

Vaccination of BALB/c Mice with an Alhydrogel Adjuvanted Whole Cell *Trichomonas vaginalis* Formulation

Jeffrey D. Smith

A thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the
MSc degree in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

© Jeffrey D. Smith, Ottawa, Canada, 2014

Abstract

A human safe, Alhydrogel adjuvanted whole cell *Trichomonas vaginalis* vaccine was tested for efficacy in a BALB/c mouse model of vaginal infection. Additionally, the systemic and local immune response were measured.

Vaccination reduced incidence and increased clearance of infection, and induced both systemic and local humoral immune responses. CD4⁺ cells were detected in vaginal tissues following intravaginal challenge with *T. vaginalis*, but were not seen in uninfected mice. CD4⁺ cells were detected more often, earlier, and in greater numbers in vaccinated vaginal tissues compared to unvaccinated controls. Presence of CD4⁺ T cells following infection can have significant implications of increasing HIV susceptibility and transmission.

These data suggest that the vaccine induces local and systemic immune responses, and confers significantly greater protection against vaginal challenge than unvaccinated vaginal challenge. These data support the potential for a human vaccine against *T. vaginalis* infection that could also impact the incidence of HIV infections.

Acknowledgements

I am indebted to the time and financial commitment provided by Dr. Gary Garber to support me through my research experience. He has provided the necessary encouragement to triumph obstacles and complete additional contributions to the body of scientific literature. The process has led to the positive development of research skills, writing skills and work ethics.

Sarah Cudmore and Shahed Ardalan had been integral to my learning and success for this project. To each of them I give thanks for enduring my array of questions and handling my peculiar personality.

The advice and guidance from Dr. Kathryn Wright, Dr. Paul MacPherson, and Dr. Lionel Filion was well received and helpful in many respects but not limited to how to present my data, understanding limitations of implications, and developing experiments.

Nicole Trudel, Fay Draper, Victoria Stewart, and Cathy Moreau were fantastic working their magic in the backgrounds to make sure that the administrative aspects of my education were under control. Easy, friendly answers to my questions along the way contributed to a low stress experience.

Lastly, I cannot leave out the support received from my family that allowed me to pursue my interest in science and research.

Table of Contents

Abstract	II
Acknowledgements	III
Table of Contents	IV
List of Abbreviations	VII
List of Figures	X
List of Tables	XII
Section 1. Introduction	1
1.1 Prevalence of <i>Trichomonas vaginalis</i>	1
1.2 Principle Features of <i>Trichomonas vaginalis</i>	1
1.3 <i>Trichomonas vaginalis</i> virus	2
1.4 Pathogenesis	3
1.4.1 Adherence	3
1.4.2 Modes of Cytotoxicity	5
1.4.3 Microflora and <i>Trichomonas vaginalis</i> Infection	6
1.4.4 Proteases	6
1.5 Iron Acquisition and Gene Regulation	8
1.6 Polyamine Metabolism	9
1.7 Immunoglobulins during Infection	9
1.8 Cytokines during Infection	11
1.9 Transmission of <i>Trichomonas vaginalis</i>	13
1.10 Trichomoniasis	14
1.10.1 Male Trichomoniasis	14

1.10.2 Female Trichomoniasis	15
1.10.3 Pregnancy and <i>Trichomonas vaginalis</i> Infection	15
1.10.4 HIV Transmission and Susceptibility	16
1.11 Diagnostic Testing	17
1.12 Treatment of Trichomoniasis	19
1.13 Animal Models of Vaccination	21
1.14 Aluminum Hydroxide Adjuvant	23
1.15 Eliciting Immunity to Vaginal Challenge	24
1.16 Experimental Rationale	25
1.17 Hypotheses	26
1.18 Objectives	26
Section 2. Materials and Methods	28
2.1 Media	28
2.2 Strains	28
2.3 Cell Preparation and Inoculation	29
2.4 Vaccine Preparation	30
2.5 Animals and Animal Care	31
2.6 Estrogen Treatment	31
2.7 Vaginal Washes	31
2.8 Collection of Vaginal Immunoglobulin	32
2.9 Blood Collection	33
2.10 Enzyme-Linked Immunosorbent Assay	33
2.11 Flow Cytometry	34

2.12 Immunohistochemistry	36
2.13 Statistical Analyses	38
Section 3. Results	40
3.1 Challenge and Infection Status	40
3.2 Serum and Vaginal Immunoglobulins	43
3.2.1 Serum IgG and IgG Subclasses	43
3.2.2 Vaginal IgG and IgA	44
3.3 CD4 ⁺ and CD8 ⁺ Splenocyte Proliferation	52
3.4 Vaginal Histology	69
3.4.1 CD4 Antibody	69
3.4.2 CD8 Antibody	70
3.4.3 CD19 Antibody	70
Section 4. Discussion	78
4.1 <i>Trichomonas vaginalis</i> Infection and Immunity	78
4.2 Serum Immunoglobulin and Vaginal Immunoglobulin	80
4.3 <i>Trichomonas vaginalis</i> Antigen Stimulation of CD4 ⁺ T cells and CD8 ⁺ T Cells	83
4.4 Lymphocytes in the Local Vaginal Tissue	85
4.5 Conclusions	88
References	91

List of Abbreviations

AP	Adhesion protein
AIDS	Acquired immunodeficiency syndrome
BSA	Bovine serum albumin
BspA	Boiling stable protein A
BV	Bacterial vaginosis
CD	Cluster of differentiation
CDF	Cell detaching factor
CFC	Cytokine flow cytometry
CFSE	Carboxyfluorescein succinimidyl ester
CP	Cysteine protease
DAB	3,3'-diaminobenzidine
DMSO	Dimethyl sulfoxide
DTP	Diphtheria, tetanus, and pertussis
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FDA	Food and Drug Administration
FIA	Freund's incomplete adjuvant
HBV	Hepatitis B virus
HIB	<i>Haemophilus influenzae</i> B

HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	<i>Herpes simplex virus</i>
ICAM-1	Intercellular adhesion molecule 1
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
Lf	Lactoferrin
LFA-1	Lymphocyte function-associated antigen 1
LG	Lipoglycan
LPG	Lipophosphoglycan
M17	Leucyl aminopeptidase family
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MIP3 α	Macrophage inflammatory protein 3 alpha
MLC	Minimal lethal concentration
MRS	Man, Rogosa, and Sharpes medium
NF- κ B	Nuclear factor kappa-light-chain enhancer of activated B cells
ODC	Ornithine decarboxylase
OPD	o-phenylenediamine
OVA	Ovalbumin
p38 MAPK	p38 mitogen-activated protein kinase
PBL	Peripheral blood lymphocyte

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline and 0.005% Tween 20
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
Pmp	Polymorphic membrane protein
RNI	Reactive nitrogen intermediates
SLPI	Secretory leukocyte protease inhibitor
STI	Sexually transmitted infection
TBS	Tris buffered saline
Tf	<i>Tritrichomonas foetus</i>
THP-1	Tamm-Horsfall protein 1
TLR	Toll-like receptor
TMA	Transcription mediated amplification
TNF- α	Tissue necrotic factor alpha
TYI	Trypticase, yeast extract, iron
Tv	<i>Trichomonas vaginalis</i>
TvFIM1	Tv Fimbrin 1
TvTSP6	Tv Tetraspanin 6
TVV	<i>Trichomonas vaginalis virus</i>
VEC	Vaginal epithelial cell
VSP	Variable surface protein
WHO	World Health Organization

List of Figures

Figure 1. Serologic IgG response on day -35 (3 weeks post-prime vaccination) of one experiment as measured by ELISA.	46
Figure 2. Serologic IgG response on day -7 (3 weeks post-boost vaccination) of one experiment as measured by ELISA.	47
Figure 3. Serologic IgG response on day 14 (2 weeks post-infection) of one experiment as measured by ELISA.	48
Figure 4. Serologic IgG response on day 28 (4 weeks post-infection) of one experiment as measured by ELISA.	49
Figure 5. Vaginal IgG response on days 1 (A), 3 (B), 7 (C), 14 (D), 21 (E), and 28 (F) (multiple time points post-infection) of one experiment as measured by ELISA.	50
Figure 6. Vaginal IgA response on days 1 (A), 3 (B), 7 (C), 14 (D), 21 (E), and 28 (F) (multiple time points post-infection) of one experiment as measured by ELISA.	51
Figure 7. Positive control flow cytometry data following ConA stimulation of splenocytes.	54
Figure 8. Negative control flow cytometry data following colchicine inhibition of splenocytes.	55
Figure 9. Sample of flow cytometry data following <i>T. vaginalis</i> antigen stimulation of splenocytes.	56
Figure 10. Proliferation of CD4 ⁺ T cells 1 day post-infection.	57
Figure 11. Proliferation of CD4 ⁺ T cells 3 days post-infection.	58

Figure 12. Proliferation of CD4 ⁺ T cells 7 days post-infection.	59
Figure 13. Proliferation of CD4 ⁺ T cells 14 days post-infection.	60
Figure 14. Proliferation of CD4 ⁺ T cells 21 days post-infection.	61
Figure 15. Proliferation of CD4 ⁺ T cells 28 days post-infection.	62
Figure 16. Proliferation of CD8 ⁺ T cells 1 day post-infection.	63
Figure 17. Proliferation of CD8 ⁺ T cells 3 days post-infection.	64
Figure 18. Proliferation of CD8 ⁺ T cells 7 days post-infection.	65
Figure 19. Proliferation of CD8 ⁺ T cells 14 days post-infection.	66
Figure 20. Proliferation of CD8 ⁺ T cells 21 days post-infection.	67
Figure 21. Proliferation of CD8 ⁺ T cells 28 days post-infection.	68
Figure 22. Immunohistochemical negative control tissues (primary antibody labelling omitted).	71
Figure 23. Immunohistochemical positive control tissues.	72
Figure 24. Positive immunoperoxidase staining with CD4 antibody for rare and few CD4 counts in murine vaginal tissue.	73
Figure 25. Positive immunoperoxidase staining with CD4 antibody for some CD4 count in murine vaginal tissue.	74
Figure 26. Immunoperoxidase staining was not seen when using CD8 antibodies in murine vaginal tissue day 1, 3, 7, 14, 21, and 28.	75
Figure 27. Immunoperoxidase staining was not seen when using CD19 antibodies (B cell) in murine vaginal tissue day 1, 3, 7, 14, 21, and 28.	76

List of Tables

Table 1. Overview of dates on which experimental procedures were performed.	39
Table 2. <i>Lactobacillus acidophilus</i> and <i>Trichomonas vaginalis</i> recovery rates detected by positive culture following vaginal wash.	42
Table 3. Detection of CD4 ⁺ cells in murine vaginal tissue sections following immunoperoxidase labelling of CD4 antigen.	77

1. Introduction

1.1 Prevalence of *Trichomonas vaginalis*

The most prevalent curable and non-viral sexually transmitted infection (STI) in the world is an under-recognized protozoan parasite, *Trichomonas vaginalis*. Global estimates of *T. vaginalis* incidence and prevalence are each greater than the combined incidence and prevalence estimates of other more commonly recognized STI including *Chlamydia trachomatis*, *Neisseria gonorrhoea*, and syphilis. Moreover, *T. vaginalis* has the third highest global prevalence next to *Herpes simplex virus* (HSV) and human papilloma virus (HPV) (WHO, 2012). Recent estimates from 2008 reports measured an incidence of 276 million and a prevalence of 187 million *T. vaginalis* infections, an increase of 11.5% and 22.2%, respectively from estimates of 2005 (WHO, 2011). This likely underestimates the actual incidence and prevalence of *T. vaginalis* since the infection is non-reportable, often asymptomatic, and usually is self-limiting in males.

It is necessary to identify and treat infections given the correlations of *T. vaginalis* infection with maternal and fetal health including premature membrane rupture, preterm labor, and low birth weight, increased human immunodeficiency virus (HIV) transmission and susceptibility, and other deleterious sequelae, such as pelvic inflammatory disease and cervical dysplasia. However, current standard detection methods are lacking and screening does not occur, which leads to spread of disease. Effective, safe vaccination would be an ideal intervention to control the increasing incidence of *T. vaginalis* infections.

1.2 Principle Features of *Trichomonas vaginalis*

Microscopy studies have described the surface and internal features of *T. vaginalis*

(Benchimol, 2004). The trophozoite form is pear-shaped when in culture with motility governed by four anterior flagella that induce a unique and distinctly recognizable twirling motion when viewed under a light microscope. A fifth flagellum runs from the anterior to the posterior portion of the trichomonad adhered to the surface forming an undulating membrane that is supported by a costa that underlies the undulating membrane. The pear shape is most pronounced by an axostyle originating from the base of the anterior flagella that rolls up to form a protruding point at the posterior of the trichomonad. The overall size is around 10 μm by 7 μm , roughly the size of a white blood cell, but may change depending on the environmental circumstance. These circumstances include contact with epithelial cells inducing an amoeboid form, and environmental stress which induces a pseudocyst form characterized by a rounded cell with internalized flagella, but no true cyst wall. The most notable internal structure of *T. vaginalis* is the hydrogenosome. This structure is the equivalent to the eukaryotic mitochondrion. However, hydrogenosome enzymes are highly reliant on iron and as a result *T. vaginalis* requires significantly higher levels of iron for survival than other microbial organisms.

1.3 *Trichomonas vaginalis* virus

Totiviridae family, double stranded RNA, icosahedral virus-like particles have been detected in various *T. vaginalis* isolates, but not all isolates (Goodman et al., 2011; Wang and Wang, 1986). These virus-like particles have been identified to be in close proximity with the Golgi complex and plasma membrane of *T. vaginalis*, and budding from the Golgi (Benchimol et al., 2002). The exact role or purpose of *Trichomonas vaginalis* virus (TVV) in *T. vaginalis* is not clear. Phenotypic variation of a highly immunogenic protein, P270, occurs

under iron dependent conditions of *T. vaginalis* infected with TVV but not without TVV (Alderete, 1999). Fluorescent antibody studies show greater surface P270 expression in low iron conditions. Under iron supplemented conditions a variable P270 surface expression pattern was reported that is similar to P270 surface expression on *T. vaginalis* isolates without TVV in both iron replete and iron deplete conditions (Alderete, 1999; Goodman et al., 2011). A role of TVV in pathogenesis is unclear (Goodman et al., 2011) and its modulation of surface immunogens could be a factor in development of a *T. vaginalis* vaccine with cross-isolate protection.

1.4 Pathogenesis

Our current knowledge of *T. vaginalis* pathogenesis is riddled with many unknowns. The study of pathogenesis has primarily used female experimental animal models or data obtained from human vaginal infections. Infection in males has received less attention due to the difficulty of studying *T. vaginalis* pathogenesis in the male urinary tract compared to vaginal infection. Additional knowledge of *T. vaginalis* pathogenesis could aid in selection of antigens for subunit vaccines.

1.4.1 Adherence

T. vaginalis can adhere to vaginal epithelial cells (VEC). Cytoadherence is controlled by a wide range of proteins, some of which have additional functions beyond cytoadherence. A list of identified cytoadherence proteins include adhesion proteins (AP) 23, 33, 51, 65-1 and 120, BspA-like, M17-like, VSP-like, Pmp, TvLG, and various surface and secreted cysteine proteases (TvCP30, TvCP62, and TvCP65) (Figuerola-Angulo et al., 2012). AP are

upregulated upon binding to VEC and is in association with the trichomonad assuming an amoeboid shape with interdigitation of filopodia and pseudopodia at discrete sites directed towards the epithelial cell (Arroyo et al., 1993). These protrusions may be related to the only fimbrin family protein identified in *T. vaginalis*, TvFIM1. TvFIM1 associates with fibrous actin bundles and increases actin polymerization. This occurs at protrusions that are generally at the leading edge at the apical tip containing the flagella and contributes to movement across cell surfaces (Kusdian et al., 2013). Recently published data describes a tetraspanin family membrane protein TvTSP6 that is potentially involved in migration through extracellular matrix and movement across epithelial cells. TvTSP6 is located on flagella, but redistributes to the plasma membrane on the side of contact of *T. vaginalis* to epithelial cells. Furthermore, it plays a role in adherence (de Miguel et al., 2012). TvFIM1 and TvTSP6 help *T. vaginalis* move through the mucosal barrier and allow movement across the vaginal epithelia. Cytoskeletal factors are not the sole mechanism of overcoming the extracellular matrix. Secreted proteases with mucinase functionality have been identified (Lehker and Sweeney, 1999). Other genes with unknown function or those that are involved with transcription and translation are upregulated during adherence (Kucknoor et al., 2005).

