described in Chapter 6. A comparison of viable organisms to quantifiable MG G37

genomes was analyzed by employing the MG309 qPCR assay (Refer to Chapter 2) for *in vitro* growth curve quantification. For all animal experiments, five-week-old female Swiss Webster mice (Harlan Sprague Dawley) received a subcutaneous injection of either estradiol cypionate (0.25mg; Depo-estradiol, Pfizer) or medroxyprogesterone acetate (3mg; Sicor) 7d and 1d prior to MG inoculation as described in Chapter 6. Included in each independent study, mock-infected mice were hormone treated but received only the PBS vehicle.

Determining *M. genitalium* viability from swab and tissue specimens

In preliminary studies, we investigated whether MG was disseminating from the vagina to upper reproductive and peripheral joint tissues by attempting to culture MG from infected tissues. Following estradiol or progesterone treatment and MG inoculation, MG viability was determined from vaginal swabs (5d PI), reproductive tract tissues (3-4 wk PI) and knee tissues collected aseptically into sterile PBS. Viability was determined following extensive mincing of each tissue type and then placing the entire tissue into Friis FB for outgrowth at 37°C for 21d. The Friis FB growth medium contained penicillin and streptomycin to prevent the growth of commensal flora from vaginal swab and tissue samples or environmental contaminants. Any tested specimen showing a change in medium color, indicating microbial growth, was sampled for molecular identification using PCR and DNA sequencing as described below.

PCR quantification and molecular typing of *M. genitalium*

Reproductive tract and knee tissues were aseptically dissected from each mouse after soaking and rinsing the fur with 70% ethanol. Dissected tissues were thoroughly minced into 0.5mL DNA lysis buffer (Qiagen) and stored at -20°C until DNA extraction. DNA extraction and PCR quantification of MG from mouse tissues was accomplished as using the MG309 quantitative PCR assay described in Chapter 2. Careful measures, including thorough washing of dissection instruments after each tissue dissection, were taken to reduce the possibility of contamination among tissues or from cage bedding.

Accurate identification of MG from animal specimens was accomplished using multiple molecular techniques. First, the presence of MG DNA from mouse swabs, reproductive tract or knee tissues was determined using the MG309 qPCR assay described above. Next, 5 MG309 PCR-positive tissue samples were selected at random for verification of MG identity by PCR and DNA sequencing of the bacterial 16s rDNA using the 27F and 518R primers described previously [141]. As an additional confirmatory test to these samples, a nested PCR was completed that first targeted a ~600bp region of the MG191 gene [55] and then a 281bp hypervariable subfragment [77]. Mouse swab and tissues that showed a color change in the Friis medium (n=16) were also verified using this paradigm. Sequences of the resultant PCR products were analyzed using the basic local alignment search tool (BLAST) and compared to sequences generated from the inocula.

RESULTS

M. genitalium G37 established viable infection of the reproductive tract and knees

Detection of viable MG from mouse swabs and tissues was done only in preliminary studies because PCR was subsequently determined to be a more sensitive and informative detection method. To address the potential discrepancy between viable and PCR titers, we first determined the ratio of MG G37 genomes to viable organisms in order to validate the quantitative PCR outcomes as an accurate measure of MG burden. The ratio of genomes:viable MG organisms was, on average, approximately 1:1 (Fig. 32). The samples collected immediately after seeding of fresh Friis FB medium showed roughly a 10-fold difference between viable titers and genomes (Fig. 32) likely attributable to a subset of non-viable organisms from the frozen stock.

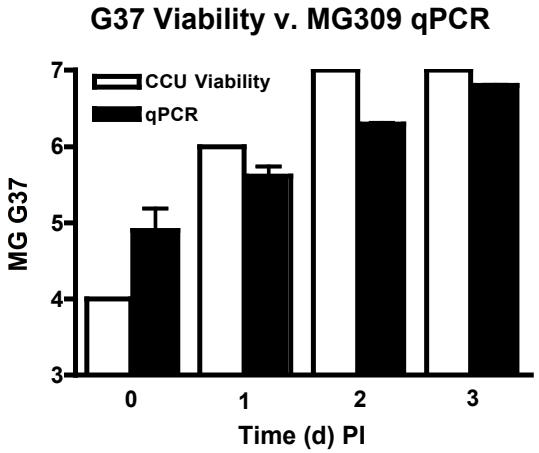


Figure 32. Quantitative comparison of viable MG titers to PCR. MG G37 was inoculated into Friis FB medium. Samples were collected daily for 3d after seeding and titrated for viability using a color changing unit assay (CCU). In parallel, MG genomes were quantified using the MG309 qPCR assay as described in Chapter 2. The ratio of viable organisms to genomes was approximately 1:1 at each sampling time except immediately after seeding.

Following inoculation of Swiss Webster mice, viable MG was recovered from tissues of infected animals after thorough mincing and incubation at 37°C in Friis medium for up to 21d. Viability was determined by a dramatic color change of the Friis FB medium from red to yellow and formation of adherent microcolonies characteristic of MG growth. In a single study (n=5) using progesterone-treated mice, MG G37 was recovered from vaginal swabs (d5; 40%, Table 8), the upper genital tract (21d PI; 40%, Table 8) and from the knees (21d PI; 60%, data not shown) of inoculated mice. In estradiol-treated mice, 2 independent studies (n=9) resulted in detection of live MG from d5 swabs (30d PI; 33%, Table 8), upper genital tract tissues (30d PI; 11%, Table 8) but not from any knee tissues (data not shown).

Table 8. Viability of MG G37 in vaginal swabs and upper genital tract tissues from progesterone- and estradiol-treated mice.

MG G37	Vag. Swab[a]	Upp. GT[b]
Progesterone[c]	2/5 (40%)	2/5 (40%)
Estradiol[d]	3/9 (33%)	1/9 (11%)

Indicated proportions are # of mice/group that were verified positive for viable MG from the indicated specimen type following 21d incubation in Friis medium.

[a]Vaginal swabs were collected 5d PI.

[b]Upper genital tract tissues were collected aseptically 3-4wk PI.

[c]Single study, n=5.

[d]**Two independent studies, n=9**

Following a color change of the Friis medium and formation of adherent microcolonies, the outgrowth was verified to be MG by PCR and sequencing of the MG 16s rRNA gene [141] and a 281bp hypervariable region of MG191 [77]. Using the BLAST (NCBI) algorithm and manual sequence alignment, purified PCR products showed >97% sequence identity to MG (reference strain GenBank Acc. No. L43967). A single polymorphism within MG191 of strain M2300, not present in G37, was preserved in all M2300 outgrowth samples ensuring no samples were cross-contaminated. No color changes were observed from any tissues of mock-inoculated mice (n=9). However, the prevalence of MG viability was relatively low among each tissue type and provided no information as to the microbial load within each tissue. Therefore, we employed the highly sensitive and specific MG309 qPCR assay (Refer to Chapter 2) for the remainder of the presented studies.

***M. genitalium* colonized mouse upper genital tract tissues**

Based on the observation that MG is present at low levels in the vagina for up to 11 weeks after inoculation, we next evaluated whether MG colonizes upper reproductive tract tissues. In four independent studies, progesterone- (n=33) or estradiol-treated (n=20) Swiss Webster mice were inoculated intravaginally with MG G37 and then humanely euthanized at selected time points for collection of upper genital tract tissues. Dissected tissues were processed for PCR-based quantification of MG load. In progesterone-treated animals, the cumulative rate of ascending infection was 21% (7/33;

Fig. 33A) among 4 independent studies and varied from 0% in two studies to as high as 66% in one experiment. In estradiol-treated mice, ascending MG infection was observed in 65% of inoculated mice (13/20; Fig. 33A) with rates varying from 33 to 100% among the 4 studies. A strong, but insignificant trend indicated that estradiol treatment resulted in enhanced upper tract colonization relative to progesterone.

However, the MG burden in upper genital tract tissues from PCR-positive animals was not significantly different between estradiol- and progesterone-treated mice (Fig. 33B) averaging $2.8x10^3$±1300 and $1.6x10^3$±640 MG genomes/tissue, respectively. Of all PCR-positive upper genital tract samples from estradiol-treated mice, the majority of isolates were from uterine tissues with fewer from the cervix or oviducts (Fig. 33C).

M. genitalium disseminated to the knees

MG has been detected in human synovial tissues of arthritic knees [135-137] and from synovial fluid of the temporomandibular joints [138, 139]. To test whether MG disseminates to the knees of female mice following vaginal inoculation, in four independent experiments, knees were collected for PCR quantification from animals humanely euthanized 3-4 weeks PI. In progesterone-treated animals, dissemination to one or both knees was observed in 21.2% (7/33) of inoculated animals (Fig. 34A). Among independent studies, rates of knee colonization varied from 8-60%.