Aside from vaginal epithelial cell adherence, *T. vaginalis* is capable of adherence to fibroblasts and muscle cells in deeper tissues of the vagina as well as binding to erythrocytes and bacteria (Alderete et al., 2001; Vilela and Benchimol, 2012). Binding to cells may be a precursor to phagocytic activity and is mediated by various proteins, sugars and lipoglycans (Vilela and Benchimol, 2011). Many cell types and organisms have been identified to be phagocytized by *T. vaginalis* including lactobacilli, vaginal epithelial cells, leukocytes, erythrocytes, and *Neisseria gonorrhoea* (Francioli et al., 1983; Rendón-Maldonado et al.,

1998). *T. vaginalis* has been shown under microscope to tear cells. This is likely a mechanism for the destruction of cells too large to completely phagocytize (Midlej and Benchimol, 2010). Phagocytosis and destruction of cells can function to acquire nutrients or access deeper layers of the vaginal epithelium.

1.4.2 Modes of Cytotoxicity

Targeting adherence proteins as a subunit vaccine could help prevent attachment and contact mediated pathogenesis but not contact independent pathogenesis. Tissue damage is host cell specific and mediated by both contact independent and contact dependent mechanisms. *T. vaginalis* is capable of damaging human but not bovine vaginal epithelial cells. Furthermore, although fibroblast adherence has been identified, cell specific damage was not observed to this cell type (Gilbert et al., 2000). In vitro damage to epithelial monolayers has been demonstrated under contact dependent conditions. A greater adherence to vaginal epithelial cell monolayers was associated with greater monolayer detachment. Although higher virulence in mouse experimental models correlated to increased adherence indices, in vitro cytotoxicity did not correlate with experimental pathology in vivo (Escario et al., 1995). Cell detaching factor (CDF) is a very large secreted factor detected in *T. vaginalis* supernatants that contributes to virulence and correlates with clinical presentation. CDF is active under pH conditions greater than 5 and production of CDF is decreased by estrogen, but does not affect the viability of trichomonads. This data suggests the secreted factor CDF is a component of contact independent cytotoxic mechanisms employed by *T. vaginalis* (Garber et al., 1991).

1.4.3 Microflora and *Trichomonas vaginalis* Infection

Microflora in the vagina is affected by *T. vaginalis* infections. Typically, an increase in vaginal pH to greater than 5 occurs following infection. This may suggest a decrease in lactobacillus colonies (Lehker and Alderete, 2000). *Lactobacillus acidophilus* is a normal commensal bacteria of the vagina in the majority of women (Ravel et al., 2011). The number of lactobacilli are notably low in women with *T. vaginalis* infections (Brotman et al., 2012). Comprehensive studies examining microflora before and after infection are required to determine interaction between *T. vaginalis* and vaginal bacteria. Still, disturbance of lactobacilli and other microflora may be an indication of the presence of *T. vaginalis* destroying and digesting vaginal microflora in addition to host cells for nutrient acquisition. In fact, some *Lactobacillus spp.* have been shown to decrease adherence of various *T. vaginalis* isolates conferring a protective role (Phukan et al., 2013). Moreover, bacterial vaginosis (BV) related species of bacteria are unaffected by *T. vaginalis* infection. Data has been shown that BV spp. such as *Atopobium vaginae* and *Gardnerella vaginalis* with *T. vaginalis* contribute to amplify chemokine responses, induce inflammation, increase recruitment of CD4 cells, and promote viral co-infections (Fichorova et al., 2013).

1.4.4 Proteases

T. vaginalis derived proteases are important for its survival and pathogenesis. Proteases can be surface enzymes or secreted factors (Alderete et al., 2001; Lockwood et al., 1987). Proteases and cysteine proteases (CP) are variably regulated by iron levels. Notably, an increased production of CP39 and CP65 under low iron conditions correlates with increased host cell apoptosis (Alvarez-Sánchez et al., 2007; Kummer et al., 2008; Sehgal et

al., 2012). On the other hand, conditions of high iron can also upregulate other CP and AP and increase cytotoxicity and adherence (Arroyo and Alderete, 1989; Hernández et al., 2011). An explanation for this dynamic is that under low iron conditions the organism must express fewer AP to ensure motility across the epithelium in a search for iron sources prior to menstruation, the period of lowest lactoferrin availability (Sehgal et al., 2012). This is supported by data that CP39 found in patient vaginal washes can play a role in acquiring nutrients by degrading collagens I, III, IV and V, human fibronectin, human hemoglobin, and human immunoglobulins (Ig) A and G (Hernández-Gutiérrez et al., 2004).

Proteases can be regulated and protect against host defenses. During menstruation iron is available in abundance from erythrocytes and lactoferrin. Also, menstrual blood contains complement proteins that are capable of lytic activity against *T. vaginalis* when added to culture under in vitro conditions (Alderete et al., 1995). Protection against in vitro complement mediated lysis was conferred by supplementation of menstrual fluids with lactoferrin. Since lactoferrin is added as an iron source, these results suggest iron availability is an important regulator of *T. vaginalis* proteases that cleave a required element of the alternative complement pathway, C3 protein (Alderete et al., 1995). Moreover, treatment of *T. vaginalis* lysates and supernatants with CP inhibitors decreases Ig degradation. The proteolytic activity on Ig is pronounced in patients infected with *T. vaginalis* versus uninfected patients (Provenzano and Alderete, 1995). Proteases may have multiple roles of adherence, cytotoxicity, or immune evasion, however some proteases such as TvGP63 are solely involved in cytotoxicity (Ma et al., 2011). Multifunctional roles of proteins are common in *T. vaginalis*.

1.5 Iron Acquisition and Gene Regulation

Iron acquisition is absolutely necessary for *T. vaginalis* survival. Iron plays a role in energy production as well as gene regulation. Notably, normal or increased iron availability is correlated with increased *T. vaginalis* pathology and HeLa cell adherence compared to trichomonads grown in iron deficient media (Ryu et al, 2001). Three hundred and thirty-six iron regulated genes were identified from a transcriptomic approach, about half were upregulated under iron rich conditions and half were upregulated under iron depleted conditions (Horváthová et al., 2012). Regulation of protein expression under low iron condition results in longer generation times, lower cytoadherence, lower protein synthesis, and increased production of a 136 kDa lactoferrin-binding receptor protein (Garcia et al., 2003; Lehker and Alderete, 1992; Lehker et al., 1991).

Ferric iron is chelated by extracellular glycoproteins of the human host immune system. This limits the direct availability of free iron (Sehgal et al., 2012). The two major sources of iron are erythrocytes and lactoferrin. Since the vaginal environment may be rich with erythrocytes and lactoferrin, but at other times depleted, *T. vaginalis* has evolved mechanisms to acquire iron from both sources.

Iron acquisition from erythrocytes is both mediated by contact-dependent phagocytosis, and secreted factors that lyse erythrocytes. Phagocytosis of erythrocytes provides iron and lipids (Lehker et al, 1990). Secreted factors were able to lyse erythrocytes in a contact independent mechanism and under high pH conditions, where the hemolytic activity peaked at a pH of 5.8 (Fiori et al., 1996). AP51 and AP65 may function dually as adhesion molecules and as heme- and hemoglobin-binding proteins (Ardalan et al., 2009).

Receptor-mediated uptake of lactoferrin is also a major source of iron acquisition

(Hirt et al., 2011). Like erythrocytes, the amount of lactoferrin fluctuates during the menstrual cycle (Sehgal et al., 2012). Holo-lactoferrin (holo-Lf) is iron saturated lactoferrin. Three proteins have been reported as lactoferrin-binding proteins. These are a 136 kDa lactoferrin-binding receptor protein (Lehker and Alderete, 1992), and 178 kDa and 75 kDa surface proteins that bind only the holo-Lf form of lactoferrin, not apo-Lf (iron free lactoferrin) or holo-transferrin (another iron carrier protein) (Alderete et al., 1995; Sehgal et al., 2012). The number of lactoferrin receptors increases by 2.5 fold and binding of holo-Lf increases 1.6 fold under iron depleted conditions.

1.6 Polyamine Metabolism

Polyamine metabolism by ornithine decarboxylase (ODC) is important for cytotoxicity. Inhibition of ODC results in a lack of putrescine synthesis and secretion. The inhibition is linked to growth arrest of trichomonads, increased cell adherence and decreased cytotoxicity. The latter two conditions are contradictory to previous studies wherein increased adherence was associated with greater cytotoxicity, an interesting nuance of polyamine metabolism and virulence in *T. vaginalis* (Escario et al., 1995).

1.7 Immunoglobulins during Infection

Studies regarding the levels of Ig have been conducted in experimental and human models. Kaur and colleagues (2008) investigated serum and vaginal Ig levels of 6 patients with symptomatic and 4 patients with asymptomatic intravaginal trichomoniasis. Serum IgG, IgM and IgA were detected in 66%, 50%, and 100% of symptomatic patients, and 50%, 0%, and 50% of asymptomatic patients. Serum IgM was detected only in symptomatic patients.

IgG subclasses were evaluated as well. IgG₁₋₄ were detected in 33.3%, 50%, 66%, and 16.6% of symptomatic cases, and only IgG₃ was detected in 25% of asymptomatic cases. Vaginal washes contained IgG and IgA in both symptomatic (100% and 66%) and asymptomatic (25% and 25%), but no IgM in either patient groups. From the IgG subclasses in vaginal washes all symptomatic patients had detectable IgG₁, but no asymptomatic patients had detectable IgG₁. IgG₂ and IgG₃ were detected in some of symptomatic and asymptomatic while no IgG₄ was detected in either. Although percent of Ig detection varied, the ELISA absorbance values were not significantly different. However, a significant difference in the detection of vaginal IgG₁ of symptomatic cases may suggest a role for vaginal IgG₁ in establishing symptoms (Kaur et al., 2008). Speculatively, the presence of serum IgM could suggest that phenotypic variance may occur in the change from asymptomatic to symptomatic infection leading to the detection of newly expressed, highly immunogenic antigens and thus production of early response IgM in serum.

Sharma and colleagues (1991) treated patients with asymptomatic and symptomatic *T. vaginalis* infections while measuring the change in serum and vaginal IgA. A decrease in both serum and vaginal IgA following treatment was noted. Adaptive response to infection is capable of mounting a specific humoral immune response, but it is ineffective at clearing infections and may play a role in symptomatology (Sharma et al., 1991). Nevertheless, it is known that reinfection following metronidazole treatment of human trichomoniasis is not prevented by the prior infection.

Mice challenged with clinical isolates of *T. vaginalis* from symptomatic and asymptomatic patients demonstrated greater Ig response from experimental infection with symptomatic isolates. Additionally, symptomatic isolates established greater parasite loads

in the vagina of infected mice (Yadav et al., 2005). Data from a different group reported the opposite that asymptomatic isolates induced higher Ig than symptomatic isolates (Paintlia et al., 2002). At this point the role of Ig following infections in unvaccinated mice in abating symptoms or infection is still unclear. However, a vaccination study in mice found detectable vaginal IgA, no serum IgA, serum IgG and significantly higher vaginal IgG compared to controls that had no detectable serum IgG or IgA, vaginal IgA and very low vaginal IgG (Abraham et al., 1996). These studies are limited as cell mediated immunity was not assessed by Abraham, and clearance was not assessed by Yadav and Paintlia.

1.8 Cytokines during Infection

The milieu of cytokines and cellular infiltration can help us understand the natural response to *T. vaginalis* infection and the mechanisms employed to evade the immune response. A glycoconjugate lipoglycan (LG) is the predominant lipid-anchored molecule on *T. vaginalis*. Human galectin-1 has been found to be a receptor for *T. vaginalis* LG (TvLG) (Okumura et al., 2008). TvLG plays a role in adherence, but more importantly is capable of proinflammatory activation of epithelial cells from endocervical, ectocervical, and vaginal epithelial cell origin (Singh et al., 2009). Specifically the ceramide phospho-inositol core induced IL-8, MIP3 α , low IL-6, NF- κ B and activated ERK1/2 signaling. Lipophosphoglycan (LPG) from the related bovine trichomonad, *Tritrichomonas foetus*, did not stimulate a response (Singh et al., 2009). Combined, these cytokines and activated signaling intermediates result in attraction of neutrophils and macrophages, indicative of acute and chronic inflammation, and lymphocytes. Recruitment of neutrophils results in more IL-8 production that is induced by stimulation of the infiltrating neutrophils with *T. vaginalis*

secretory products. These secretory products also induce IL-8 production from mast cells (Nam et al., 2011a; Nam et al., 2011b). Zariffard and colleagues report TNF- α is produced through TLR4 responsive cells treated with genital tract secretions of women with trichomoniasis significantly more than women without vaginal infection (350 pg/mL versus 44 pg/mL) (Zariffard et al., 2004). From this data it is evident that the innate immune response is initiated by *T. vaginalis* infection. IL-12 and TNF- α are, however, inhibited over time during infection as the levels of these cytokines diminish through a mechanism of NF- κ B inhibition by *T. vaginalis* (Chang et al., 2004). A recent study of cytokines from cervicovaginal lavage found higher levels of IL-22 and IL-17 in patients with trichomoniasis compared to uninfected controls (Makinde et al., 2013). IL-22 functions to induce antimicrobial peptides and maintains epithelium. IL-17 increases inflammatory response, recruits innate cells, synergistically functions with TNF- α , and is known for its role in delayed type hypersensitivities, a cell mediated response.

The recruitment of neutrophils and macrophages is subverted by induction of apoptosis. Neutrophils undergo a reactive oxygen species-dependent caspase-3 activation (Song et al., 2008) and macrophages undergo a Bcl-x_L-dependent apoptotic pathway reliant on p38 MAPK signaling cascade (Chang et al., 2006). Reactive nitrogen intermediates (RNI) produced by macrophages are greater in vaginal washes of asymptomatic infections compared to symptomatic infections. Furthermore, higher RNI correlates with lower parasitic load (Malla et al., 2004). Symptomatic isolates have higher parasitic loads which may contribute to the ability to reduce RNI released by macrophages through increased apoptosis better than asymptomatic *T. vaginalis* isolates with lower parasitic loads (Malla et al., 2004).

IL-2 and IFN- γ , a Th1 type response, were higher in asymptomatic isolate-infected mice versus symptomatic isolate-infected mice (Malla et al., 2007; Paintlia et al., 2002). A better understanding of the role of the cellular arm of immunity in *T. vaginalis* infection is required.

Biopsy of human endometrium and cervical smears from patients infected with *T. vaginalis* show significant influx of plasma cells, B cells, and CD3 T cells versus healthy controls (Kiviat et al., 1985; Reighard et al., 2011). Adaptive immune responses are present during infection yet do not provide protection (Petrin et al., 1998).

Overall, *T. vaginalis* is capable of subverting innate and adaptive mechanisms during natural infection. Yet, vaccinated experimental infection models are capable of mounting adaptive immune responses and have significant clearance of infection and reduced duration of infection compared to their unvaccinated counterparts (Abraham et al., 1996). So despite the highly adapted capability of *T. vaginalis* to thrive in the vaginal environment it is still feasible to attain protection against infection through vaccination. With the variety of proteins identified involved in pathogenesis there is a wide variety of potential antigens available for subunit vaccines.

1.9 Transmission of *Trichomonas vaginalis*

Transmission is almost exclusively by sexual contact. From a screening of young women in Ndola, Zambia, *T. vaginalis* infections in virgins were found that may be related to bathing habits or other cultural behaviours (Crucitti et al., 2011). Case reports in literature of transmission to children during birth are rare likely because *T. vaginalis* is not reportable, but vertical transmission is a known risk and can manifest as respiratory, nasopharyngeal

and gut infections (Al-Salihi et al., 1974; Bruins et al., 2013; Smith et al., 2002). Virgin and peri-natal populations are low risk. Individuals at a higher risk of *T. vaginalis* infection include female sex workers, injection drug users, are of black descent, having history of multiple STI, are less educated, are impoverished, or have multiple sex partners (Hollman et al., 2010; Shuter et al., 1998; Sutton et al., 2007).