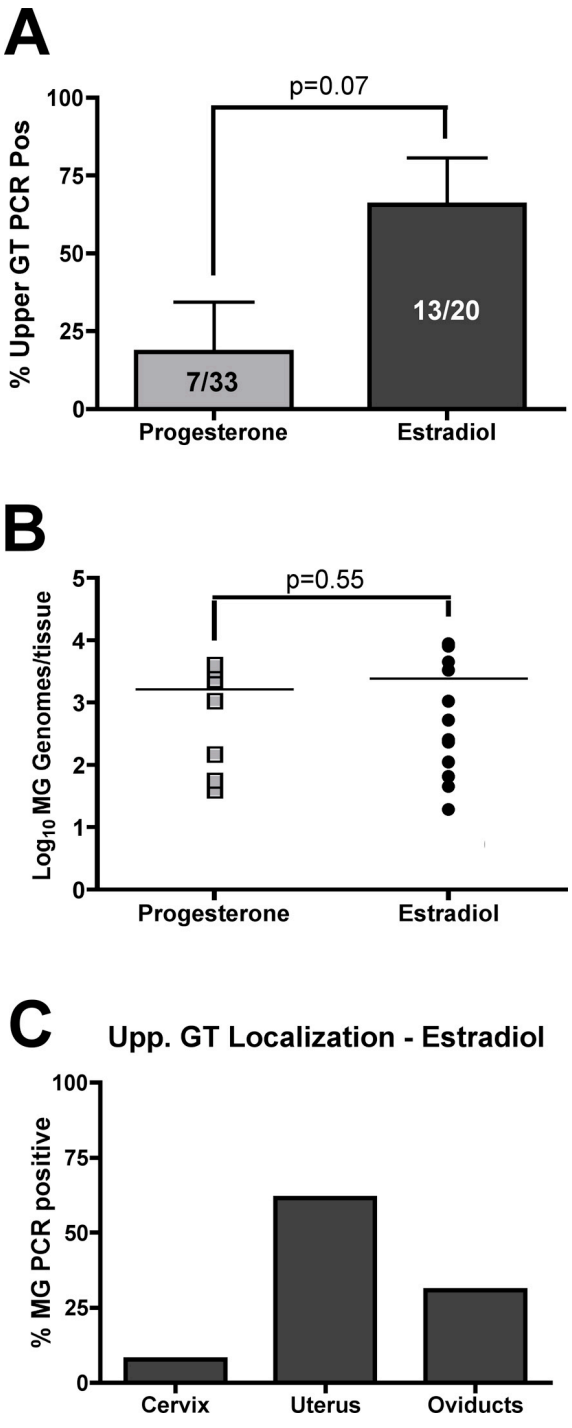


Figure 33. Upper genital tract colonization and localization. Upper genital tract tissues (cervix, uterus and oviducts) were collected separately from estradiol (n=20) or progesterone-treated (n=33) SW mice 3-4 weeks following inoculation with MG G37. Tissues were processed for PCR quantification of MG DNA loads. (A) Among 4 independent studies, the rate of ascending infection (% PCR positive mice) and (B) the MG burden were calculated following either progesterone or estradiol treatment. (C) Specific localization of MG within the upper reproductive tract of estradiol-treated mice also was evaluated and is expressed as the percent of PCR-positive samples from each tissue type among all upper genital tract isolates.

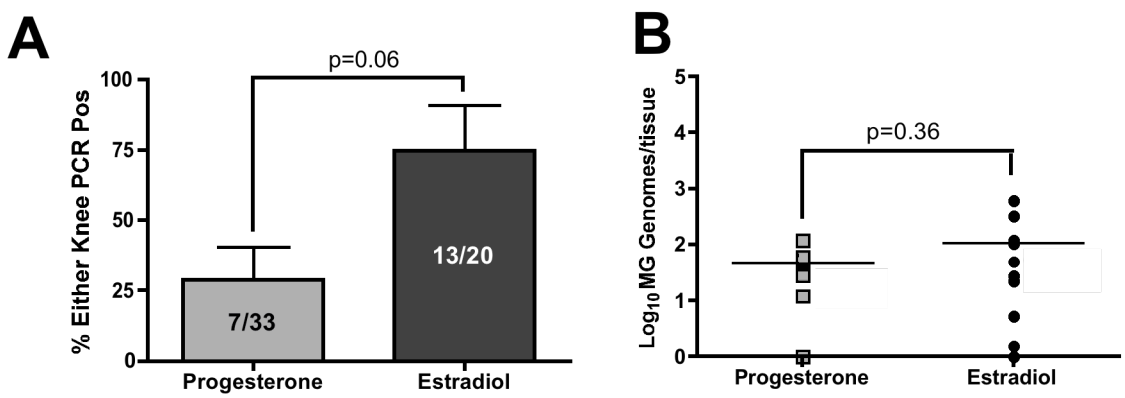


Figure 34. Dissemination of MG G37 to the knee tissues. Knee tissues were dissected from estradiol (n=20) or progesterone-treated (n=33) SW mice 3-4 weeks following inoculation with MG G37. MG DNA loads were determined by PCR-based quantification. (A) Among 4 independent studies, the rate of ascending infection (% PCR positive mice) and (B) the MG burden (expressed as MG genomes/tissue) were calculated following either progesterone or estradiol treatment. Comparisons of the mean prevalence and MG burden of dissemination to the knees between estradiol- and progesterone-treated groups were accomplished using the Student's t-test.

In estradiol-treated mice, MG dissemination to the knee tissues was observed in 65% (13/20) of inoculated mice with rates varying from 33-100% among the studies (Fig. 34A). Again, a strong trend toward increased dissemination following estradiol treatment was observed but the difference, compared to progesterone, was not significant. The average MG DNA load from a single knee was 110±40 genomes/tissue from estradiol-treated animals and 50±10 genomes/tissue in progesterone-treated animals but, similar to the upper genital tract, the mean DNA loads were not significantly different (Fig. 34B, p=0.36; Student's t test).

To evaluate then kinetics of MG dissemination in estradiol-treated mice, MG G37 was inoculated intravaginally and then tissues were collected kinetically on d1, d3 and d7 PI for PCR quantification. No MG was detected in any tissue 1d PI, but MG DNA was detected in upper genital tract tissues from 1/3 mice 3d PI (460 genomes/tissue) and then in 2/3 mice (30±25 genomes/tissue) by d7 PI (Table 9). MG DNA was isolated from both knee joints of 3/3 estradiol-treated mice as early as 7d PI (150±50 genomes/tissue; Table 9. No MG DNA was detected from mock-infected animals dissected in parallel (n=3 at each time point). Importantly, the overall prevalence of MG detection in genital and knee tissues were the same but not all animals with detectable MG in reproductive tract tissues also had MG in the knees. Based on these results we next used the lower passage Danish M2300 strain to establish whether a more contemporary isolate with controlled passage history would show any significant differences in reproductive tract and knee colonization.

***M. genitalium* strain M2300 infection dynamics were similar to G37**

Based on enhanced dissemination following estradiol treatment, we first evaluated M2300 viability from vaginal swabs and upper genital tract tissues. In two independent studies (n=9) where estradiol-treated mice were inoculated intravaginally with MG M2300, viable MG was isolated from vaginal swabs (33%), upper genital tract tissues (30d PI; 11%) and from knees (30d PI; 11%) of inoculated mice (Fig. 35A).

Table 9. Kinetics of dissemination and PCR quantification of MG G37 in the upper reproductive tract and knee tissues following estradiol treatment and intravaginal inoculation.

Hours PI	24	72	168
MG G37			
Vagina[a]	3/3[b] ($3.5x10^4$±3206)[c]	1/3 (240)	2/3 (166±66)
Upper GT[d]	0/3	1/3 (462)	2/3 (29±25)
Knees[e]	0/3	0/3	3/3 (127±59)
Mock-Inf.			
Vagina	0/3	0/3	0/3
Upper GT	0/3	0/3	0/3
Knees	0/3	0/3	0/3

[a]Value obtained from vaginal swabs

[b]Indicated proportions are # of PCR-positive mice/group at each sampling time.

[c]PCR titers in parentheses are expressed as mean±SEM MG genomes/tissue.

[d]Upper GT represents mouse upper genital tract tissues collected and processed as a single tissue for PCR quantification.

[e]Knees represent the proportion of mice that were MG PCR-positive in both knees.

Because PCR afforded enhanced detection of MG G37 compared to culture, we tested whether viable titrations and quantitative PCR titers were similar for M2300 as well. In early and log-phase growth viable titrations were nearly identical to qPCR titers (Fig. 35B) obtained in parallel.

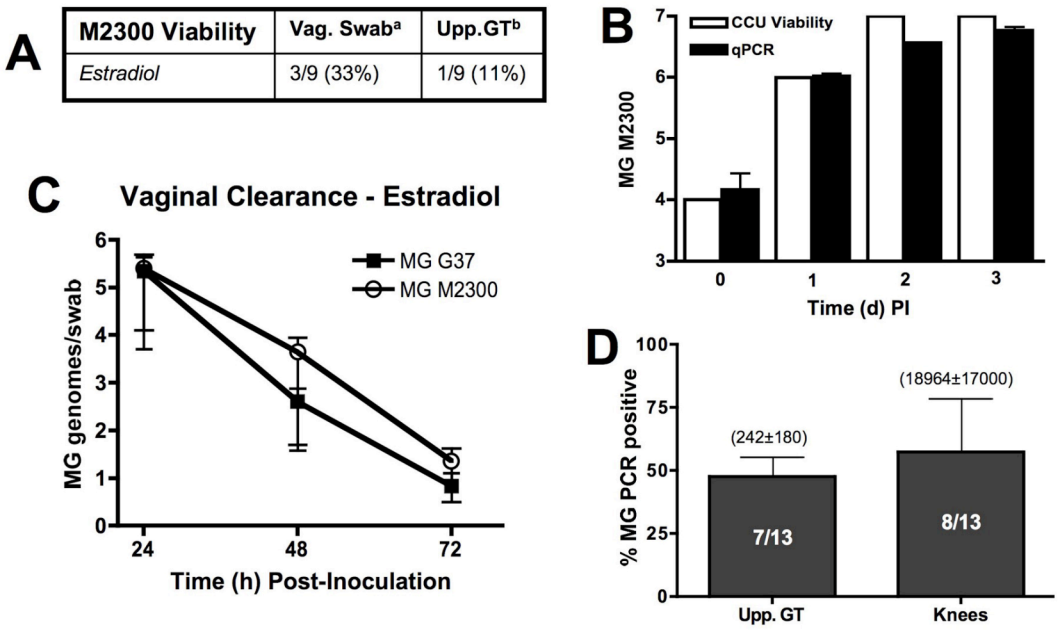


Figure 35. Comparison of MG strains G37 and M2300. SW mice were treated with estradiol and then inoculated with MG strain G37 or M2300. (A) Vaginal clearance of MG was monitored by PCR of vaginal swabs obtained 24-72h following inoculation (n=5 mice/group). (B) Among 3 separate studies (n=13), ascending infection to the upper genital tract tissues or dissemination to the knees was quantified following dissection of mice 3-4 weeks PI and MG PCR. Values in parentheses indicate the mean ± SEM of PCR titers (MG genomes/tissue) among all PCR-positive samples.

Therefore, we next compared vaginal clearance and dissemination prevalence for G37 and the low-passage M2300 strain in estradiol-conditioned Swiss Webster mice (n=5/group) using qPCR. Vaginal clearance of G37 and the M2300 strain was similar (Fig. 35C) and confirmed the findings from the previous G37 studies. Among three independent studies using MG M2300, the rate of ascending infection was roughly 50%

(7/13) averaging $2.4x10^3$±180 genomes/tissue (Fig. 35D). Collectively, 61% (8/13) of mice had MG PCR-positive knee tissues with an average DNA load of $1.9x10^4$±17000 genomes/tissue (Fig. 35D) indicating the observed results were not specific to G37 and that M2300 inoculation resulted in significantly higher DNA loads in the knee compared to the G37 strain ($p<0.05$; Student's t-test).

To verify the specificity of the MG309 qPCR assay, 5 randomly selected tissue samples were verified to contain MG DNA by PCR and sequencing of the complete MG 16s rRNA gene and a hypervariable region of MG191. Using the BLAST algorithm and manual sequence alignment, purified PCR products showed >97% sequence identity to MG (reference strain GenBank Acc. No. L43967, data not shown). Detection of the single nucleotide polymorphism within the MG191 gene of M2300 compared to G37 indicated that the detected DNA was due to the inoculum and no cross-contamination had occurred.

DISCUSSION

Despite the growing body of literature implicating MG as a cause of genital disease in women, no experimental evidence existed for the capacity of MG to establish infection in upper genital tract tissues. In our studies, MG was detected, by both viable outcome measures and PCR, in vaginal swabs, upper genital tract tissues and the knees of female mice following vaginal inoculation. This is the first report of ascending MG infection of the mouse reproductive tract and dissemination to the knees providing strong

experimental evidence that, following vaginal exposure, MG can establish long-term infection of upper reproductive tract and knee tissues. These findings provide important rationale for the continued development of experimental models that address the capacity and mechanisms of MG to cause reproductive tract disease in women.

Estradiol treatment prior to MG inoculation consistently led to increased rates of ascending reproductive tract infection (Fig. 33) and dissemination to the knees (Fig. 34) relative to progesterone-treated animals. The apparent discrepancy between our results and a previous report of estradiol-induced resistance to vaginal MG infection [61] could be attributed to the regimen, dosage and formulation of estrogen delivery. Also, differences in the G37 passage history and the sensitive PCR-based detection method used in our studies likely contributed to the observed disparity. It remains unclear why some species, such as *M. pneumoniae* and *M. pulmonis*, require progesterone treatment for infection while others, including the genital pathogen *M. fermentans*, only colonize the genital tract following estrogen treatment [61]. Interestingly, estradiol treatment increases the susceptibility of balb/c mice to *N. gonorrhoeae* infection [142] while progesterone enhances murine susceptibility to *C. trachomatis* [143] and *C. muridarum* [144]. Despite the similar cell-associated phenotypes of these bacterial species, it is evident that systemic manipulation of sex hormones dramatically affects the susceptibility, duration of infection and pathological outcomes in murine STI models. Importantly, natural changes in hormone levels or oral contraceptive usage are known to affect transmission of other STI in humans and it may now be possible to study these important factors for MG infection and pathogenesis.

The G37 strain was isolated originally from the male urethra [8] but, despite minimal passage in our laboratory, it is unknown how phenotypically similar our strain is to that used in the previous murine study [61] or to the currently circulating clinical strains. In extensive genetic characterization of mgpB and mgpC, two genes within the MgPa operon contain extensive variation among several laboratory and clinical MG strains [55]. Similarly, variable numbers of tandem repeat elements have been observed in the MG309 putative lipoprotein gene [95] collectively suggesting that MG is capable of efficient genetic variation that may produce alternate pathogenic manifestations based upon survival niche selection.

Importantly, the laboratory strains used in our studies were adapted for efficient growth in cell-free medium. This might explain the clearance of many organisms from the vagina by 3d PI leaving only a subset of organisms able to establish long-term infection. However, infection with the limited passage Danish M2300 strain resulted in similar rates of vaginal clearance and prevalence of upper tract and peripheral tissue colonization compared to G37 (Fig. 35). A notable difference between G37 and M2300 was the significantly increased mean knee titer following M2300 infection suggesting that culture adaptation may affect pathogenesis in the mouse model (Fig. 35D). It will be important to compare the pathogenic potential of G37 to specific clonal derivatives and lower passage, clinical isolates to better understand the mechanisms of persistence and the capacity to induce long-term inflammation at genital and synovial sites.

Our studies primarily utilized Swiss Webster mice because the outbred genetic background more closely models infection of the heterogeneous human population. A

possible drawback to using Swiss Webster mice is the reduced availability of genetic variants compared to the C57bl/6 or balb/c genotypes to investigate host genes involved in infection. In addition, considerable variation in the prevalence of upper genital tract and peripheral tissue colonization was seen among our studies suggesting that reproducibility of the model might be increased by using an inbred mouse strain. In limited studies, we also evaluated the susceptibility of estrogenized balb/c, ICR and C57bl/6 mice to vaginal and upper genital tract colonization. Similar rates of vaginal colonization (See Chapter 6) and dissemination prevalence (data not shown) were observed indicating that the reported paradigm likely is adaptable to alternate strains of mice.

Although real-time PCR is a highly sensitive detection method, the PCR data provided no information on bacterial viability as only genomes are quantified. We did investigate and demonstrate MG viability in vaginal swabs, reproductive tract and peripheral joint tissues but long-term studies of bacterial viability were discontinued because PCR was determined to be a more sensitive detection method, afforded a quantitative measure of MG load and was more amendable to larger animal studies. The apparent discrepancy between PCR detection and viable MG outgrowth observed in our studies might be analogous to the misunderstood difficulties in isolating the viable organism from human clinical specimens. However, it also cannot be ruled out that viable isolation might have been enhanced if tissues were cultured for more than 21d or co-cultured with human cells as described previously [13].

Considering the findings presented in Chapters 6 and 7, we have established the relevant parameters for a reproducible small animal model to investigate the capacity of MG to cause urogenital tract disease in women. Because progesterone- and estrogen-dominated animals were susceptible to MG infection, it should now possible to model co-infections in the genital tract. The observation that MG can localize to the knee and other joints following intravaginal exposure is intriguing and warrants continued investigation into the potential implications for inflammatory arthritides. The mechanisms for MG dissemination currently are not understood. It is noteworthy that, although not addressed thoroughly in all of our studies, MG was detected by PCR in the blood of vaginally inoculated mice suggesting that hematogenous spread throughout the mouse via leukocytes could be possible. Finally, the results presented herein provide important rationale for causal associations and reproductive tract disease by demonstrating the potential for upper genital tract infection following vaginal exposure. Future studies utilizing this model will help to uncover important factors and mechanisms of female reproductive tract disease and allow for evaluation of prospective therapeutic interventions to address the global epidemic of STI.