1.10 Trichomoniasis

1.10.1 Male Trichomoniasis

Men are carriers of *T. vaginalis* since the clinical spectrum of trichomoniasis in males is often asymptomatic and usually self-limited (Krieger, 1995). However, this data needs to be repeated using more sensitive diagnostic techniques. Reports of asymptomatic *T. vaginalis* infection in men range from 14-77.3%, however, over 50% of *T. vaginalis* infections in males is an accepted average of the true number of asymptomatic infections (Hobbs et al., 2006; Krieger, 1995; Krieger et al., 1993; Schwebke and Hook, 2003; Seña et al., 2007). About 60% of asymptomatic infections will become symptomatic within 10 days, while 60% of all infections will be self-resolving within 2 weeks. It is rare to have an infection extend beyond 1-3 months without symptoms. Symptoms of trichomoniasis in men is similar to other genital tract infection and include discharge, dysuria, frequent micturition, urethral irritation, urethral pain, lower abdominal pain, balanoposthitis, prostatitis, pruritis and epididymitis (Krieger, 1995; Krieger et al., 1993; Weston and Nicol, 1963). Benign hyperplastic prostate tissue growth has been associated to be sustained with *T. vaginalis* infection through induction of inflammatory cytokines discussed previously (Mitteregger et al., 2012). Infertility is also a concern for chronic infections in men (Fichorova, 2009).

1.10.2 Female Trichomoniasis

T. vaginalis infection is chronic in women. Prevalence of infection is 10 times higher in women than men, and infections are frequently asymptomatic, though not as often as asymptomatic infections in men (WHO, 2012). Following a variable incubation period, the women who are symptomatic present similarly to other STI leading to presuppositions of diagnosis often without adequate testing. Yellow, malodorous discharge, genital itching and erythema, colpitis macularis, endometritis, vaginitis, and high vaginal pH are typical symptoms. Sequelae also include infertility, cervical cancer, pelvic inflammatory disease, implications on fetal health, and increased transmission and susceptibility of HIV (Hesseltine et al., 1942; Fichorova, 2009; Moodley et al., 2002; Pastorek II et al., 1996; Wølner-Hanssen et al., 1989; Zhang et al., 1995).

1.10.3 Pregnancy and *Trichomonas vaginalis* Infection

Pregnant women are at risk of preterm birth, premature membrane rupture, and delivery of low birth weight babies (Cotch et al., 1997; Fichorova, 2009). The factors contributing to negative fetal health outcomes are not known. A serine protease inhibitor, secretory leukocyte protease inhibitor (SLPI), is significantly decreased in women with *T. vaginalis* infection. SLPI, normally present on mucosal surfaces, can inhibit inflammatory response proteases. SLPI is cleaved to a non-functional protein during *T. vaginalis* infection. The decrease of SLPI is associated with weakened human amnion and chorion. SLPI cleavage is elicited from cultures of *T. vaginalis* as well as cell free culture filtrates, is *T. vaginalis* load-dependent, and is isolate dependent (Draper et al., 1995a; Draper et al.,

1995b; Draper et al., 1998; Huppert et al., 2013). Treatment of *T. vaginalis* infections in pregnant women may not be advisable in light of recent findings wherein treatment has not reduced probability of preterm labor, but instead may increase preterm labor (Johnston and Mabey, 2008; Klebanoff et al., 2001). Further studies are needed to reaffirm this relationship using a standard metronidazole treatment regime. Nevertheless, causation of increased preterm labor is speculated to be triggered by inflammatory responses in response to detection of antigens from dying *T. vaginalis* and potential *T. vaginalis* virus harbored within *T. vaginalis* (Klebanoff et al., 2001). Studies are required to confirm these speculations. Diagnosis and treatment before or early in pregnancy may be advisable.

1.10.4 HIV Transmission and Susceptibility

HIV transmission and susceptibility for both men and women infected with *T. vaginalis* is a major concern. HIV infection increases susceptibility to *T. vaginalis* infection (odds ratio 2.12) (Mavedzenge et al., 2010). *T. vaginalis* and HIV coinfections are associated (odds ratio 1.22 in men, 1.31 in women) (Mhlongo et al., 2010). The relationship between these STI highlights issues in populations such as Africa where treatment is inaccessible or unaffordable, thus propagating infection at a greater rate. Odds ratio of acquiring HIV when infected with *T. vaginalis* ranges from 1.52 and 2.74 (Mavedzenge et al., 2010; McClelland et al., 2007; Van Der Pol et al., 2008). Based on a 1.8 odds ratio of acquiring HIV, a model of assessing coinfections in the United States attributed 2% of all HIV infections to *T. vaginalis* infection (Quinlivan et al., 2012).

Reasons for HIV acquisition include damage to epithelial layer due to inflammation, increased monocytic cell susceptibility to HIV, and higher probability of bacterial vaginosis

or colonization of abnormal flora (Draper et al., 1998; Guenthner et al., 2005; Quinlivan et al., 2012; Thurman and Doncel, 2011). Increased transmission of HIV is likely due to higher viral load shedding into the genital tract or genital ulcers (Paz-Bailey et al., 2010). Higher viral load was a predictor of *T. vaginalis* infection, and treatment of *T. vaginalis* decreased HIV RNA detected in vaginal secretions and decreased frequency of shedding (Anderson et al., 2012; Kissinger et al., 2009; Muzny and Schwebke, 2013; Wang et al., 2001). Lower CD4 counts (40-140 and 150-250 cells/mL) were associated with *T. vaginalis* infection (Nweze and Mouneke, 2011). Finally, higher rates of *T. vaginalis* and HIV coinfection were found in pregnant women compared to women who are not pregnant ($p < 0.05$), an alarming finding considering vertical transmission of HIV (de Lemos and Garcia-Zapata, 2010).

1.11 Diagnostic Testing

Standard diagnostic testing includes microscopic wet mount analysis of a vaginal sample or culture. The most sensitive method of detection is through the use of transcription mediated amplification (TMA) or PCR. A comparison of these four diagnostic methods was conducted by Nye and colleagues (2009). *T. vaginalis* was detected in 16.2-28.7% of vaginal swabs. PCR was positive more than 1.75 times compared to wet mount. Sensitivity of wet mount, culture, PCR, and TMA were 54.6%, 75%, 83%, and 96.6%. PCR and TMA of urine samples had sensitivities of 76.1 and 87.5%. Urine samples are typically not collected due to the low number and viability of trichomonads in the sample which makes wet mount identification or culture growth difficult. However, PCR and TMA are effective tools when urine samples are obtained. In men, detection ranged from 4.0% to 13.4%. Only one third of the infections were detected by culture. Urethral swabs for detection of *T. vaginalis* returned

sensitivities of 28.6%, 54.8%, and 95.2% for culture, PCR, and TMA, respectively. Again, urine samples were analyzed by PCR and TMA with 47.6% and 73.8% sensitivity (Nye et al., 2009). Wet mount is not applicable for men due to low recovery of trichomonads from any sampling method (Brill, 2010). Wet mount, culture, PCR and TMA sensitivity from the Nye et al (2009) study is consistent with other data, though PCR sensitivity is variable depending on the specific primers used (Crucitti et al., 2003; Freeman et al., 2010; Huppert et al., 2007; Patil et al., 2012; Paul et al., 2012; Roth et al., 2011; Van Der Pol et al., 2006; Wendel et al., 2002). This data emphasizes the need to establish better diagnostic standards. Furthermore, due to the common asymptomatic harboring of the infection syndromic approaches to ordering diagnostic tests can result in missing three quarters of infections and should be avoided (Garcia et al., 2012; Yin et al., 2008).

Better diagnostic standards would entail a fast and inexpensive method of screening patients visiting a STI clinic. The major barrier to overcome at this time is the cost of PCR or TMA and the resources or skills required are not readily available. Wet mount involves simple microscopy. Culture involves simple media inoculation. Low sensitivity hinders wet mount diagnostics while culture requires up to a week to determine a positive result. The sensitivity of culture is acceptable under low resource circumstances, but the time delay between testing and diagnosis can result in lack and delay of treatment that allows disease transmission. New PCR and TMA tools have both recently received FDA approval for use in clinical settings. Both PCR and TMA are very rapid, point of care diagnostics. The OSOM Trichomonas Rapid Test (Genzyme Diagnostics, Cambridge, Massachusetts) is a TMA immunochromatographic test strip that can be used for self-obtained sample testing with sensitivity greater than 83% and greater than 97% specificity (Jones et al., 2013; Workowski

et al., 2010). The OSOM Trichomonas Rapid Test cannot be used for testing males. Unfortunately, the test is too expensive for application in low resource settings. These settings, such as Africa, are typically the areas of need for better diagnostic techniques as 20% of global incidence and 20% of global prevalence of *T. vaginalis* infection resides (WHO, 2012) in this continent alone. Cost of testing and treatment is yet another reason to produce an affordable vaccine.

1.12 Treatment of Trichomoniasis

Standard treatment of trichomoniasis is a 2 g single orally administered dose of metronidazole or tinidazole. Alternatively, metronidazole may be prescribed 500 mg twice daily, orally for 7 days. These compounds are nitroimidazole derivatives. Their mode of action is passive diffusion into the trichomonad and localization in the hydrogenosome. Enzymes of the hydrogenosome reduce the prodrug releasing nitro radicals. Typically nitro radicals cause damage to chromosomes in anucleated organisms. *T. vaginalis* has a nucleus and instead the damage is likely caused to proteins and protein trafficking (Dunne et al., 2003). Unfortunately metronidazole resistance has been detected as early as 1959. In 2001 and 2006, up to 5-10% of *T. vaginalis* isolates were suspected to have some level of metronidazole resistance (Schwebke and Barrientes, 2006; Upcroft and Upcroft, 2001). Resistance rates will only continue to increase and be more prevalent. A study from 2012 tested *T. vaginalis* isolates from six US cities and detected low level metronidazole resistance (MLC 50-100 ug/mL) in 4.3% of isolates (Kirkcaldy et al., 2012). Whether or not symptomatic infections or asymptomatic infections are correlated with metronidazole resistance has not been studied. It would be interesting to know whether or not asymptomatic

isolates are more likely to be metronidazole resistant than symptomatic isolates. If asymptomatic isolates were more likely to be metronidazole resistant we would see an increase in metronidazole resistant infections due to a lack of treatment of asymptomatic infections that stems from no screening procedures for *T. vaginalis* infections. A modification of the hydrogenosomal enzyme pyruvate:ferredoxin oxidoreductase involved in reduction of metronidazole is suspected to contribute to resistance (Dunne et al., 2003). Alternative treatment options have limited data to suggest effective replacement of nitroimidazoles (Muzny and Schwebke, 2013). However, modification of the expression of other proteins could also contribute to metronidazole resistance (Leitsch et al., 2012).

Reinfections are common up to one year following treatment and frequently are asymptomatic (Johnston and Mabey, 2008). Reinfection is common when partners are not screened following diagnosis of *T. vaginalis*. A study of male partners of *T. vaginalis* infected women detected 73.2% of men to be infected (Hobbs et al., 2006). Without partner treatment these women are at high risk to be reinfected which can present like a treatment failure. Also, treatment success should be assessed following treatment. Despite adherence to the drug treatment regime and despite no sexual contact during or after treatment, cases of *T. vaginalis* infections have been speculated on rare occasion to fall to subclinical levels, but remain present after completion of treatment (Gatski and Kissinger, 2010; Peterman et al., 2009). Treatment failure, inability to obtain diagnosis and/or treatment, non-adherence to treatment, lack of partner management, and high risk behaviours warrant the need for alternative interventions to *T. vaginalis* infections. Vaccination of at risk populations is a viable goal, which would circumvent the problems listed previously.

1.13 Animal Models of Vaccination

Experimental vaccination of cattle against the related bovine infection

Trichomonas foetus was a successful example of a viable trichomonad vaccination. Both methods of whole cell *T. foetus* and subunit antigen TF1.17 as a subcutaneous prime and boost vaccination provided significant reduction of duration of infections in heifers (Corbeil et al., 2001; Kvasnicka et al., 1989). The duration of infection was two weeks shorter and clearance correlated with peak levels of vaginal IgG and IgA. Intranasal immunization of heifers did not confer significant protection compared to unvaccinated controls. Intranasal immunization differed from subcutaneous vaccination by the presence of intravaginal IgG₁. This data can be taken to be evidence of the significance of a humoral immune response in clearance of *T. foetus* and conceptually is relevant to the initial design of vaccination against *T. vaginalis*. Additionally, IgE response may be important in facilitating systemic IgG transport across the genital epithelium (Corbeil et al., 2005). Typically, damage and symptoms from *T. foetus* infection occur at 7-9 weeks post-infection. Clearance following vaccination in most cases occurred prior to 6 weeks of infection (Corbeil et al., 2001). *T. foetus* vaccination studies were motivated by the significant financial impact of loss of calves due to spontaneous abortion of fetuses carried by heifers in the cattle industry (Corbeil, 1994). Public interest to develop vaccines for human *T. vaginalis* infections is limited despite the serious impact on maternal and fetal health as well as the transmissibility of HIV.

A bovine model of *T. vaginalis* infection is costly, burdensome and prohibitive as a disease model. However, it is testament to the success of trichomonal vaccines. A mouse model of experimental intravaginal infection exists as a cheap alternative that uses hormonal

and microflora modifications to more appropriately mimic the human vaginal environment. Female BALB/c mice have been used previously in experimental infection experiments to investigate immune responses (previously discussed), and vaccines against *T. vaginalis*. The current mouse model employs a prime-boost subcutaneous vaccination schedule followed by intravaginal inoculation of *Lactobacillus acidophilus* and estrogenization (Abraham et al., 1996). Microflora adjustment results in a decreased vaginal pH from the production of lactic acid, but also plays a role of duration of infection in mice (McGrory and Garber, 1992; Meysick and Garber, 1992). Estrogenization synchronizes mice to estrus, and significantly increases initial infection rates of mice, although, estrogen affects the production of immunoglobulins (Cappuccinelli et al., 1974; Meysick and Garber, 1992). Estrus and menstrual cycle variation of intravaginal conditions may be necessary to consider when testing vaccine efficacy of experimental intravaginal infection (Corbeil et al., 1985).

The current vaccine model uses an adjuvant that is toxic if used in humans and therefore forbidden for use in humans. Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) are potent immunostimulators of the Th1 and Th2 arms of adaptive immunity. Freund's adjuvant has served as the gold standard for experimental animal immunization. In the *T. vaginalis* vaccination experiments of Abraham and colleagues (1996), a vaccine consisting of a subcutaneous FCA prime and FIA boost vaccination 4 weeks apart with infection 4 weeks following boost infection (see Table 1), there was a significant decrease of initial *T. vaginalis* infection and significant decrease of duration of *T. vaginalis* infection in vaccinated mice compared to controls. Furthermore, higher serum IgG, and higher vaginal IgG and IgA were detected in vaccinated mice versus controls (Abraham et al., 1996). This experiment defined the ideal infectious inoculation

dose of *T. vaginalis*, demonstrated natural infection does not confer natural immunity, and most importantly demonstrated successful prevention and clearance of infection following vaccination.

1.14 Aluminum Hydroxide Adjuvant

An adjuvant safe for use in humans needs to be tested. Aluminum hydroxide is an ideal adjuvant to be tested. Aluminum hydroxide is a FDA approved adjuvant currently employed in various vaccines such as hepatitis A/B, DTP, HIB, HPV and pneumococcus. Over eighty years have passed since the discovery of aluminum hydroxide as an adjuvant. Although the adjuvant has been in long term usage, only recently a mode of action has been proposed.

Flach and colleagues (2011) defined an interaction of aluminum salts (alum) that is specific to dendritic cells (DC). An alum tipped cantilever was measured for attractive forces towards DC2.4 and THP-1 dendritic cells. Lipid sorting ensued on the surface of DC in addition to attractive forces exerted on the cantilever. The lipid sorting resulted in activation of Syk and PI3K signal cascades and endocytic delivery of antigen adhered to aluminum crystals without phagocytosis of the aluminum crystals themselves. No antigen transfer mechanisms are suspected as MHCI cross-presentation is absent and only MHCII presentation follows. Binding of DC to CD4⁺ T cells was significantly increased in alum stimulated DC versus controls. Increased binding is mediated by upregulated ICAM-1 and LFA-1 binding. The stronger binding elicits a more robust activation of CD4⁺ T cells in contact with primed DC (Flach et al., 2011). IL-4 plays a major role for the activation of CD4⁺ T cells and production of Th2 antibodies because IL-4 deficient mice vaccinated with

OVA adsorbed to alum produced an antibody profile similar to Th1 adjuvant FCA/FIA which included an increased IgG_{2a} response (Brewer et al., 1996). The data above disputes previously proposed mechanisms involving a depot effect and role of inflammasomes for adjuvanticity (Franchi and Núñez, 2008; Kool et al., 2008; Marrack et al., 2009).