CHAPTER 8: PRELIMINARY TESTING OF IMMUNOSTIMULATORY *M. GENITALIUM* PROTEINS AS MICROBICIDES

OVERVIEW

Microbicides are a modern class of antimicrobial compounds designed for topical application and prevention of STI. Several types of compounds designed for vaginal application have been explored as candidate microbicides including those that target the pathogen and those that work through modulation of the local host environment. Compounds that target the host mucosal immune system have an inherent advantage in that a single formulation may provide protection from multiple pathogens simultaneously. We have focused our efforts on a new class of microbicides that target the host innate immune system, termed immunomodulators. These compounds, when delivered prior to or just after a pathogen exposure, enhance the host immune response thereby preventing establishment of infection. In Chapter 4, we showed that 3 antigenic MG membrane proteins efficiently activated the innate immune response through ligation of TLR2/6 heterodimers. In this chapter, we test the hypothesis that recombinant MG proteins can be formulated as immunomodulating microbicide components to provide protection from STI. We undertook these studies using a well-described mouse model of genital HSV-2. Interestingly, despite reproducible modulation of the host innate response no significant protection was observed. Using FSL-1, an immunostimulatory lipopeptide derived from *Mycoplasma salivarium*, a significant enhancement to HSV-2 resistance was observed and was investigated further for optimal dosing and treatment regimen.

INTRODUCTION

Microbicides are a relatively new and attractive approach to prevent infection by sexually transmitted pathogens. Importantly, this strategy does not prevent transmission of the pathogen such as with a condom but, instead, either kills the organism directly or prevents infection of the vaginal or rectal mucosa following exposure. Microbicides are a particularly formidable option for STI prevention because they have the benefit of being female-initiated, an important concern considering the disproportionate burden of STIs in women [31].

Although many approaches to microbicide development have been or are currently in clinical trials, no FDA-approved and licensed product is available in the USA. Such strategies have included detergent-based compounds to disrupt the microorganism's cell membrane (e.g. nonoxynol-9), some that provide a physical barrier preventing pathogens from reaching target cells (e.g Carraguard®, Cyanoviran®, cellulose sulphate and PRO 2000®) and others that act by maintaining an acidic pH in the vagina (e.g. Acidform®, BufferGel® and *Lactobacillus crispatus*) thereby providing protection against infection (Reviewed in [145]). A newer class of microbicides, termed immunomodulators, includes those compounds that target epithelial and immune cells of the host mucosa to enhance the local immune responses (e.g. TLR agonists). One significant benefit of these strategies is the potential to provide simultaneous protection against multiple STI with a single formulation. All of these formulations can be selectively applied directly to the vaginal or rectal mucosa and therefore require careful

considerations for development as a commercial product including ease of use, discreetness and safety following repetitive usage.

In Chapter 4, we presented results showing that MG encodes several membrane proteins that elicit potent TLR-mediated cytokine elaboration from reproductive EC. In these studies, purified recombinant forms of MG307, MG309 and MG338 were found to specifically activate the innate immune response through ligation of TLR2/6 heterodimers (See Fig. 21, Chapter 4). Therefore, these inflammatory proteins were next prioritized for testing as immunomodulating microbicides. Evaluation of microbicide utility was approached with two important goals: 1) The capacity to elicit transient immunomodulation following vaginal delivery, and 2) The capacity to provide enhanced protection against HSV-2 challenge. A lethal challenge mouse model of genital HSV-2 disease was employed for these studies because the model is well established, is amendable to large animal studies and has been used in our laboratory for testing of several other candidate microbicides [146].

In our studies, we found that vaginal delivery of purified MG307, MG309 and MG338 resulted in transient modulation of host cytokines providing good rationale for efficacy testing. Interestingly, only pre-application of recombinant MG309 provided a significant increase in the time to death following HSV-2 challenge. None of the applied proteins attenuated disease severity or enhanced survival proportions. Studies in parallel using purified FSL-1, a *Mycoplasma salivarium*-derived TLR2/6-agonist showed that pre-treatment enhanced the survival rate of treated mice and significantly increased the threshold of successful infection. In addition, no immunotoxicity was observed at

peripheral lymph nodes even after repetitive vaginal application (WA Rose and RB Pyles, Submitted for Publication). Collectively, these findings indicated that the selected inflammatory recombinant MG proteins were not effective microbicide components as tested. However, FSL-1 provided good evidence for attenuation of HSV-2 disease and warrants continued efficacy and safety testing.

MATERIALS AND METHODS

Recombinant *M. genitalium* proteins and TLR agonists

We tested 3 antigenic, TLR2/6-activating MG proteins (MG309, MG307 and MG338) for utility as immunomodulating microbicides. Each of the tested proteins was cloned into the pROEX HtB expression plasmid, fused with a N-terminal 6-his tag and then purified on Nickel resins as described in Chapter 4. Purified proteins were verified free of any contaminating *E. coli* endotoxin using the Pyrotell Limulus amebocyte lysate assay (Associates of Cape Cod). The synthetic lipopeptide Pam2CGDPKHPKSF (FSL-1) derived from *Mycoplasma salivarium* (InvivoGen) is a TLR2/6 agonist (See Chapter 4) and was used as a control for immunomodulation and tested independently for efficacy and safety as a microbicide.

Animals and intravaginal immunomodulation

All procedures involving animals were approved by the UTMB Institutional Animal Care and Use Committee. Female outbred Swiss Webster mice (Harlan)

weighing 20-24g were housed in AAALAC-approved quarters with food and water provided ad libitum. Animals were pre-treated with a subcutaneous injection of progesterone (150 mg/kg/dose; Depo-medroxyprogesterone) 7d and 1d prior to HSV-2 inoculation. In order to provide rationale for efficacy testing in mice, purified recombinant MG proteins and FSL-1 were first tested for their ability to transiently modulate the host immune response. Purified rMG307, rMG309, rMG338 and FSL-1 were delivered intravaginally (1.0μg) to Swiss Webster mice (n=5/group at each time point). Vaginal lavages were collected at 6 and 24h PI for cytokine quantification via the BioPlex CBA assay using the mouse 23-Plex panel (BioRad) as described in earlier chapters. In parallel, mice receiving only PBS were lavaged at the indicated times to establish a baseline for comparison. Quantitative comparison of secreted cytokines was done using ANOVA where significances were indicated when $p<0.05$ compared to values from PBS animals.

The HSV-2 model of genital disease

To test the capacity of purified recombinant MG307, MG309, and MG338 proteins to independently provide protection against HSV-2 disease, mice were pre-treated with 1.0μg/20uL of the protein 6h prior to a lethal challenge with $1x10^4$ plaque forming units (pfu) of HSV-2 strain 186. This challenge dose, a 100 times greater than the LD_{50}, was selected to permit statistical comparisons between treated and control groups. PBS treated mice were followed in parallel to serve as controls. Inoculated animals were monitored daily for health status and development of symptomatic disease,

including hair loss and erythema, for 14d PI. Survival was monitored up to 21d PI. Animals that developed severe disease signs, including hind limb paralysis or encephalitic disease, were humanely euthanized to prevent suffering.

Evaluation of FSL-1-induced resistance to HSV-2

For efficacy studies, FSL-1 was diluted in PBS and used at the following concentrations (2ug/20uL, 4ug/40uL and 6ug/60uL) as specified for each experiment. For initial studies, selected doses of FSL-1 were delivered vaginally (2, 4 or 6ug/mouse) at selected times prior to or following HSV-2 challenge (10^4pfu/mouse). Dosing was based on FSL-1 solubility in the PBS vehicle and the volumetric constraints of the vagina resulting in a maximum delivery of 6ug. FSL-1-induced resistance to HSV-2 infection was quantified using the infectious dose 50% (ID_{50}) shift paradigm [146]. Briefly, FSL-1 doses of 2 or 6ug/mouse were delivered at indicated times and then groups of mice were challenged with viral inoculums of 10^2, 10^3 or 10^4 pfu (n=10). Mice receiving only PBS prior to inoculation were followed in parallel. Infected animals were identified by plating of vaginal swabs collected 2d after viral challenge [147]. Following infection, animals were assessed daily for disease signs and survival as noted above.