1.15 Eliciting Immunity to Vaginal Challenge

Vaccination to elicit protection in the vagina has been an ongoing challenge. The mucosal associated lymphoid tissue of the intestines differs from the vaginal mucosal immune system. Primarily the difference is the lack of distinct, organized lymphoid tissues (Neutra et al., 1996). Lymphoid aggregates detected in human uterine endometrium consists of a B cell core, surrounded by CD8⁺ T cells, and additional monocytes and macrophages on the outermost ring. There is an inherent lack of CD4⁺ T cells in these aggregates. CD4⁺ T cells are, however, distributed in other areas of the vaginal epithelium (Johansson et al., 1999). Aggregates were not detected in postmenopausal women (Huston et al., 2012). Hormonal changes also affect the composition of localized immune cells and their function, thus affecting the response elicited to invading organisms including antibody production and antigen presentation. Vaginal immunization is affected by the stage of menstrual cycle while intranasal immunization is not. However, intranasal is weak at stimulating cell mediated immunity. Parenteral and intramuscular vaccination have been successful at inducing Ig and cell mediated immunity (Huston et al., 2012). For this study, a subcutaneous injection is ideal for its ease of administration and has been effective with Freund's adjuvant (Abraham et al., 1996).

Exploitation of the common mucosal immune system has been employed in a

number of vaccination studies which measured the levels of local and systemic antibodies induced through various routes of immunization including vaginal, rectal, intranasal, parenteral and subcutaneous. Induction by non-vaginal and non-rectal routes were successful in inducing significant systemic Ig. Cervical and vaginal secretions of Ig were produced via all routes of immunization with the exception of rectal immunization. (Kozlowski et al., 2002; Thapar et al., 1990a; Thapar et al., 1990b; Wu et al., 2000). Targeted iliac lymph node immunization is a method of immunization of animals in a region of internal and external iliac lymph nodes, the draining lymph nodes of the genital and reproductive tract. This technique produced significant secretory IgA and IgG antibodies following boost vaccination in the rectum, urine, vagina, and urethral/seminal washings of macaques. Serum IgA and IgG were also detected. Induction of CD4⁺ T cells was important for this effect (Lehner et al., 1999). The importance of stimulating both local and systemic immunity is under debate as the source of Ig may be partially a transudation from circulation (Brandtzaeg, 1997; Johansson and Lycke, 2003). Moreover, cervical secretions are likely the source of the majority of IgA rather than that produced by the vaginal epithelium (Kutteh et al., 1996).

1.16 Experimental Rationale

Aluminum hydroxide-adjuvanted vaccination induces a Th2-biased immune response. Based on the previous discussion, an antibody response is likely ideal for opsonisation and induction of complement activation, and neutralizing surface and secreted cytopathic and immunoevasion factors such as AP or CP. Antigen adsorbed to aluminum hydroxide can stimulate production of IgA and IgG in the vagina to varying levels

depending on the route of immunization. Vaginal immunization is significantly less effective than parenteral immunization or subcutaneous vaccination with alum (Thapar et al., 1990a; Thapar et al., 1990b). In the studies of Thapar and colleagues (1990a; 1990b), subcutaneous vaginal IgA returned to baseline within 16 weeks, however, other immunization sites excluding vaginal immunization had long lasting IgA beyond 24 weeks post-immunization. Nevertheless for this short term vaccination and infection study the data suggests the feasibility of employing a prime-boost subcutaneous vaccination schedule using live, whole cell *T. vaginalis* antigen adjuvanted with aluminum hydroxide (formulated as Alhydrogel) (Brenntag Biosector, Frederikssund, DK).

1.17 Hypotheses

Live, whole cell *T. vaginalis* adjuvanted with aluminum hydroxide in a subcutaneous prime-boost vaccination will stimulate vaginal protection leading to decreased infection rates and increased clearance.

Measures of humoral and cell mediated immunity from systemic and local tissues will reveal distinct responses in terms of vaccinated versus unvaccinated groups.

1.18 Objectives

1. To apply an adjuvant approved for use in humans in the *T. vaginalis* mouse model of vaginal infection.
2. To measure systemic and local antibodies.
3. To measure spleen lymphocyte proliferation to *T. vaginalis* antigen following infection of vaccinated and unvaccinated groups.

4. To identify lymphocyte infiltration in vaginal tissue following infection of vaccinated and unvaccinated groups.

2. Materials and Methods

2.1 Media

Trichomonas vaginalis was cultivated in TYI broth which was prepared based on Diamond's TYI broth (Diamond et al., 1978). Following pH adjustment to 6.5 and autoclaving, a vitamin mixture (MEM vitamin solution, Gibco, Grand Island, NY) was added (2.7 mL/100 mL TYI broth). For passaging axenic cultures TYI broth with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) was used. This medium will be referred to as simply TYI. When culturing vaginal washes to isolate *Trichomonas vaginalis*, the TYI was supplemented with 0.3 mg/mL Penicillin/Streptomycin (Gibco), 2.5 ug/mL Fungizone (Gibco), and 10 ug/mL Gentamicin (Gibco), to suppress the growth of normal vaginal flora.

Lactobacilli MRS broth (MRS) (Difco Laboratories, Detroit, MI) was made according to manufacturer's guidelines. MRS was used for axenic cultures of *Lactobacillus acidophilus*. MRS was supplemented with 20 ug/mL metronidazole (Baxter, Toronto, ON), and 5 ug/mL ciprofloxacin (Sandoz, Quebec, CA) when culturing vaginal washes for *L. acidophilus* detection.

2.2 Strains

Trichomonas vaginalis clinical isolate OC15 was derived from a woman with clinical vaginitis. The isolate was axenically cultured and then stored in either -80°C or liquid nitrogen. Stock isolates were grown in screw cap culture tubes slanted on a rack containing 10 mL of TYI and passaged every 3 days up to 9 passages, at which time a new frozen sample was cultured to maintain its pathogenicity. Cultures were incubated (Forma II Incubator, Thermo Electron Corporation, Marietta, OH) in an environment of 5% CO₂ and

37°C.

Lactobacillus acidophilus (ATCC 4356, American Type Culture Collection, Rockville, MD) was cultured from -80°C axenic cultures in the morning into 10 mL MRS in a screw cap culture tube slanted on a rack in an incubator set to 5% CO₂ and 37°C. In the evening 10 uL from the morning culture was seeded into both a culture tube with 10 mL fresh MRS and a 250 mL Erlenmeyer flask with 100 mL MRS, and incubated overnight. The evening 10 mL *L. acidophilus* culture was used to seed the next morning's 10 mL MRS culture tube culture repeating the above process to propagate the culture.

2.3 Cell Preparation and Inoculation

Axenic cultures of live, motile *Trichomonas vaginalis* were collected into 50 mL conical tubes and centrifuged (Sorval RT1, Thermo Scientific, Rochester, NY) at 2,100 rpm for 10 min at 4°C. The supernatant was discarded and the pellet washed by resuspension in 30 mL PBS (pH 7.4) for three washes. Following the final centrifugation, the pellet was resuspended in 1 mL of either PBS or TYI (preparation for vaccination or intravaginal challenge, respectively) and counted using a 1:100 dilution of the suspension in a trypan blue solution and pipetting a small volume onto a hemocytometer. A concentration of 1×10^7 trichomonads per mL was required for vaccination, diluted from stock concentration with PBS. A concentration of 2.5×10^7 trichomonads per mL was required for vaginal challenge, diluted from stock concentration with prewarmed TYI. Intravaginal challenge was completed on day 0/-1 with 20 uL inoculum volume.

Log phase *L. acidophilus* was measured using a spectrophotometer (Genesys 20, Thermo Spectronic, Rochester, NY) the morning of inoculations from a 100 mL overnight

culture. A cuvette containing 1 mL of log phase *L. acidophilus* was read at 650 nm, with a cuvette containing MRS only as a blank. An OD of 0.5-0.7 indicated adequate phase of growth. The 100 mL volume was transferred to two 50 mL conical tubes and spun at 4,000 rpm, for 10 minutes at 4°C. The supernatant was discarded and the pellet washed three times with PBS. The final pellet was resuspended in 1.5 mL of prewarmed MRS. Mice were given 20 uL intravaginal inoculations on day -7/-6.

2.4 Vaccine Preparation

One milliliter of a *T. vaginalis* stock culture with a concentration of 1×10^7 trichomonads per mL was combined with each 1 mL of Freund's Complete Adjuvant, Freund's Incomplete Adjuvant (Difco Laboratories), or Alhydrogel (Brenntag Biosector, Frederikssund, DK) preparation. Alhydrogel preparation was made using stock 10 mg Al/mL Alhydrogel and diluted to 0.75 mg Al/200 uL or 0.50 mg Al/200 uL with PBS and pH adjusted to 7.4, measured with a pH meter (Accumet Basic 04870, Fisher Scientific), using 1N acetic acid. An additional control vaccination group contained a solution of 0.75 mg Al/200 uL Alhydrogel without live trichomonads. All Alhydrogel vaccine preparations were mixed on a rotating incubator (Orbit Environ-Shaker, Lab-Line, US) set at 25 rpm and 37°C for 1.5 hours. Concentration of aluminum was decided based on acceptable aluminum concentrations for injection into mice.

Mice were vaccinated subcutaneously with 200 uL of inoculum in a single injection containing 1×10^6 trichomonads injected into the left or right regio costalis once for prime (day -56), and once on the opposite side for boost vaccination (day -28). Sham vaccinations did not contain trichomonads, but did contain adjuvant. Unvaccinated groups received no

injections on either day. Inoculum regimen and key procedure dates (Table 1) followed previously established protocols (Abraham et al., 1996) in our lab.

2.5 Animals and Animal Care

Female BALB/c mice 17-19 g were obtained from Charles River (Montreal, Canada) and housed in the animal care facilities at the University of Ottawa Roger Guindon Campus (Ottawa, CA). Mice were divided in groups of four with food and water provided ad libitum. These groups were randomly allocated to either receive vaccination or sham vaccination, or remain unvaccinated. All procedures performed on mice were approved by the Animal Care Committee of the University of Ottawa, Faculty of Medicine for ethical consideration, protocol number BMI-108.

2.6 Estrogen Treatment

Mice were injected with 0.5 mg estradiol valerate (Watson's Pharmacy, Ottawa, ON) subcutaneously in the left or right regio umbilicalis once on day -9 and once on the opposite side day -2. Estrogen was used to induce estrus in the mice.

2.7 Vaginal Washes

Vaginal washes were performed on the mice to determine the status of *L. acidophilus* colonization or *T. vaginalis* infection. *L. acidophilus* washes were conducted on day -1, and *T. vaginalis* washes on day 7, 14, and 28. Day 21 washes were also performed for mice in the cell proliferation, vaginal immunoglobulin and histology sacrifice study groups (Table 1). On the indicated days 25 uL of either MRS (for lactobacilli culture

isolation) or TYI (for *T. vaginalis* culture isolation) were pipetted gently and repeatedly in the vagina of each mouse, then collected into an individually labeled culture tube containing either MRS with antibiotics or TYI with antibiotics. The wash was repeated one additional time following the first wash with the same volume and repetitions, and collected into the same culture tube. Culture tubes were incubated at 37°C, in 5% CO₂ and assessed for growth after 4 days. A positive result for *L. acidophilus* was determined by naked eye examination of the culture tube for bacterial growth. A positive result for *T. vaginalis* was determined by microscopic examination of culture tube growth for motile trichomonads.

2.8 Collection of Vaginal Immunoglobulin

Vaginal washes for the collection of immunoglobulin (Ig) were performed on select mice on different dates depending on their sacrifice date. Day -1/1/3/7/14/21/28 are the dates on which samples were obtained; however, mice were not sampled at every date. The vaginal wash procedure, including a workup of vaginal mucus to obtain additional vaginal immunoglobulin, is described elsewhere (Parr and Parr, 1997). In brief, 50 uL of PBS was pipetted gently and repeatedly up to 10 times in the vagina of mice using a wide bore pipette tip. The liquid was aspirated and transferred to a microfuge tube containing 1 uL of a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO). An additional wash was repeated for a final volume of 100 uL. This volume was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was extracted and replaced with an additional 100 uL of fresh PBS with 1 uL protease inhibitor cocktail, then rotated in an incubator at 20 rpm for 2 hours at 4°C. This supernatant was collected and stored with the previous 100 uL vaginal wash. Then a final 100 uL of fresh PBS with 1 uL protease inhibitor cocktail was added and the steps

repeated as described. The final 300 uL volume was stored at -20°C until use.

2.9 Blood Collection

Mice were bled from the saphenous vein if not being sacrificed, or exsanguinated by cardiac puncture if being sacrificed. In the first case blood was collected directly from the bleeding vein using a microvette (CB 300, Sarstedt, DE) to obtain up to 300 uL of blood. For cardiac punctures the blood was extracted from the heart following puncture with a needle and syringe, then transferred to a microvette directly from the syringe, needle removed. Blood samples were set at room temperature for 2 hours, then centrifuged for 20 minutes at 2,000 g. Serum was pipetted from the collection tubes into microtubes and stored at -80°C until use.

2.10 Enzyme-Linked Immunosorbent Assay

A previously prepared laboratory preparation of OC15 lysate (sonicated whole cell OC15 with known protein concentration) was coated overnight on 96 well, high binding cell culture plates (Costar 3595, Corning, NY) at a concentration of 2 ug/mL OC15 lysate in a total volume of 50 uL. Plates were covered, wrapped in foil, and set at 4°C overnight. Wells were emptied and washed three subsequent times using PBS-T wash buffer (PBS, 0.005% Tween 20). Plates were dried by tapping upside down on paper towel. One hundred microliters of blocking buffer (PBS-T, 1% bovine serum albumin) were added to each well for blocking. The plates were either kept overnight at 4°C, 2 hours at room temperature, or 1 hour at 37°C. Emptying wells, washing, and drying steps were repeated. For serum samples, the wells were then filled with 100 uL of PBS-T with 0.1% BSA. To each filled well a

volume of 0.5 uL of undiluted serum was added. For vaginal wash samples, 40 uL of PBS-T with 0.1% BSA was added to each well. Then, from the 300 uL total vaginal wash sample volume a volume of 60 uL was added to each well. The plates were covered, wrapped in foil, and incubated for 2 hours at room temperature. Dumping, washing, and drying steps were repeated. Peroxidase-conjugated goat anti-mouse monoclonal antibodies were added in a total volume of 100 uL PBS-T to each well at indicated dilutions: 1:10,000 IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:5,000 IgG_{2a} (Jackson ImmunoResearch Laboratories), 1:7,000 IgG₁ (Jackson ImmunoResearch Laboratories), and 1:7,000 IgA (Invitrogen Molecular Probes, Frederick, MD). The plates were covered, wrapped in foil, and incubated for 2 hours at room temperature. Dumping, washing, and drying steps were repeated. Urea buffered o-phenylenediamine (OPD) (Sigma-Aldrich) was added to each well at a total volume of 100 uL, after which plates were covered and wrapped in foil. Following 25 minute room temperature incubation the plates were read at 450 nm on a Biorad Microplate Reader (Model 3550, Hercules, CA). A stopping solution was added to vaginal wash ELISA consisting of 25 uL 3M HCl. These plates were read at 490 nm. All ELISA plates for serum contained a positive and negative control as well as blank wells that underwent all steps with the exception of addition of serum. Vaginal wash plates used a serum based positive control for total IgG, but no controls were available for IgA.