RESULTS

Recombinant *M. genitalium* proteins and FSL-1 elicit transient acute-phase immunomodulation

Following vaginal application of recombinant MG307, MG309, MG338 and purified FSL-1, differential immunomodulation was observed. The immunomodulation by all 3 recombinant MG proteins was identical and characterized by significant vaginal secretion of IL-1α, IL-1β, GM-CSF, G-CSF, IL-1α, IL-6, IL-12(p40), TNF-α, KC (mouse IL-8), MIP-1α, MIP-1β and MCP-1 relative to control mice. Similar to the MG proteins, FSL-1 also induced secretion of IL-6, IL-12(p40), TNF-α, MIP-1α and MIP-1β. In addition, cytokines that were induced by FSL-1 but not any of the MG proteins included IFN-γ and IL-2. The observed immunomodulation was significant when measured 6h following application of the candidate compound but was back to baseline levels after 24h indicating a transient effect. As a comparator for the relative magnitude of induction, IL-1β is presented in Figure 36 as a representative acute-phase cytokine. Although significant relative to control mice, the inductions by rMG307, rMG309 and rMG338 were consistently lower in magnitude than FSL-1 treated mice. These findings provided rationale for testing of microbicide efficacy using the HSV-2 mouse model.

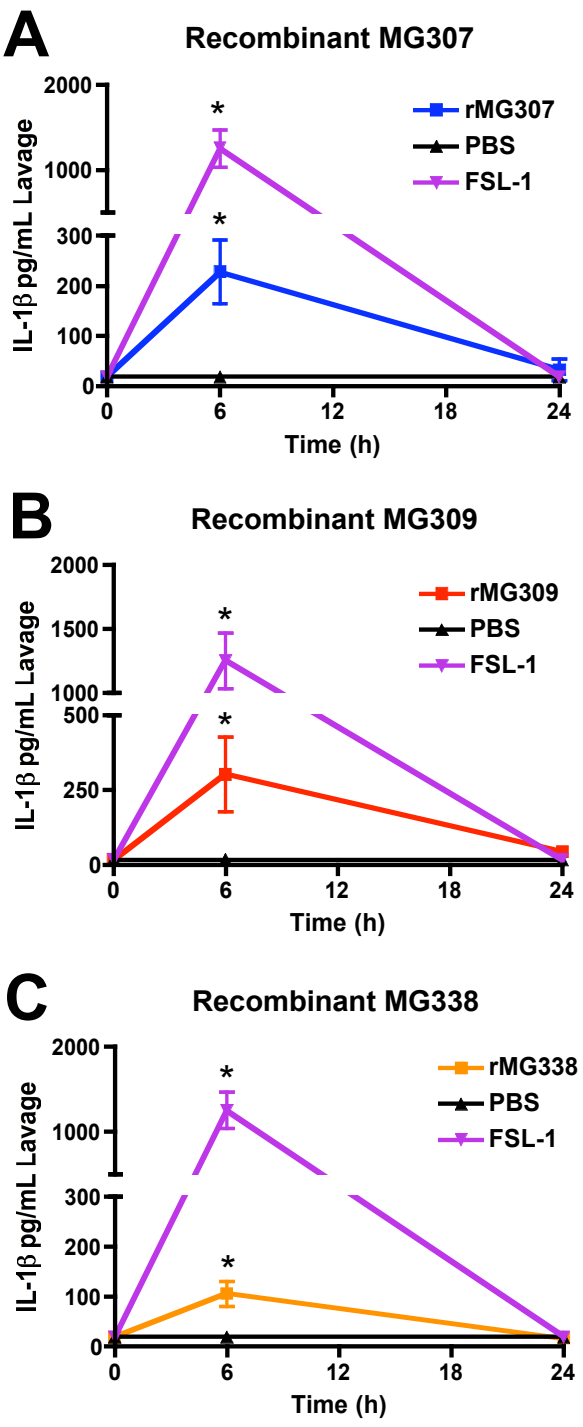


Figure 36. Transient immunomodulation following intravaginal application of recombinant MG proteins. Purified recombinant MG307, MG309 and MG338 were delivered intravaginally (1.0μg) to Swiss Webster mice (n=5/group). Vaginal lavages were collected at 6 and 24h PI for cytokine quantification. Purified FSL-1, a TLR2/6 agonist, was delivered in parallel as a positive control for TLR-mediated immunomodulation. *, $p<0.05$ compared to PBS using ANOVA.

Vaginal application of *M. genitalium* proteins does not provide attenuation of HSV-2 disease

After demonstrating that purified recombinant MG proteins elicit transient immunomodulation when applied to the mouse vagina, we next evaluated whether vaginal application provided protection from subsequent HSV-2 challenge. Using progesterone-treated Swiss Webster mice, vaginal delivery of purified rMG307, rMG309 rMG338 or FSL-1 (1ug) 6h prior to HSV-2 challenge ($1x10^4$ pfu) did not provide any

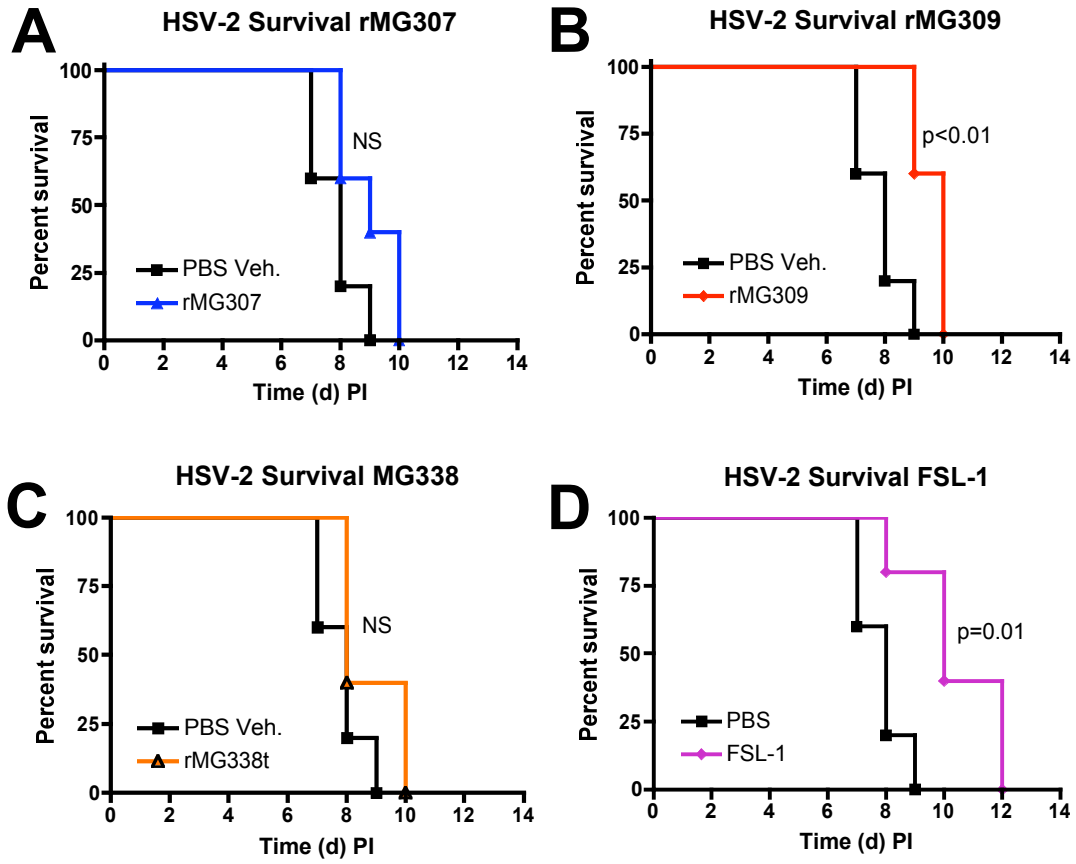


Figure 37. HSV-2 survival proportions following vaginal pre-treatment with recombinant MG proteins. Purified recombinant MG307, MG309 and MG338 (1.0µg) were delivered intravaginally to Swiss Webster mice 6h prior to a lethal HSV-2 challenge (n=5/group). Inoculated mice were monitored daily for disease signs and survival. NS, not significant. Statistical significance was determined using log rank analysis of survival proportions.

significant attenuation of HSV-2 disease (data not shown). Mice treated with rMG309 did show a significantly increased survival time following inoculation but 100% of mice still succumbed to infection (Fig. 37B). Similarly, FSL-1-treated mice showed a significant increase in survival time but not an increase in the overall proportion of survival (Fig. 37D). Because the FSL-1 pre-treatment using a 1ug dose enhanced survival by 3d, we next undertook a more thorough investigation of FSL-1 dosing and treatment regimen to enhance the observed HSV-2 disease attenuation.

Vaginal delivery of FSL-1 delayed onset of HSV-2 disease

Vaginal application of FSL-1 delivered 6 or 24h prior to HSV-2 challenge significantly delayed the onset of disease signs and concomitantly increased the mean time to survival compared to mice receiving only PBS prior to viral challenge (Table 10). Based on the potential utility as a vaginal microbicide, FSL-1 (2ug/dose; 4ug total) was also delivered 1h prior and 1h after HSV-2 challenge to determine an optimum time for application relative to pathogen exposure. Interestingly, this treatment paradigm did not attenuate HSV-2 disease or survival compared to control mice (Table 10). To evaluate whether repetitive dosing enhanced attenuation of HSV-2 disease, FSL-1 was delivered as 3 consecutive doses (2ug/each; 6ug total) 6, 5 and 4h prior to HSV-2 challenge. Repetitive dosing was then compared to a single dose of equal concentration (6ug) delivered 6h prior to viral challenge. Only the single 6ug dose of FSL-1 provided significantly improved outcomes compared to PBS or FSL-1 (2ug) delivered 1h prior and 1h after HSV-2 challenge (Table 10). These data were collected with the significant

collaboration of William A. Rose in our laboratory and, at the time of writing this dissertation, have been submitted for publication to *Antiviral Research*.

Table 10. Vaginal application of FSL-1 significantly delayed HSV-2 disease development and increased survival times.

FSL-1 and vehicle groups[a]	Time to symptoms[b] (days)	Survival time[c] (days)	Survival[d] (%)
FSL-1 2ug 24h prior	8.0 (6.4-9.7)[e,f]	9.4 (7.8-11.0)[e,f]	1/10 (10)
FSL-1 2ug 6h prior	9.0 (8.1-9.9)[e,f]	10.1 (8.9-11.3)[e,f]	0/10 (0)
FSL-1 2ug 1h prior, 1h after	6.0 (5.5-6.4)	7.3 (6.8-7.7)	0/10 (0)
FSL-1 2ug 6h, 5h, 4h prior	6.4 (5.9-7.0)	9.1 (7.0-11.2)	1/9 (11)
FSL-1 6ug 6h prior	8.5 (6.7-9.9)[e,f]	10.0 (8.4-11.6)[e,f]	4/10 (40)
PBS vehicle control	5.8 (5.0-6.4)	7.2 (6.9-7.6)	0/8 (0)

[a]Vaginal application of FSL-1 (2ug) or PBS at indicated times relative to HSV-2 challenge.

[b]Mean (95% confidence interval) time to onset of disease signs

[c]Mean (95% confidence interval) survival time after viral inoculation

[d]Proportion of surviving mice per group (percent survival).

[e]p<0.05 compared to vehicle control (ANOVA, Dunnett's Test).

[f]p<0.05 compared to FSL-1 2ug 1h prior, 1h after (ANOVA, Dunnett's Test).

Vaginal FSL-1 application increased resistance to HSV-2

Having identified 2 FSL-1 treatment regimens that delayed the onset of HSV-2 disease signs (delivered 24 and 6h prior to inoculation), the ID_{50} shift paradigm [146] was then used to quantify the increased resistance to HSV-2 afforded by FSL-1 pre-treatment. Briefly, FSL-1-treated animals were distributed randomly into subgroups (n=10) for challenge with escalating viral doses (10^2, 10^3, or 10^4 pfu; Table 11). Of the tested doses (2 or 6ug) and times (6 or 24h prior to challenge), the 2ug FSL-1 dose applied 24h prior to HSV-2 challenge resulted in a significant shift in the infectious dose 50% (ID_{50}=760pfu) compared to PBS vehicle treated control animals (ID_{50}=31pfu). Among the animals that were infected, this dose of FSL-1 also resulted in a significant increase in the survival proportion. When FSL-1 (2ug) was delivered 6h prior to HSV-2 challenge, the ID_{50} (260pfu) was increased 10-fold and the LD_{50} (660pfu) was increased 10-fold (Table 11) relative to untreated mice. Similar outcomes were observed when 6ug FSL-1 was delivered 6h prior to viral challenge (data not shown).

DISCUSSION

In this chapter, we attempted to exploit 3 TLR-activating MG proteins for use as immunomodulating microbicides against HSV-2 genital disease. In Chapters 3 and 4 we showed that recombinant proteins encoded by MG307, MG309 and MG338 very efficiently activated TLR2/6 heterodimers to activate the innate immune response.

Table 11. FSL-1 application provided enhanced resistance to HSV-2 challenge in mice.

HSV-2 dose	FSL-1 2ug 24h prior[a]		FSL-1 2ug 6h prior		vehicle control	
(pfu)	%Infected[b]	%Survival[c]	%Infected	%Survival	%Infected	%Survival
$1x10^1$	ND[d]	ND	ND	ND	30	70
$1x10^2$	30	70	10[e]	90	70	50
$1x10^3$	50[e]	50[e]	100	40	100	0
$1x10^4$	90	10	100	0	ND	ND
ID_{50}[f]	760 (80-7000)		260 (10-6000)		31 (10-80)	
LD_{50}[g]	760 (80-7000)		660 (300-1000)		68 (7-700)	

[a] Vaginal application of FSL-1 (2ug) or PBS at indicated times relative to HSV-2 challenge.

[b] Percentage of mice with infectious virus in d2 vaginal swabs.

[c] Percentage of mice that survived HSV-2 vaginal challenge up to d14 PI.

[d] ND, not determined.

[e] $p<0.05$ compared to vehicle control (Fisher's exact test).

[f] Viral inoculum required to infect 50% of the mice, ID_{50} (95% confidence interval).

[g] Viral inoculum necessary to cause death in 50% of mice; LD_{50} (95% confidence interval).

Specifically, these proteins were shown to activate secretion of several immunopotentiating cytokines from human vaginal and cervical EC providing good rationale for evaluation as vaginally applied microbicides.

Vaginal and cervical EC express robust levels of TLR2, 3, 5, 6 and CD14 with low levels of TLR1, 4 and 7-9 [42]. Understanding how vaginal EC respond to topically applied therapeutics is of utmost importance considering the vaginal and ectocervical mucosa would likely be the primary target for immunomodulating compounds. In previously-published studies from our laboratory and others, modulation of the host innate immune response has proven to be a effective approach for providing enhanced protection against experimental HSV-2 infection. Specifically, artificial stimulation of TLR3 via dsRNA (Poly I:C) provides a significant delay in onset of HSV-2 disease signs [148, 149]. Similarly, synthetic oligonucleotides containing un-methylated CpG motifs have also shown significant anti-herpetic activity [148-150] collectively providing good rationale for TLR-based microbicides. Importantly, comparison of these agonists and their responses has indicated that the pattern of cytokines necessary for anti-herpetic efficacy is consistent with FSL-1 immunomodulation and suggests that host-targeted therapies are a viable approach to STI prevention.

In our studies, pre-treatment with recombinant MG proteins did not afford any protective advantage when mice were subsequently challenged with HSV-2 (See Fig. 37). Treatment with FSL-1, however, did show significant attenuation of HSV-2 disease severity (Tables 10 and 11). Despite all of the tested agonists being validated TLR2/6

agonists, application of recombinant MG proteins to the mouse vagina elicited secretion of several cytokines that were not in concordance with animals treated with FSL-1. Following vaginal delivery, FSL-1 led to the transient induction of IL-2, IL-12p40, IFN-γ, and MIP-1α but no cytokines associated with a T_H2-biased immune response. The recombinant MG proteins elicited vaginal secretion of several pro-inflammatory cytokines including IL-1β, IL-6, IL-12(p40), TNF-α, KC (mouse IL-8). In addition, several cytokines implicated in monocyte/macrophage recruitment and stimulation were induced including GM-CSF, G-CSF, MIP-1α, MIP-1β and MCP-1 suggesting that application of MG proteins might not be inducing the appropriate immunomodulatory response necessary for HSV-2 resistance.

In studies of other TLR agonists, vaginal application of un-methylated CpG-containing oligonucleotides resulted in elaboration of IFN-γ, IL-12, IL-18 and RANTES into vaginal secretions [151, 152]. Similarly, the cytokine profile elicited by Poly I:C delivery to the mouse vagina was similar to CpG and FSL-1 and included secretion of IL-1α, IL-1β, IL-6, IL-12, IFN-γ, IFN-β, MIP-1α, RANTES [146]. Although not measured following vaginal delivery of the recombinant MG proteins, type I interferons appear to be an important factor for effective anti-herpetic responses as Poly I:C and FSL-1 both induce robust secretion of IFN-α or IFN-β. Collectively, the presented results from studies in our lab and from others [151, 153-159] suggest that an effective immunomodulatory profile of cytokines would include IL-2, IL-12(p40), IFN-β, IFN-γ and MIP-1α. The marked differences between the recombinant MG proteins and the

other effective anti-herpetic agonists may explain the lack of protection afforded by prophylactic treatment using these proteins.