2.11 Flow Cytometry

Mice were euthanized at days 1/3/7/14/21/28 and spleens harvested. Spleens were cut and crushed on a dish with 1 mL PBS, then poured onto a 100 um cell strainer seated over a 50 mL conical tube containing 5 mL PBS. The dish and syringe barrel used to crush the

spleen were rinsed with 3 and 2 mL PBS, respectively and poured onto the cell strainer. The syringe barrel was used to gently press the contents on the cell strainer through the strainer. The syringe barrel and strainer were rinsed with 2 and 3 mL of PBS, respectively, and discarded. Five milliliters of the cell filtrate was slowly added on top of each of three 15 mL conical tubes containing 5 mL prewarmed Lympholyte M (Cedarlane Labs, Burlington, ON) taking care to form two distinct layers. The three tubes were centrifuged at 2,000 rpm for 30 minutes at room temperature. The resulting buffy coat layer was extracted carefully from each tube and added to a new 15 mL conical tube containing 10 mL PBS. The tube was centrifuged at 1,600 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10 mL PBS as a wash phase. Centrifugation and washing was repeated one additional time. The final pellet was resuspended in 1 mL PBS and counted using the same method for counting trichomonads described above by hemocytometer. From the resuspended pellet stock 1×10^7 cells were combined with 1 mL carboxyfluorescein succinimidyl ester (CFSE) with dimethyl sulfoxide (DMSO) in PBS and 0.1% BSA as per manufacturer's instructions (Invitrogen, Eugene, OR). The tube of cells was incubated in an enclosed 37°C, water bath for 10 minutes. CFSE is light sensitive and care must be taken to avoid samples being exposed to light. The CFSE incorporation reaction was stopped by addition of 10 mL of very cold PBS, then centrifuged at 1,600 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet washed and centrifuged for 2 additional non-chilled PBS washes. The final pellet was resuspended in 200 uL RPMI mixture (RPMI medium 1640 (Gibco), 10% FBS, 1% penicillin/streptomycin, 50 uM/mL beta-mercaptoethanol (Sigma-Aldrich), 0.04 ug/mL IL-2 (R&D Systems, Minneapolis, MN)) and recounted as described. A final concentration of 1×10^6 cells/mL was required. These cells

were added to polystyrene round bottom tubes in 100 uL volumes with either 0.00025 ug ConA (Sigma-Aldrich), 0.1 ug colchicine (Sigma-Aldrich), 1.4 ug OC15 lysate, or nothing. The cells were incubated for 4 days at 37°C, 5% CO₂. Twenty-five minutes prior to running cells on the flow cytometer (CyAn ADP Analyzer, Beckman Coulter) 1 uL each of rat anti-mouse fluorochromes for CD4 (PE-Cy 7, Cat#552775, BD Pharmingen), and CD8 (PE-Cy 5, Cat#553034, BD Pharmingen) were added to the tubes. Cells were prepared without CFSE, CD4 labelling only, CD8 labelling only, and unstained to set voltages and gates. Gating was set as appropriate to encompass the CD4 and CD8 single positive populations in their own quadrants. Analysis files were saved and additional gates were set using Kaluza flow cytometry analysis software (Kaluza Analysis Software v1.2, Beckman Coulter). The additional gates were manually set to encompass the approximate region of peaks resulting from dividing cells based on loss of CFSE. The data was analyzed to obtain the percentage of cells in a given cell division cycle number (i.e. 1st, 2nd, 3rd division and so forth). The data was then presented in graphical format to view the division kinetics of each sacrificed mouse.

2.12 Immunohistochemistry

Spleens and vaginas were collected from mice euthanized days -1/1/3/7/14/21/28. Tissues were embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) by flash freezing. The OCT covered samples were submerged in isopentane contained in a beaker seated in liquid nitrogen. The tissues were transferred to cryovials and kept in liquid nitrogen until transferred to -80°C storage. Spleen tissue was cut 7 um thick longitudinally at -20°C using a microtome (Microm HM 500 M). Vaginal tissue was cut 7 um thick across the lumen

at -19°C using a microtome. Different temperatures were required for ideal cutting quality. Serial triplicate spleen and duplicate vaginal tissues were thawed onto Superfrost Plus Gold microscope slides (Cat#22-035813, Fisher Scientific) and transferred to -80°C storage until staining procedures were completed.

An immunoperoxidase universal staining procedure for monoclonal mouse frozen tissues was used as provided by the Department of Pathology and Laboratory Medicine (University of Ottawa). Tissues were dried by constant air flow in a biological safety cabinet, then fixed by addition of -20°C anhydrous acetone for 5 minutes in a -20°C freezer. The tissues were then dried again to ensure no residual acetone. Finally, the tissues were rehydrated in Tris-buffered saline (TBS, pH 7.6) for 10 minutes. TBS was decanted and tissues were incubated for 10-20 minutes at room temperature with 1% BSA in TBS as a suppressor. Excess suppressor was decanted and diluted rat anti-mouse primary antibodies in TBS were added for the respective investigation of CD4⁺ cells (1:100) (Anti-mouse CD4, Cat#550280, BD Pharmingen), CD8⁺ cells (1:100) (Anti-mouse CD8a, Cat#550281, BD Pharmingen), and B Cells (1:50) (Anti-mouse CD19, Cat#550284, BD Pharmingen). Primary antibodies were incubated overnight at 4°C in a humidification chamber. The next day the tissues were washed twice for 5 minutes by submerging the slides in TBS. Next, biotinylated goat anti-rat IgG (1:200 in TBS) (Cat#559286, BD Pharmingen) was added to the slides for 30 minutes at room temperature in a humidification chamber. Wash steps were repeated. To quench endogenous peroxidases the slides were blocked with 6% H₂O₂ in methanol for 10 minutes. A single wash step was performed. An ABC complex kit (Cat#32052, Thermo Scientific, Rockford, IL) was incubated on tissues for 45 minutes. Two washes were performed. The peroxidase activity of the ABC kit was resolved using a

betazoid DAB chromogen kit (BioCare Medical, Concord, CA) placed on the tissues for 5 minutes, then washed with distilled water. At this point the slides were submerged in distilled water and brought to the Department of Pathology and Laboratory Medicine to counterstain, dehydrate, clear, and mount the slides. For analysis of the tissues, the experimenter was blinded from whether the vaginal tissue came from a mouse that was vaccinated, sham vaccinated or unvaccinated.

2.13 Statistical Analyses

Fisher's exact test was performed for statistical differences between infection status at day 7, 14, 21 and 28 (Microsoft Research, <http://research.microsoft.com/en-us/um/redmond/projects/MSCompBio/FisherExactTest/>). Serum data and percent of divided cells from proliferation data were analyzed by one way ANOVA with post-hoc Tukey's multiple comparisons test (GraphPad Prism v5.04, GraphPad Software, Inc.). A cutoff for vaginal Ig was set as two times the standard deviation of the absorbance readings from unvaccinated mice, where values above the cutoff are considered positive readings.

Table 1

Overview of dates on which experimental procedures were performed. The sample number describes the total number of mice on which the respective procedures were conducted. These values do not reflect the total number of mice used in this study.

Experimental Protocol	
<i>Vaccination and Infection*</i>	<i>Day of procedure</i>
Prime vaccination	-56
Boost vaccination	-28
Delestrogen	-9
<i>Lactobacillus acidophilus</i> inoculation	-7, -6
Delestrogen	-2
<i>Trichomonas vaginalis</i> challenge	0, 1
<i>Vaccination Experiments (n = 101)**</i>	
Saphenous bleed for serologic IgG	-35, -7, 14, 28
Vaginal wash for <i>Trichomonas vaginalis</i>	7, 14, 28
<i>Cell Proliferation (n = 72)**</i>	
Spleen collection	1, 3, 7, 14, 21, 28
<i>Immunohistology (n = 73)**</i>	
Tissue collection	-1, 1, 3, 7, 14, 21, 28
<i>Vaginal Immunoglobulin (n = 70)**</i>	
Vaginal wash for IgA and IgG	-1, 1, 3, 7, 14, 21, 28***

*Vaccination and Infection protocol consistent with all mice in study with the exception of unvaccinated mice which did not receive prime or boost vaccinations.

**Mice were used in more than one category (i.e. multiple mice were used for combinations of infection data, cell proliferation data, tissue collection, and vaginal Ig)

***Collection dates do not apply to all mice; variable based on sacrifice date

3. Results

3.1 Challenge and Infection Status

Female BALB/c mice from five separate experiments received a vaccination at day -56 and day -28 before vaginal infection. The mice received either subcutaneous injection of Freund's Complete adjuvant and Freund's Incomplete adjuvant (day -28) with whole cell *T. vaginalis* (Freund's - 14 mice), 0.50 mg Al Alhydrogel with whole cell *T. vaginalis* (Alum 0.50 - 26 mice), 0.75 mg Al Alhydrogel with whole cell *T. vaginalis* (Alum 0.75 - 18 mice), 0.75 mg Al Alhydrogel without antigen (Alum sham - 15 mice), or no vaccination (unvaccinated - 28 mice). Injections containing *T. vaginalis* organisms contained 1×10^6 trichomonads. Mice were treated as described in Materials and Methods Table 1 under "vaccination experiments" and "vaccination and infection". Data on the recovery rates of *L. acidophilus* or *T. vaginalis* organisms from infection experiments is summarized in Table 2.

Lactobacilli colonization was similar in all groups as determined by washes 5 days post *L. acidophilus* inoculation (day -2). Freund's vaccination had the lowest lactobacilli colonization (7/14) versus the highest colonization group, unvaccinated (22/28). However, this difference was not statistically significant. Alum 0.50, Alum 0.75 and Alum sham had lactobacilli colonization rates of 16/26, 12/18, and 10/15, respectively.

Infection status was determined on day 7, 14, and 28 by culture of vaginal washes. Unvaccinated mice had a significantly higher proportion of infections on day 7 compared to Freund's and Alum vaccinated mice ($p < 0.005$ Freund's, $p < 0.001$ Alum 0.50 and Alum 0.75). The proportion of infections in Alum sham mice on day 7 was not significantly different compared to unvaccinated or vaccinated groups.

The number of cleared infections on day 28 as compared to day 7 were as follows:

4/4 Freund's; 5/8 Alum 0.50; 3/4 Alum 0.75; 5/8 Alum Sham groups; and 5/22 unvaccinated. Vaccinated mice had fewer initial infections and clearance of a greater percent of initial infections compared to unvaccinated mice.

Table 2

Lactobacillus acidophilus and *Trichomonas vaginalis* recovery rates detected by positive culture following vaginal wash.

Organism cultured and day of culture	Number of mice with recovered organisms				
	Freund's	Alum 0.50	Alum 0.75	Alum Sham	Unvaccinated
<i>L. acidophilus</i> Wash (Day -1)	7/14 50%	16/26 62%	12/18 67%	10/15 67%	22/28 79%
<i>T. vaginalis</i> Wash 1 (Day 7)	4/14 ^a 29%	8/26 ^b 31%	4/18 ^b 22%	8/15 53%	22/28 79%
<i>T. vaginalis</i> Wash 2 (Day 14)	2/14 ^b 14%	5/26 ^b 19%	1/18 ^{b,c} 6%	6/15 40%	20/28 71%
<i>T. vaginalis</i> Wash 3 (Day 28)	0/14 ^b 0%	3/26 ^b 12%	1/18 ^b 6%	3/15 20%	17/28 ^c 61%

^a Statistically significant compared to unvaccinated (p < 0.005).

^b Statistically significant compared to unvaccinated (p < 0.001).

^c Statistically significant compared to Alum sham (p < 0.05).

3.2 Serum and Vaginal Immunoglobulins

Systemic and local antibody production is an important measure of effective induction of the humoral immune system and potential correlate of protection for vaccines. We assessed systemic IgG and IgG subclasses. Blood was collected from mice receiving Freund's (8 mice), Alum 0.50 (8 mice), or Alum 0.75 (8 mice) subcutaneous vaccination with 1×10^6 trichomonads, unvaccinated (3 mice), or Alum 0.75 (4 mice) without trichomonads (Alum sham). Blood was collected by saphenous bleed at days -35, -7, 14, and 28.

We assessed presence of total IgG and IgA in the vagina. Vaginal washes were performed on mice at days 1, 3, 7, 14, 21, and 28 following intravaginal challenge with *T. vaginalis*. Mucus from vaginal washes was pelleted and an extraction process was repeated twice to obtain antibodies contained within the mucus. PBS used in the extraction process was combined with the first supernatant collected from the centrifugation of the vaginal wash. Protease inhibitors were combined in PBS to reduce endogenous protease activity upon the vaginal antibodies. The number of mice sampled at each date varied based on sacrifice and date.

3.2.1 Serum IgG and IgG Subclasses

Figure 1, 2, 3, and 4 illustrate a single representative experiment of the serologic IgG response in mice treated as described in Materials and Methods Table 1. To account for a lack of a suitable standard when using a whole cell vaccine approach with different vaccine formulations a single plate was run for each isotype tested. This approach avoids complications of plate to plate variation and antigen heterogeneity. Mice immunized with

adjuvant and trichomonads had significantly higher serum total IgG and IgG₁ ($p < 0.05$) at all sample time points, except day -35 (post-prime vaccination) for Alum 0.75, compared to unvaccinated and Alum sham groups. Although Alum 0.75 day -35 serum total IgG was not statistically significant compared to controls, an IgG response was present in Alum 0.75 vaccinated mice compared to absence of IgG in controls (Figure 1A). High serum total IgG and IgG₁ was maintained 28 days following intravaginal *T. vaginalis* challenge in the Freund's, Alum 0.50, and Alum 0.75 groups. Total IgG and IgG₁ detection was nearly absent at each time point for unvaccinated and Alum sham groups. Serum IgG_{2a} responses were not detected in unvaccinated or Alum sham groups, but could be detected in some of Freund's, Alum 0.50 and Alum 0.75 vaccinated groups. Freund's vaccinated groups had higher mean IgG_{2a} absorbance values than either Alum vaccinated groups; however, this mean was only significantly different from the Alum 0.75 vaccinated group on day -7. The cumulative effect of subcutaneous immunization of mice with adjuvant and *T. vaginalis* antigen was a pronounced total IgG and IgG₁ response with variable IgG_{2a} production.

3.2.2 Vaginal IgG and IgA

Figure 5 illustrates data from the vaginal immunoglobulin experiment group with respect to total vaginal IgG. Figure 6 illustrates vaginal IgA. Each data point represents a single mouse. For both total IgG and IgA a cutoff was set the mean plus two standard deviations calculated from the absorbances of unvaccinated mice. Points above this value were considered positive. IgG positive values were obtained on day 1, 3, 7, 14, 21 and 28 for Freund's; day 1, 3, 7, 14 and 21 for Alum 0.50; day 1, 3, 7, 14 and 21 for Alum 0.75; day 3, 21 and 28 for unvaccinated; and day 3 and 7 for Alum sham. IgA positive values were

obtained on day 7 for Freund's; day 7 and 21 for Alum 0.50; day 1 and 7 for Alum 0.75; day 14 and 21 for unvaccinated; and day 1 and 7 for Alum sham. Total vaginal IgG responses are higher and more frequently detected as positive than vaginal IgA responses. Vaginal IgG was detected more frequently and almost exclusively from vaginal washes of vaccinated mice.