It remains less clear why the TLR2/6 agonists used in our studies differ in their immunomodulatory capacities. Using equimolar concentrations, FSL-1 elicited a transient modulation of several host cytokines that, for most cytokines, was more robust compared to each of the recombinant MG proteins (Fig. 36). FSL-1 is a short lipoylated peptide, 10 amino acids in length and is commercially synthesized and purified (Invivogen). Compared to FSL-1, the recombinant MG proteins are much larger (~20-60kd) and have significant 3-dimensional structure that likely make these proteins more susceptible to degradation in the hostile environment of the vaginal lumen. In addition, obtaining protein yields of recombinant MG307, MG309 and MG338 higher than those used in these studies was difficult. In turn, although the recombinant MG proteins elicited significant secretion of several cytokines following vaginal exposure, higher dosages at different times could enhance the attenuation of HSV-2 disease. Therefore, the lack of protein longevity in the vagina might contribute to the avoidance of prolonged inflammation but also could affect the potency of the treatment. In addition, lower intravaginal doses of FSL-1 (Fig. 37) were less efficacious for enhancement of survival following HSV-2 challenge compared to later studies that employed higher dosages (Tables 10 and 11). Interestingly, the transient cytokine response peaking 6h PI did not correlate well with the dosing of FSL-1 for effective antiherpetic outcomes. This indicates that the recruited immune cells likely provided the protective effects against HSV-2 that require up to 24h to migrate to the mucosal surface.

In summary, we have identified a specific *Mycoplasma salivarium*-derived TLR2/6 agonist, FSL-1, capable of providing increased resistance to HSV-2 infection using a murine model of genital disease. Exploitation of recombinant MG membrane proteins encoded by MG307, MG309 and MG338 as immunomodulating microbicides was not successful as tested. However, these results did provide an enhanced understanding of the immunomodulatory cytokines that seem to be necessary for inducing an effective anti-herpetic environment in the vagina. In addition to the therapeutic aspects, continued investigation of how bacteria-specific TLR agonists interact with the female reproductive mucosa may also provide important evidence for how commensal flora and other bacterial pathogens affect HSV-2 pathogenesis and treatment.

CHAPTER 9: KEY FINDINGS AND IMPLICATIONS FOR FUTURE RESEARCH

Since its original isolation in 1980 [8], *M. genitalium* has been of considerable interest as a cause of urogenital disease in both men and women. Following the firm establishment that MG is transmitted through sexual contact [4, 90] and causes NGU in men (Reviewed in [70, 134]), studies were next focused on determining whether MG is a cause of reproductive tract inflammation in women. Among several studies, significant associations between MG infection and cervicitis [21, 23, 72-74] and PID [71] have been observed. In addition, there is preliminary evidence to suggest that MG can cause salpingitis (Reviewed in [71]) and negatively impact fertility [28, 29]. This considered, we undertook a multifaceted approach to understanding the capacity and mechanisms of MG to cause inflammatory reproductive tract disease using cell culture models of human epithelia and a novel murine model developed as part of this dissertation.

In studies presented in Chapter 3, we have significantly contributed to the understanding that MG can establish intracellular infection of reproductive tract ECs. Although several other reports of intracellular localization have been cited [76, 85, 87, 88], our findings are the first experimental demonstration of intracellular survival within human vaginal and cervical ECs. We demonstrated this using the G37 type strain and the more contemporary clinical strain, M2300, that was kindly provided by Jorgen Jensen (Staten Serum Institut, Copenhagen, Denmark). These findings were shown by direct visualization using electron microscopy and then verified using an antibiotic protection assay whereby preferential localization to intracellular sites was observed. For the

antibiotic protection studies, the ME-180 cervical EC line was employed because, at the time of these studies, it was assumed that MG required a serum-containing medium for survival and replication. Subsequent experimentation showed that MG was capable of replicating in serum-free medium when co-cultured with low-pass human vaginal and cervical EC. The human vaginal and cervical EC used in these studies were obtained originally from 3 donors after which considerable time and careful measures were taken to establish an immortalized cell line for continued research [42]. Innate immune responses from these cells to several pathogens or specific TLR agonists were very similar to their primary progenitor [42]. Therefore, we are confident that the contemporary approaches used in this dissertation to understand host-pathogen interactions and evaluate host immune responses are a superior approach compared to carcinoma cell lines such as HeLa or cells of non-reproductive tract origin.

Using these human vaginal, ecto- and endocervical ECs we next showed that infection with MG G37 or M2300 results in pro-inflammatory cytokine secretion. The endocervical ECs were the most responsive followed by the ectocervical and vaginal EC. This finding may have biological significance if MG could indeed ascend the cervical os to the upper genital tract tissues (See Chapter 7) thereby causing acute or long-term inflammation. For each experiment, we quantified 27 human cytokine targets simultaneously using the BioPlex CBA assay. Of this panel that included both Th-1 and Th-2 associated cytokines, the pattern of cytokine induction was predominately Th1-biased and most consistent with recruitment and stimulation of monocytes and macrophages. Interestingly, long-term infection of cervical ECs resulted in a down-

regulation of IL-8 secretion and concomitant increase in IL-10, an anti-inflammatory cytokine. This could be due to prolonged immune stimulation of these cells whereby a tolerance is induced and the cells have shifted to an anti-inflammatory state to limit excessive inflammation [160]. Alternatively, MG might employ some active mechanism for down-regulating inflammation but considerably more evidence is needed to support his hypothesis. Collectively, these findings imply that ECs could become non-responsive during persistent reproductive tract infection. However, substantial clinical evidence for reproductive tract inflammation suggests that the mucosa are responsive and that host immune cells are likely involved in these responses.

To investigate further the potential role of immune cell recruitment to the reproductive mucosa, we employed a novel 3-dimensional culture system developed in our laboratory with William A. Rose and through collaborations with Dr. Alison Quayle (Louisiana State University Health Sciences Center, New Orleans, LA). Using this model it was possible to investigate how polarized vaginal ECs with distinct apical (representing the vaginal lumen) and basolateral surfaces interacted with and responded to acute MG infection (Refer to Chapter 3). With regard to immune cell recruitment, this model allowed for quantification of cytokine secretion from both the apical and basolateral sides of the EC multilayer to more accurately dissect the innate response.

Following EC infection, MG rapidly attached to and invaded the apical-most ECs. This was observed using scanning and transmission electron microscopy. Following 6-12h of infection, transmigration of MG through the epithelial multilayer had occurred and ultimately resulted in complete migration to the basolateral chamber of the culture setup.

Interestingly, cytokine secretion was exclusively from the basolateral side of the EC multilayer providing good evidence for recruitment of immune cells that would be present near the basement membrane. The mechanisms and clinical implications for transmigration of MG through the vaginal EC matrix are not clear. However, both in human clinical cases and experimental animal experiments discussed in Chapter 8, MG has been detected in upper genital tract tissues and peripheral joints suggesting MG can disseminate within the host. In polarized EC cultures, MG was primarily localized to intracellular vacuoles and never observed free in the cytosol. Collectively, these findings suggest that intracellular localization might also result in efficient cell-to-cell spread and afford MG a survival advantage via exploitation of the intracellular space.

The importance of intracellular survival may be linked to the ability of MG to evade host immune responses and establish long-term clinical reproductive tract infection in both men and women. Considering that macrophage recruitment seems likely based on the pattern of cytokines secretion from infected genital ECs, we first tested and discovered that MG was susceptible to macrophage phagocytosis and killing. We next determined that a significant proportion of MG was protected from effective macrophage responses when MG established intracellular infection of vaginal ECs (See Chapter 5). These findings elucidated a putative mechanism for long-term MG survival in reproductive tract tissues. Most importantly, rapid EC attachment and invasion of luminal EC of the vaginal mucosa also indicates that, following sexual transmission, establishment of vaginal infection likely occurs rapidly. In our culture models, cytokine secretion from reproductive tract ECs peaked 72h PI but intracellular localization was

visualized as early as 2h PI. Therefore, based on models of EC infection, establishment of intracellular infection and evasion of host cellular immune responses would occur before any robust innate response would be effective.

The important association of MG reproductive tract infection with HIV-1 could be of particular importance considering the enormous public health burden of HIV infections worldwide (Reviewed in [93]). Currently, there are no sound mechanisms for these associations but several important findings from this dissertation provide some putative explanations. We found that MG elicited minimal innate responses from human vaginal EC from 3 donors but ecto- and endocervical ECs secreted cytokines most consistent with recruitment and stimulation of monocytes and macrophages (See Chapter 3). Thus, reproductive tract infection by MG may result in recruitment of macrophages and other immune cells to infected tissues. Although we have demonstrated that intracellular mycoplasmas should be protected from phagocytosis, cytokine secretion by infected ECs could result in prolonged inflammation at these tissues. In turn, this could result in enhanced susceptibility to HIV-1 by mobilizing T cells and macrophages to the vaginal and cervical mucosa of which macrophages are robust potentiators of the innate response through significant cytokine elaboration. Importantly, macrophages are one of two major cellular reservoirs for latent HIV-1 infection and contribute to early-stage virus transmission and dissemination throughout the host (Reviewed in [124]).

Should MG encounter a macrophage *in vivo*, we tested and determined that MG exposure results in robust secretion of pro-inflammatory cytokines including IL-6, IL-8 and several chemokines responsible for granulocyte recruitment. The mechanisms for

this activation are likely mediated by TLR activation (See Chapter 4). IL-6 secretion is positively correlated with HIV-1 burden [122] and known to up-regulate HIV-1 replication [123]. Clinically, the MG burden in cervical specimens also is strongly associated with HIV-1 shedding [92]. Therefore, our findings suggest that direct recruitment of macrophages and T cells to the reproductive mucosa by MG infection is one explanation for the observed associations. However, continued research will be important to understand the dynamics of HIV-1 and MG co-infections of vaginal and cervical tissues.

An important consideration for many of the observations and conclusions made in this dissertation is that many of the approaches independently employed homogenous reproductive tract EC populations. These cells provided important understanding of the specific role and response of vaginal and cervical ECs to MG infection and directed us to which immune cells could be involved. However, it is well understood that the vaginal and cervical mucosa are not solely composed of ECs, but are part of a diverse multi-cellular matrix that includes immune cell populations [38, 121]. Investigation into the genital tract response and whether or not MG can cause inflammation were largely impeded by the lack of a cost-effective animal model of reproductive tract disease. Therefore, we developed a novel mouse model of MG reproductive tract infection to investigate the early events following sexual transmission and how intact mucosa respond to MG exposure.

Results from studies outlined in Chapter 6 indicated that MG G37 and M2300 could establish viable and long-term infection of the lower female reproductive tract of

mice. From these studies, it was not possible to discern whether the infection was primarily of the vagina, the cervix or upper genital tract tissues because the outcome measure was PCR detection from vaginal swabs that likely also included the ectocervix. However, the finding that MG was capable of establishing a long-term infection of reproductive tract tissues was important rationale for continued investigation. We next addressed the affect of hormonal conditioning using estradiol or progesterone for altered susceptibility to infection. We found that systemic conditioning with either estradiol or progesterone rendered the mouse vagina susceptible to long-term MG infection.

There is some controversy regarding estradiol-induced susceptibility as it was shown previously that mice are resistant to vaginal MG infection when inoculated after estradiol treatment [61]. This discrepancy could be due to our employment of a highly sensitive qPCR assay that was developed and validated as part of this dissertation (Refer to Chapter 2). We found that this method was more sensitive than culture, a finding also observed in clinical studies (Reviewed in [14]). In addition, although G37 was used in our and Taylor-Robinson's studies [61], potential differences in the passage history could result in altered phenotypes in mice. Compared to other mouse STI models, estradiol treatment increases the susceptibility of balb/c mice to *N. gonorrhoeae* infection [142] while progesterone enhances susceptibility to CT [143] and *C. muridarum* [144]. Collectively, systemic manipulation of sex hormones probably dramatically affects modeling of pathogenesis and will be an important consideration for future studies. With regard to clinical implication, our findings suggest that natural modulation of sex hormones and oral contraceptive usage could affect susceptibility to MG infection and

development of reproductive tract disease. Using this MG mouse model, it was possible to more thoroughly address these factors for reproductive tract infection and address the capacity of MG to establish upper tract infection.

Arguably the most important findings presented in this dissertation show that MG can disseminate to the upper reproductive tract tissues following vaginal inoculation (Refer to Chapter 7). Prior to this work, no experimental evidence existed for the ability of MG to ascend the cervical os and, therefore, has important implications for upper tract disease. Inflammatory upper tract syndromes including PID, endometritis and salpingitis can have significant deleterious affects on reproductive health and fertility [62]. Importantly, MG has been associated independently with each of these conditions in human patients [71]. Following inoculation of outbred Swiss Webster mice with MG G37 or M2300, colonization of the cervix, uterus and oviducts (Refer to Chapter 7) was observed and resulted in antibody responses that paralleled human responses observed clinically [28, 47]. These are important results demonstrating that, following vaginal exposure, MG can indeed establish infection of upper reproductive tract tissues and should heighten awareness to potential inflammatory sequelae.

Although MG appears to preferentially colonize the genital tract, this organism also has been a suspected cause of reactive arthritis since DNA was detected in the knee joints of arthritic patients [135-137] and in synovial fluid from temporomandibular joints [139]. Using the mouse model, we showed that vaginal inoculation of MG indeed resulted in dissemination to the knee joints in up to 65% of estradiol-treated mice (See Chapter 7) providing some experimental evidence for dissemination to synovial sites.

These data are intriguing and warrant additional research into the mechanisms and pathological consequences of synovial infection.

In summary, exposure of MG to female reproductive tract tissues appears to result in establishment of long-term intracellular infection of mucosal ECs thereby providing a survival niche and enhanced resistance to the host immune response. Investigation of the early events after MG exposure to the vaginal and cervical mucosa is important for understanding how MG establishes infection and for the targeted development of effective preventative and therapeutic measures. We still have much to learn about MG reproductive tract infections from both a clinical and experimental standpoint. A significant impediment for continued advancement of our understanding of this organism is a lack of physician awareness and universal screening of patients with relevant clinical symptoms. Based on our findings, MG appears to have the capacity to elicit inflammation in reproductive tissues that could affect reproductive health. Furthermore, the observed responses to MG in cultured ECs and animal models provide some explanation for the associations with HIV co-infections that warrant additional research and heightened clinical notoriety. To this end, future investigation of MG's role and associations with other STI will be important for understanding the complex and dynamic inflammatory syndromes of the female reproductive tract.

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VITA

Christopher L. McGowin was born 09 July 1982 in Dallas, Texas to David D. McGowin and Sharon K. McGowin. Chris developed an interest in the field of microbiology and infectious disease while in college at Texas State University in San Marcos, Texas whereby he received several awards for excellence in research of microbial ecology. During his graduate training at the University of Texas Medical Branch, Chris focused heavily on research of women's health and sexually transmitted diseases and received the UTMB Graduate School of Biomedical Sciences *Associates Scholarship Award* for exceptional academic merit. In 2007, Chris received two 1st place awards for poster presentations: one at the annual UTMB Pathology Research Symposium and the other at the UTMB Sealy Center for Vaccine Development Annual Meeting. While at UTMB, Chris delivered several invited presentations of his research including a platform presentation at the International Society of Sexually Transmitted Diseases Research in Seattle, Washington in July of 2007. Chris received a travel grant to the ISSTDR meeting and, in conjunction, also presented findings at the NIH Collaborative Research Center Meeting in Seattle, WA during this time. In 2008, Chris was nominated and awarded the 'Who's Who Among Students in American Universities and Colleges'. In 2009, Chris was awarded a travel grant from the McLaughlin Endowment for a poster presentation at the Annual McLaughlin Research Colloquium.

During his graduate education, Chris also served as a mentor to several trainees including a student from the UTMB Clinical Laboratory Sciences program and a student from Ball High School. These opportunities, combined with several guest lectures in the UTMB Clinical Laboratory Sciences program, provided Chris with valuable teaching experience.

Education

B.S., August 2004, Texas State University, San Marcos, Texas

Publications

McLean, RJC., C. Bates, CL McGowin, M. Barnes, and G. Aron. Methods of Studying Biofilms. M.A. Ghannoum and G.A. O'Toole (eds.), In Microbial Biofilms. ASM Press, Washington, D.C. Published 2004.

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Summary of Dissertation

A significant number of inflammatory reproductive tract syndromes in women have no etiologic agent identified indicating other causes must exist. *Mycoplasma genitalium* was first isolated from men with urethritis in 1980 and has since been intensely studied as a sexually transmitted pathogen of the male and female reproductive tracts. In women, *M. genitalium* is predominately associated with cervicitis and pelvic inflammatory disease but may also cause impaired fertility. Despite these significant clinical associations, no experimental evidence existed for MG to colonize female reproductive tract tissues and cause inflammation. In our studies, we found that *M. genitalium* can establish long-term intracellular infection of vaginal and cervical epithelial cells that provides protection from the phagocytic immune responses of macrophages. However, significant inflammatory cytokine secretion was observed from vaginal, ecto- and endocervical epithelial cells. The cytokine responses from human genital epithelial cells and macrophages were consistent with recruitment of immune cells to the reproductive mucosa and provided some evidence for the associations of *M. genitalium* and HIV-1 co-infection. Using a novel mouse model developed as part of this dissertation, we demonstrated that *M. genitalium* causes acute-phase inflammation and can ascend to upper genital tract tissues including the uterus and oviducts after vaginal inoculation. These findings were the first experimental demonstration that MG can disseminate to the upper reproductive tract tissues and provided important evidence for the capacity to cause disease at these sites. In summary, intracellular infection of epithelial cells appears to be an efficient means for establishment of reproductive tract infection and concomitantly provides a survival niche and enhanced resistance of *M. genitalium* to the host immune response.

Future investigation of *M. genitalium*'s role and associations with other STI will be important for understanding the complex and dynamic inflammatory syndromes of the female reproductive tract.