Figure 1

Serologic IgG response on day -35 (3 weeks post-prime vaccination) of one experiment as measured by ELISA. Total IgG (A), IgG₁ (B) and IgG_{2a} (C) were measured for groups of mice that received a subcutaneous prime vaccination of Freund's Complete Adjuvant, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). Data from this group is representative of results from ELISA conducted on subsequent groups. Significance was determined using a one-way ANOVA and a Tukey's multiple comparisons test for post hoc analysis (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

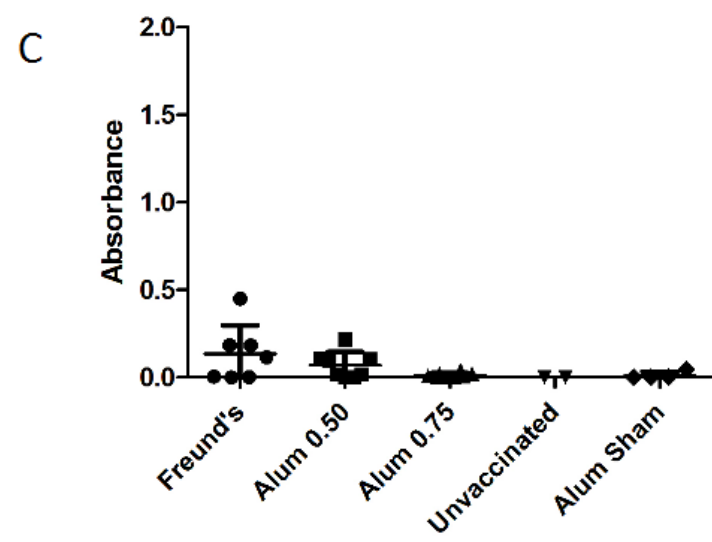
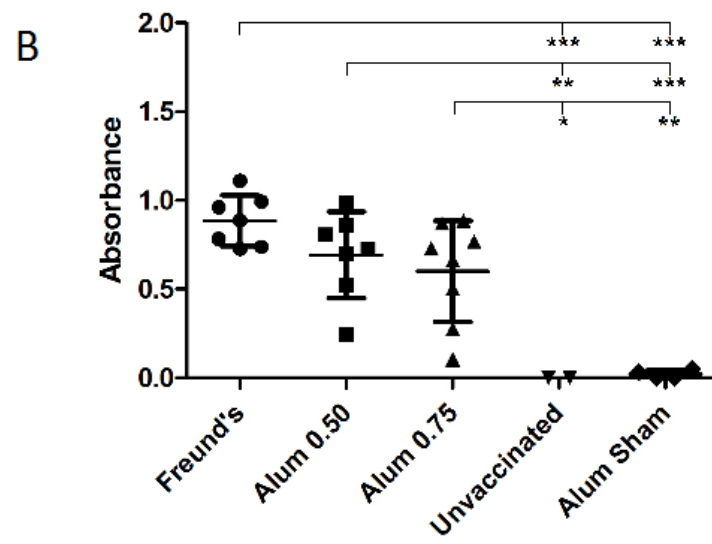
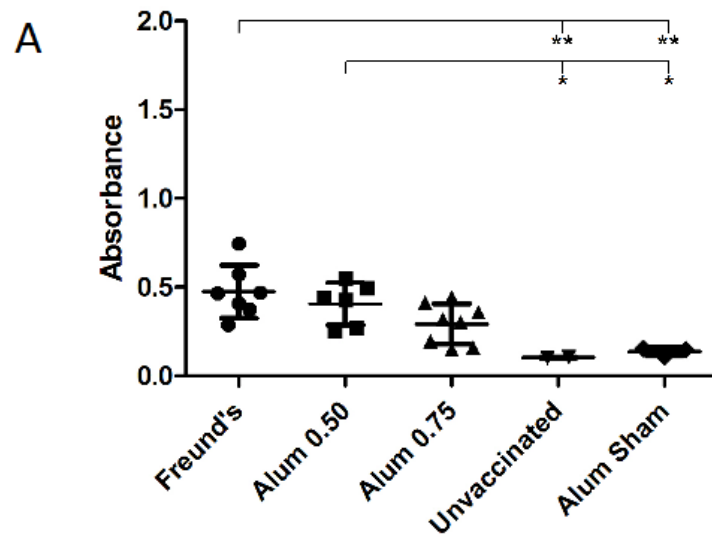


Figure 2

Serologic IgG response on day -7 (3 weeks post-boost vaccination) of one experiment as measured by ELISA. Total IgG (A), IgG₁ (B) and IgG_{2a} (C) were measured for groups of mice that received a subcutaneous prime vaccination as previously described in Figure 1 as well as a subcutaneous boost vaccination of Freund's Incomplete Adjuvant, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). Data from this group is representative of results from ELISA conducted on subsequent groups. Significance was determined using a one-way ANOVA and a Tukey's multiple comparisons test for post hoc analysis (* = $p < 0.05$; *** = $p < 0.001$).

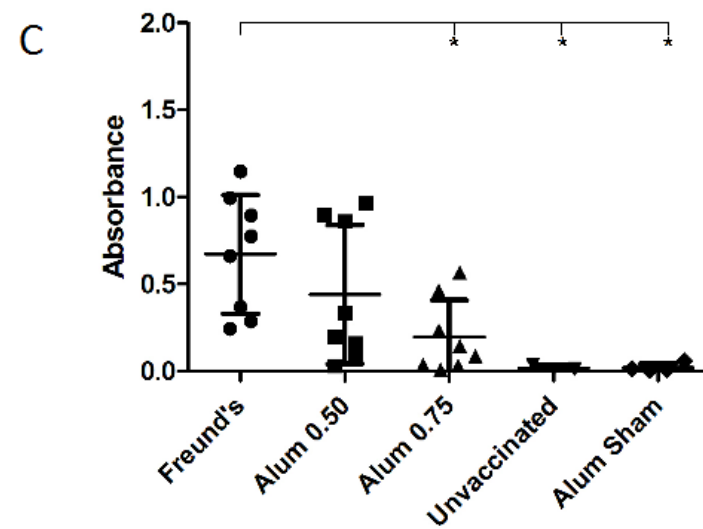
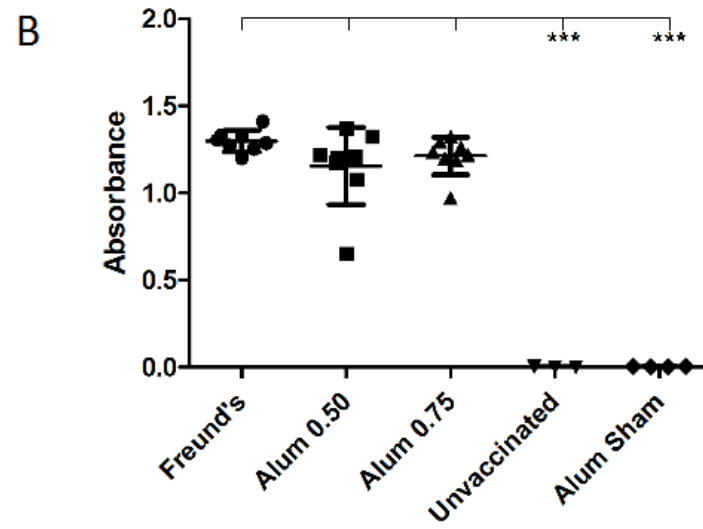
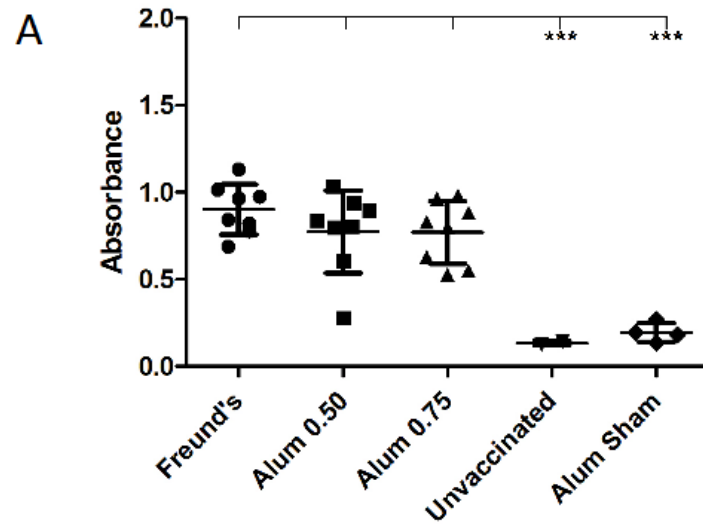


Figure 3

Serologic IgG response on day 14 (2 weeks post-infection) of one experiment as measured by ELISA. Total IgG (A), IgG₁ (B) and IgG_{2a} (C) were measured for groups of mice that received subcutaneous prime and boost vaccinations of Freund's, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). Data from this group is representative of results from ELISA conducted on subsequent groups. Significance was determined using a one-way ANOVA and a Tukey's multiple comparisons test for post hoc analysis (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

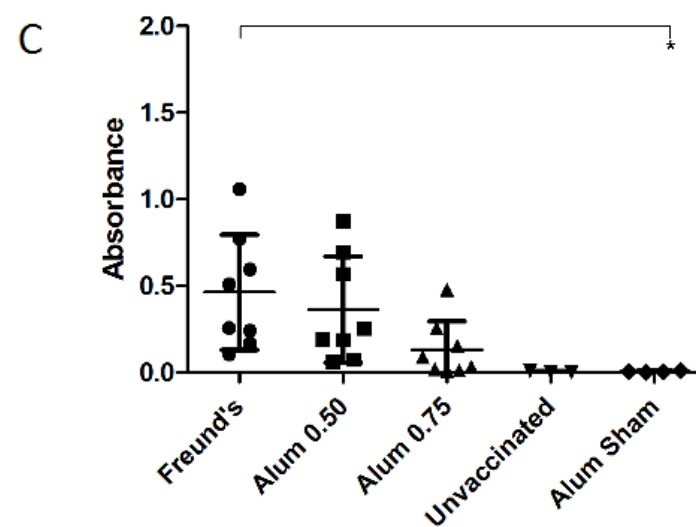
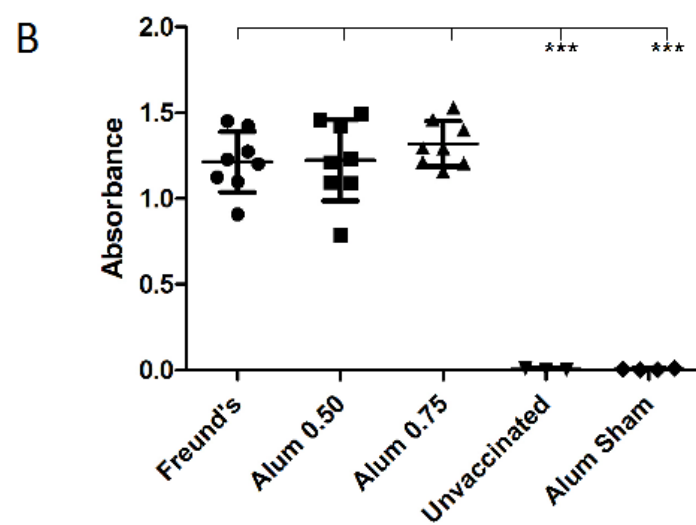
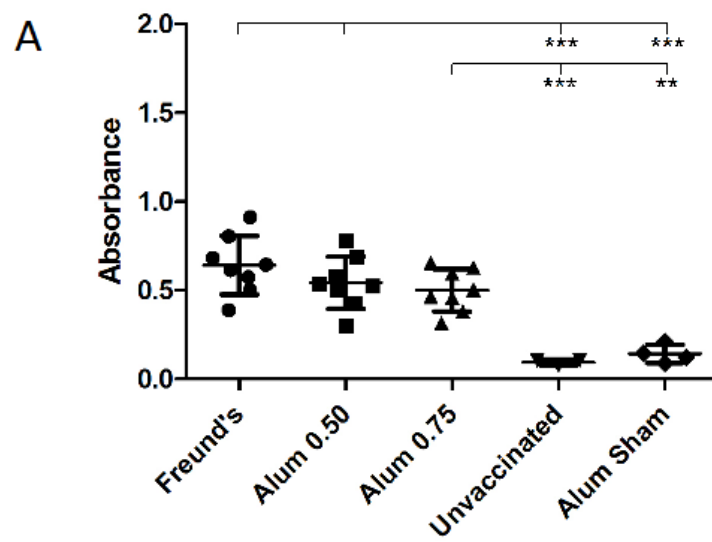


Figure 4

Serologic IgG response on day 28 (4 weeks post-infection) of one experiment as measured by ELISA. Total IgG (A), IgG₁ (B) and IgG_{2a} (C) were measured for groups of mice that received subcutaneous prime and boost vaccinations of Freund's, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). Data from this group is representative of results from ELISA conducted on subsequent groups. Significance was determined using a one-way ANOVA and a Tukey's multiple comparisons test for post hoc analysis (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

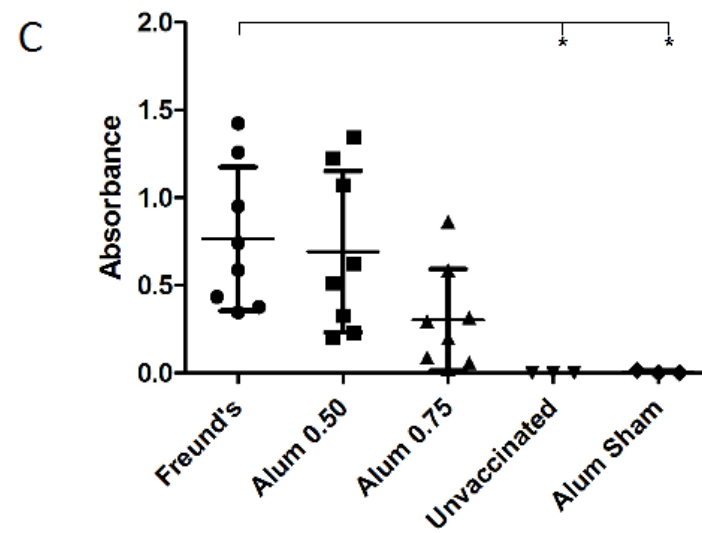
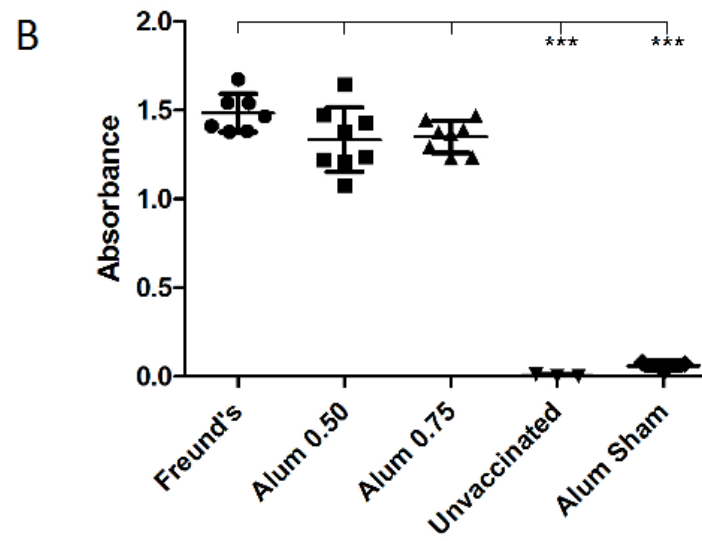
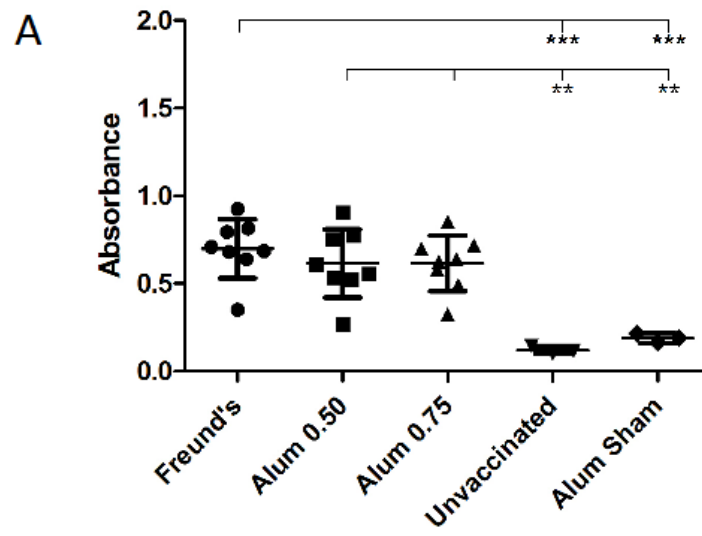


Figure 5

Vaginal IgG response on day 1 (A), 3 (B), 7 (C), 14 (D), 21 (E), and 28 (F) (multiple time points post-infection) of one experiment as measured by ELISA. Groups of mice received subcutaneous prime and boost vaccinations of Freund's, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). A cutoff of two standard deviations above the mean optical density of unvaccinated mice was used. Values above this cutoff are considered positive.

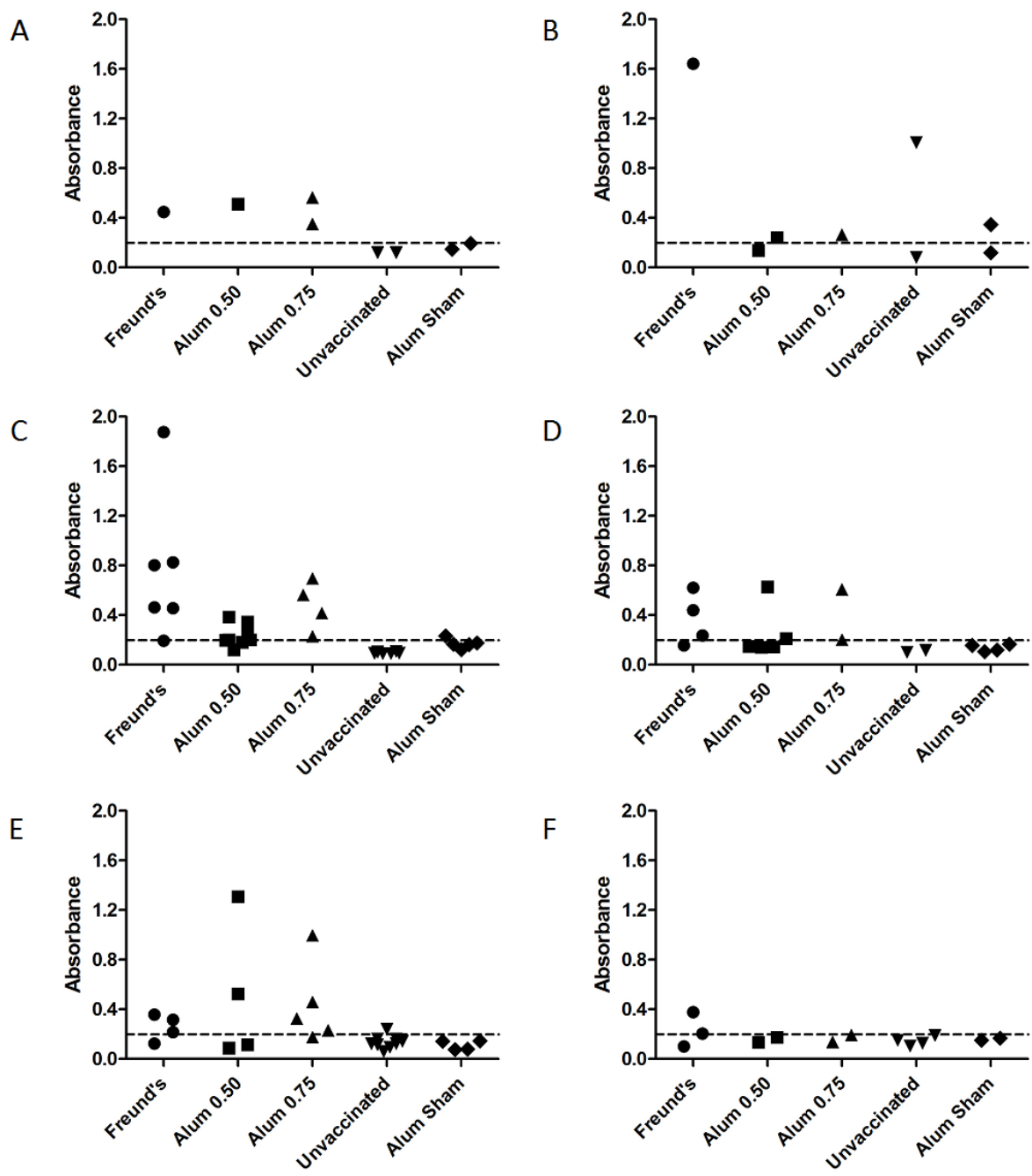
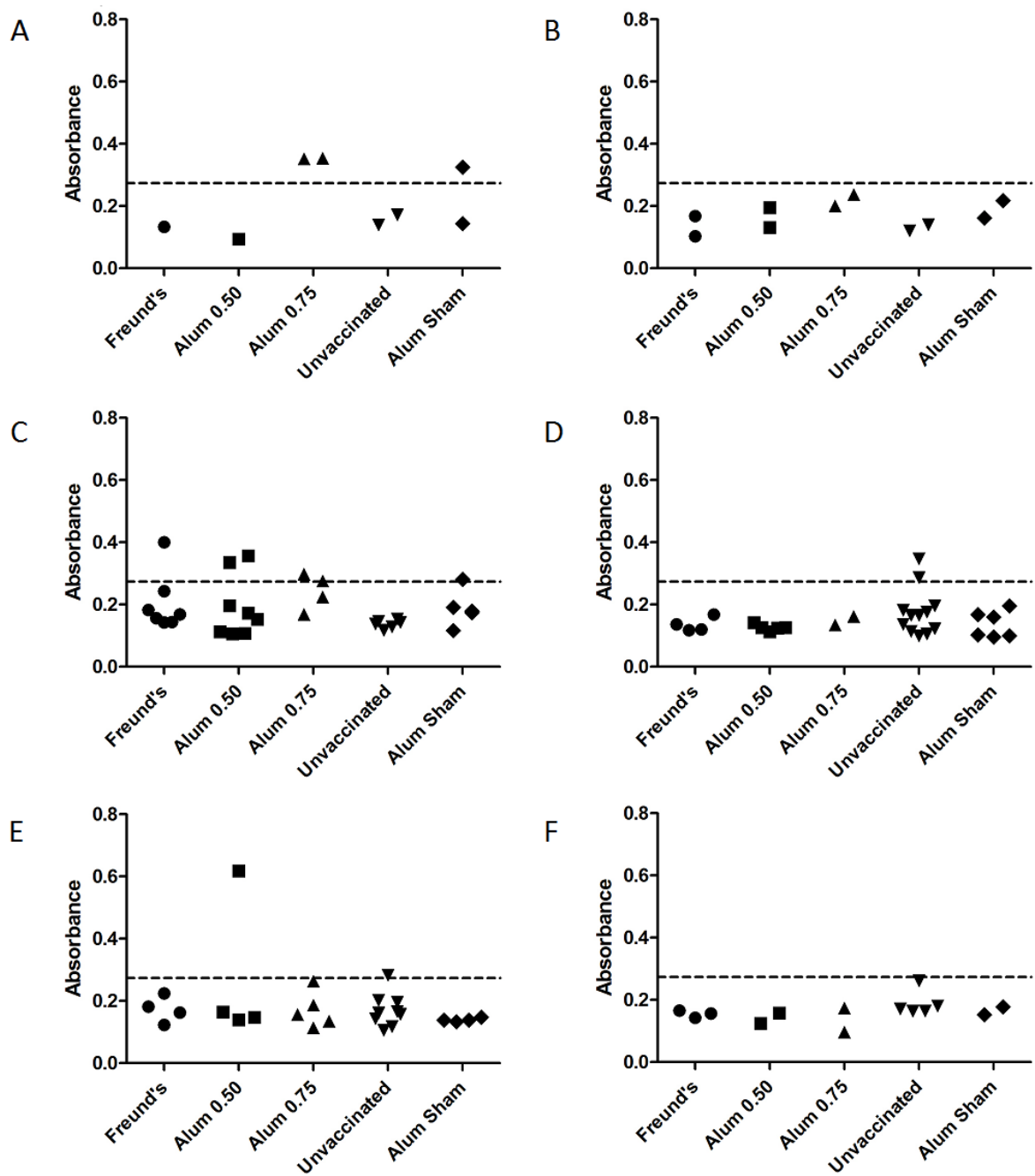


Figure 6

Vaginal IgA response on day 1 (A), 3 (B), 7 (C), 14 (D), 21 (E), and 28 (F) (multiple time points post-infection) of one experiment as measured by ELISA. Groups of mice received subcutaneous prime and boost vaccinations of Freund's, Alum 0.50, or Alum 0.75 with 1×10^6 trichomonads, unvaccinated, or Alum 0.75 without trichomonads (Alum sham). A cutoff of two standard deviations above the mean optical density of unvaccinated mice was used. Values above this cutoff are considered positive.



3.3 CD4⁺ and CD8⁺ Splenocyte Proliferation

The proliferative response of CD4⁺ and CD8⁺ T cells to *T. vaginalis* antigen on days 1, 3, 7, 14, 21, and 28 following infection in immunized and unimmunized animals was assessed. Briefly, mice were euthanized and spleens collected under sterile conditions. The lymphocytes were isolated as describe in Materials and Methods, stained with CFSE to measure proliferation and incubated with whole cell *T. vaginalis* lysate for 4 days. Samples were labelled with anti-CD4 and anti-CD8 fluorescent labels, PE-Cy7 and PE-Cy5, respectively, for distinction by flow cytometry. Data was collected and manual gating applied to each sample. Seven division cycles were gated wherein the seventh division cycle was the limit of detectable CFSE (Figure 7B). Figure 7 A-G, Figure 8 A-G, and Figure 9 A-G are representative samples of the data obtained from the flow cytometry using the Kaluza software with respect to ConA stimulation (positive control), colchicine inhibition (negative control), and an experimental sample. Data is displayed as the percentage of CD4 or CD8 cells from each division cycle (division cycle number) as seen in Figure 7H, Figure 8H and Figure 9H.

The proliferation of CD4⁺ T cells (Figures 10-15) and CD8⁺ T cells (Figures 16-21) was variable across time points, within groups and between groups. As a cell divides it multiplies exponentially as a factor of 2ⁿ, where n represents the division cycle number. Therefore, we could expect that if a population of T cells divides it would be recorded as a peak of percent of cells in a given division cycle number. The location and height of the peak depends on how many times those cells divided and how many cells divided, respectively. In reality, T cells will not necessarily divide at the same time. Still, if there are cells present that

are primed as *T. vaginalis* antigen-specific we predict that their response to antigen would induce early proliferation and the cells would undergo multiple divisions. The result would be a notable peak in later division cycles 4 to 7. Instances where the majority of the CD4⁺ or CD8⁺ population were in division cycles 4 to 7 indicating a population of CD4⁺ or CD8⁺ T cells proliferating earlier were infrequent and occurred variably across all time points (for CD4 populations: Figure 10C, Figure 12C, Figure 13C and D, and Figure 15 D; and for CD8 populations: Figure 18C, Figure 19C-E, Figure 20 C and D, and Figure 21 A, C and D). The majority of these instances were almost exclusively seen in Alum 0.75 vaccinated and unvaccinated groups. Therefore, a peak in later division cycles cannot be associated with vaccination under our assumptions above. The most common feature of the division kinetics of the splenocyte populations was greater than 40% of the total population undivided and less than 10% of the CD4⁺ or CD8⁺ T cell population in any of the division cycles, without any notable peaks. There may be a small dividing population that truly is *T. vaginalis* antigen-specific, but the background division is too high and the sample numbers are too low to determine the differences between vaccinated and unvaccinated groups.

Due to the variability of data and no explicit differences between vaccinated and unvaccinated groups no conclusions can be made. A better approach to measuring a specific response to *T. vaginalis* antigen would be to measure cytokine response using a cytokine flow cytometry (CFC) assay (Karlsson et al., 2003; Maecker et al., 2005). Cytokines to be assessed would be IL-4 (Th2 CD4) and IFN- γ (Th1 CD4 and CD8). This approach would allow a quantitative comparison of antigen specific intracellular cytokine response of CD4 and CD8 lymphocytes.

Figure 7

Positive control flow cytometry data following ConA stimulation of splenocytes. Cells were gated according to location of lymphocytes on the forward and side scatter plot (A). Then, CFSE labelling (B – seen as 'FITC') was used to set gates according to the dividing population peaks. CD8 (C) and CD4 (D) populations are distinctly labelled from background fluorescence and are uniquely labelled with little double positive labelling (E). Cell count data was obtained from gating of CFSE dividing population peaks and CD8 (F) or CD4 (G) gating. Cell numbers from each dividing population were converted to a percentage of the total cell population and plotted (H). The resulting graph demonstrates the percent of cells in the population of CD4 or CD8 cells that reach each respective division number (from 0 to 7).

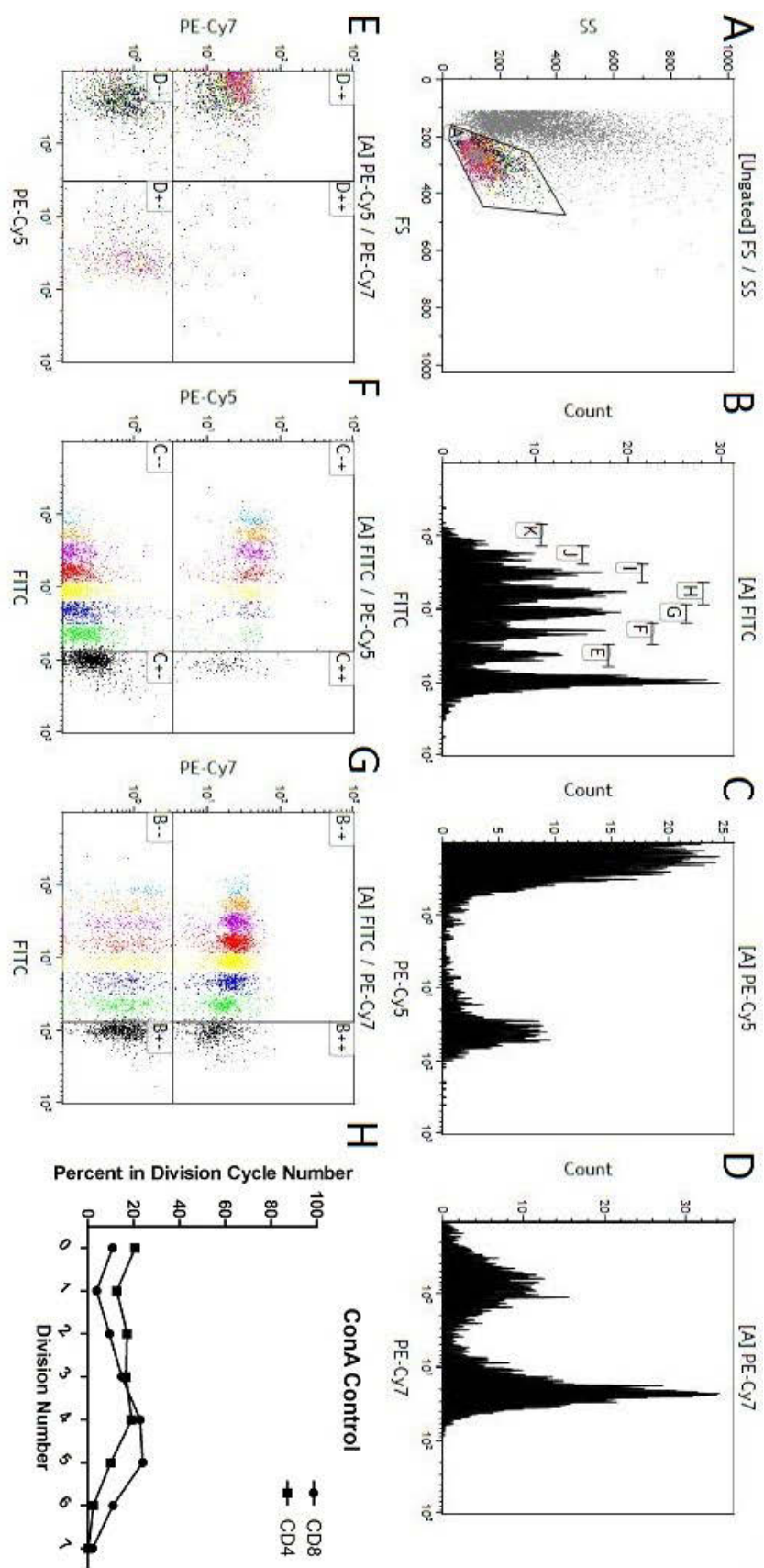


Figure 8

Negative control flow cytometry data following colchicine inhibition of splenocytes. Cells were gated according to location of lymphocytes on the forward and side scatter plot (A). Then, CFSE labelling (B – seen as 'FITC') was used to set gates according to the dividing population peaks. CD8 (C) and CD4 (D) populations are distinctly labelled from background fluorescence and are uniquely labelled with little double positive labelling (E). Cell count data was obtained from gating of CFSE dividing population peaks and CD8 (F) or CD4 (G) gating. Cell numbers from each dividing population were converted to a percentage of the total cell population and plotted (H). The resulting graph demonstrates the percent of cells in the population of CD4 or CD8 cells that reach each respective division number (from 0 to 7).

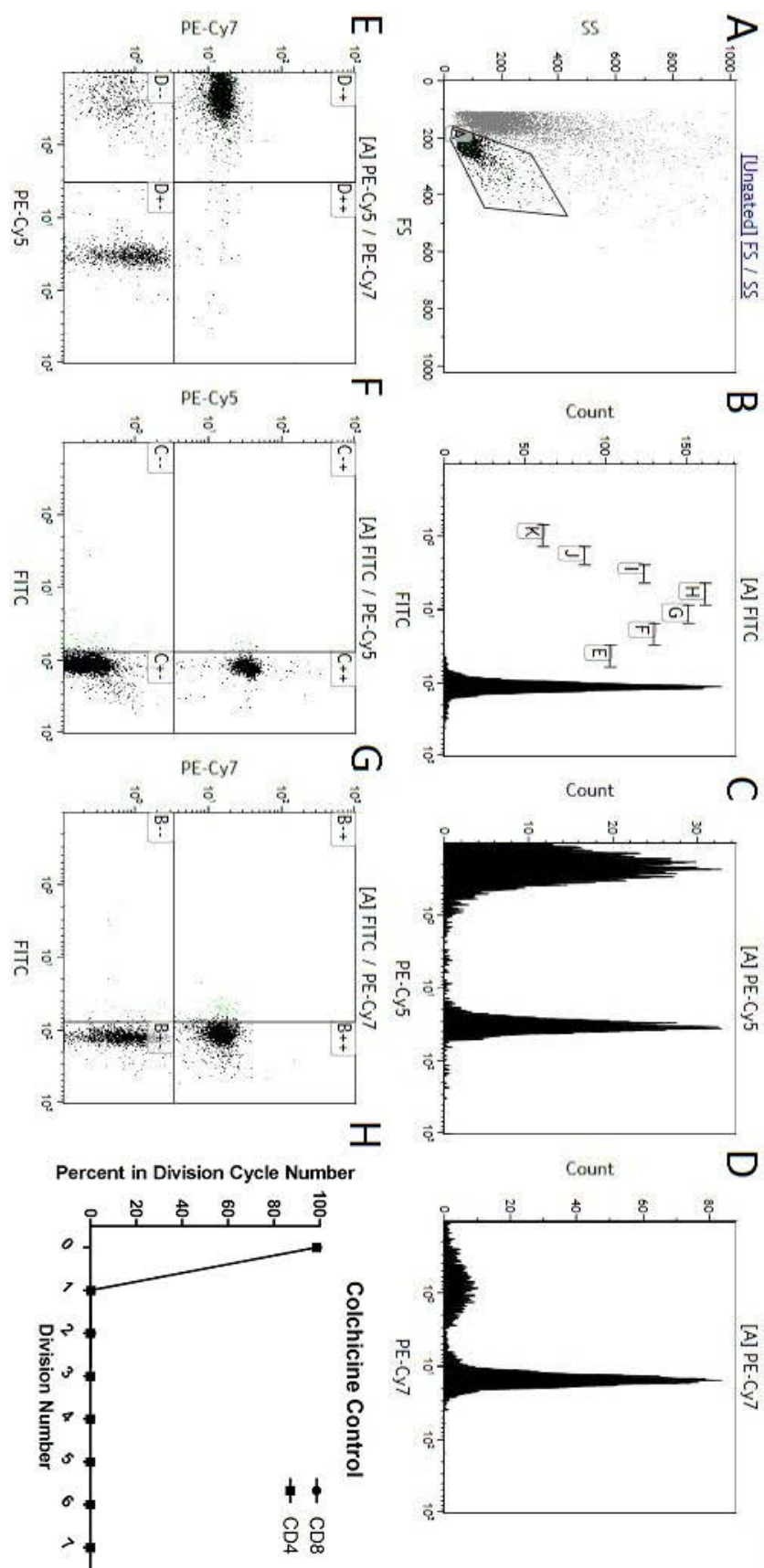


Figure 9

Sample of flow cytometry data following *T. vaginalis* antigen stimulation of splenocytes. Cells were gated according to location of lymphocytes on the forward and side scatter plot (A). Then, CFSE labelling (B – seen as 'FITC') was used to set gates according to the dividing population peaks. CD8 (C) and CD4 (D) populations are distinctly labelled from background fluorescence and are uniquely labelled with little double positive labelling (E). Cell count data was obtained from gating of CFSE dividing population peaks and CD8 (F) or CD4 (G) gating. Cell numbers from each dividing population were converted to a percentage of the total cell population and plotted (H). The resulting graph demonstrates the percent of cells in the population of CD4 or CD8 cells that reach each respective division number (from 0 to 7).

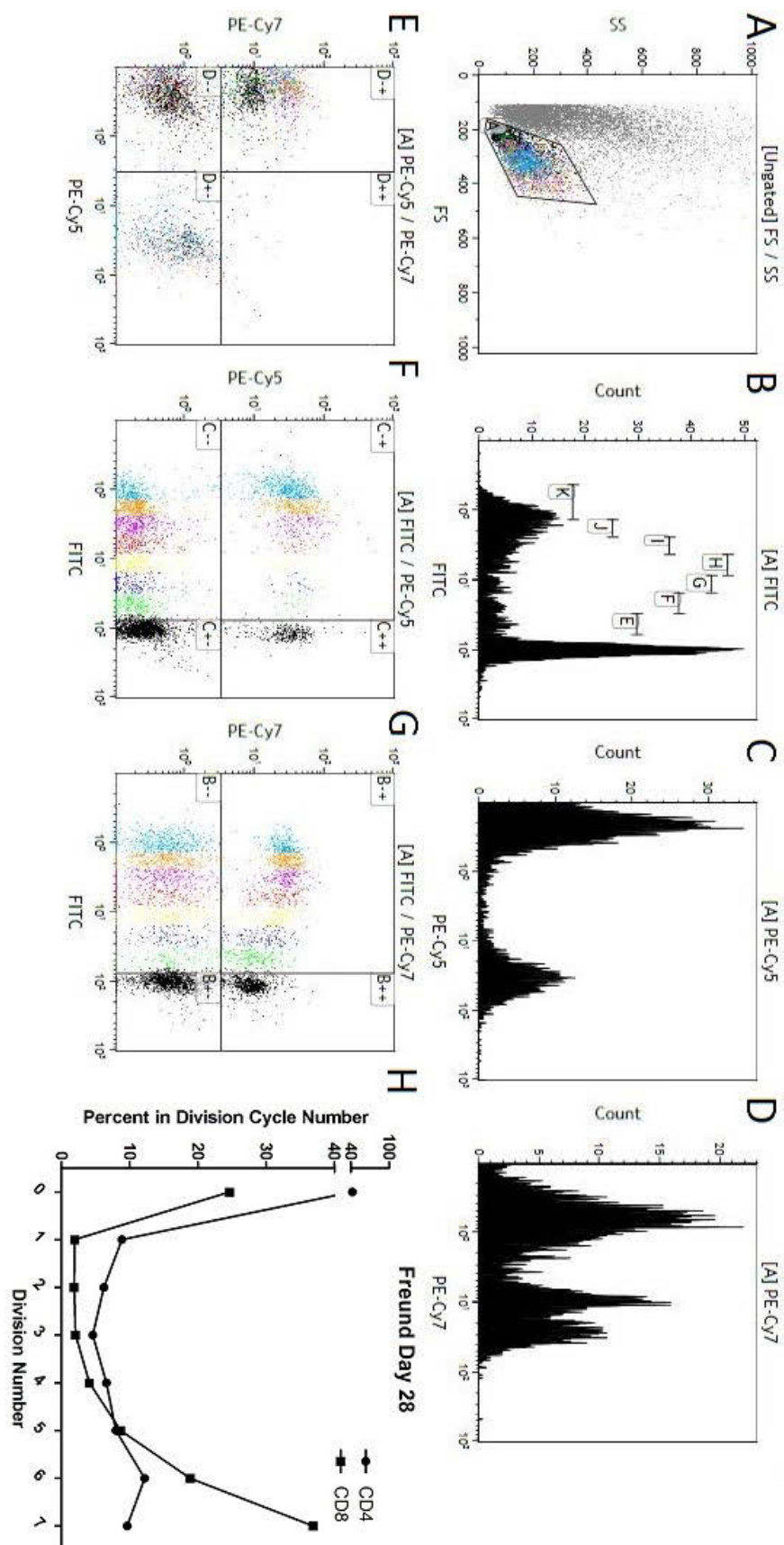


Figure 10

Proliferation of CD4⁺ T cells 1 day post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). The division kinetics of each group is similar with the exception of one mouse in Alum 0.50 and the mice in Alum 0.75 that have greater percentage of the cell population in division number 3, 4, and 5.

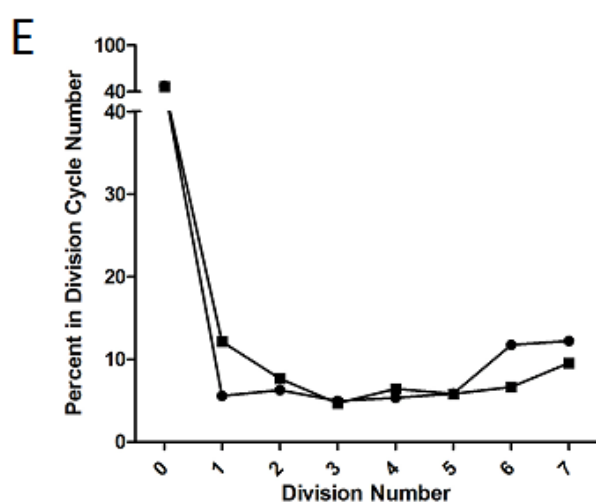
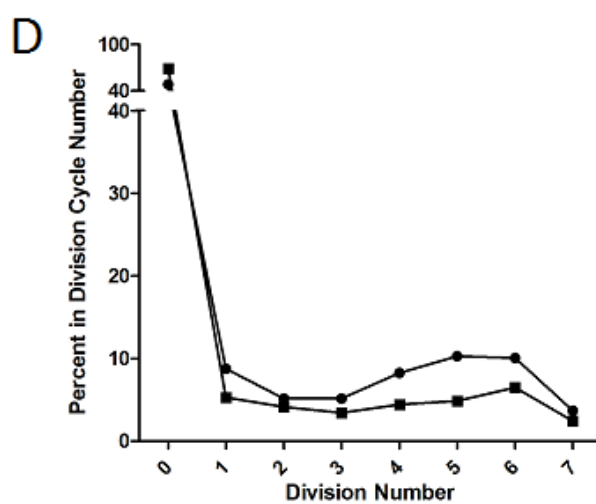
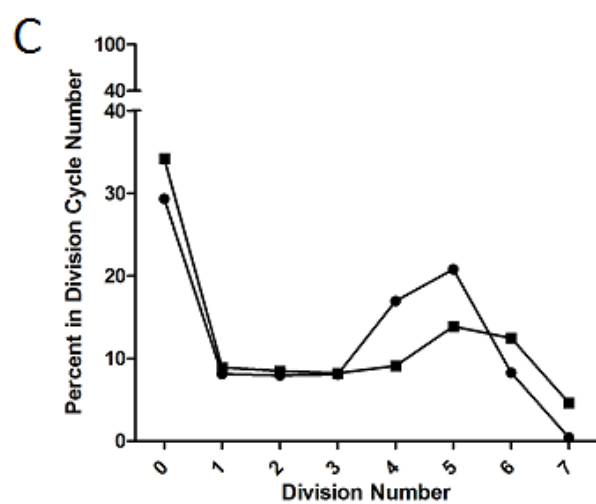
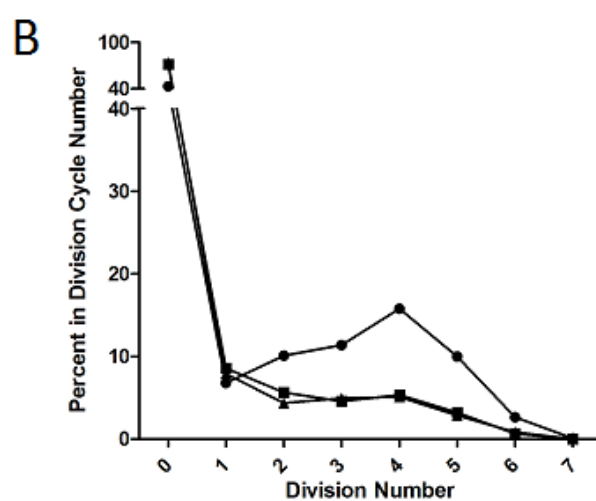
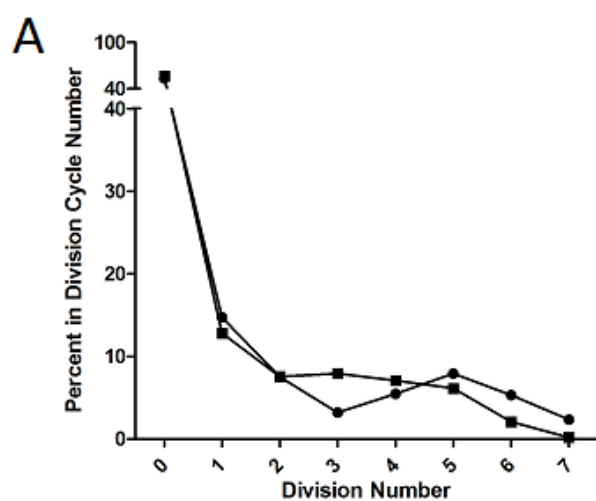


Figure 11

Proliferation of CD4⁺ T cells 3 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Alum sham group has consistent percentages of division beyond division cycle 5 whereas the other groups show a waning of cells present in the furthest divisions.

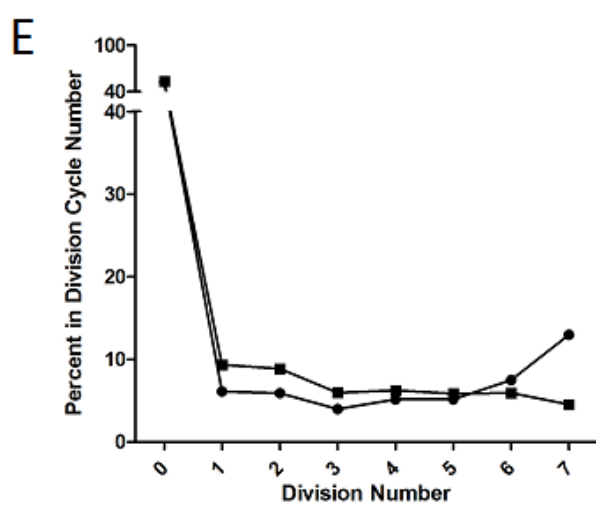
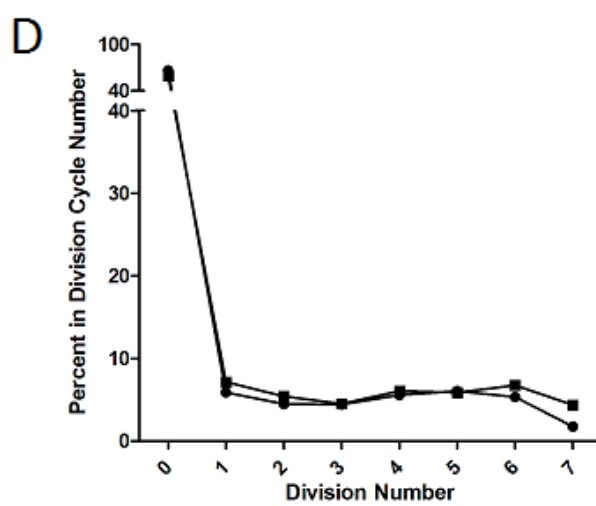
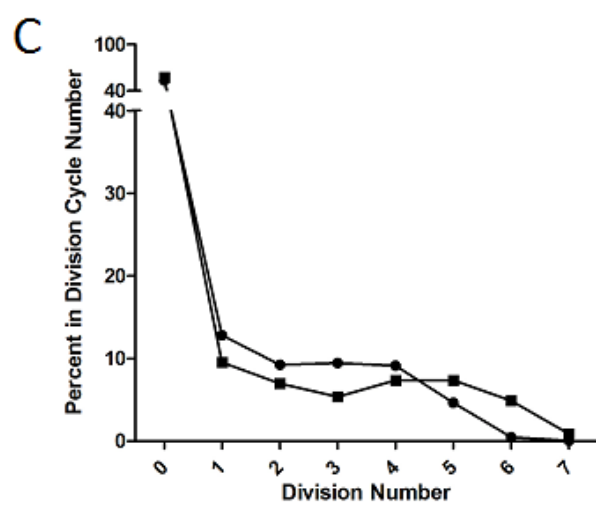
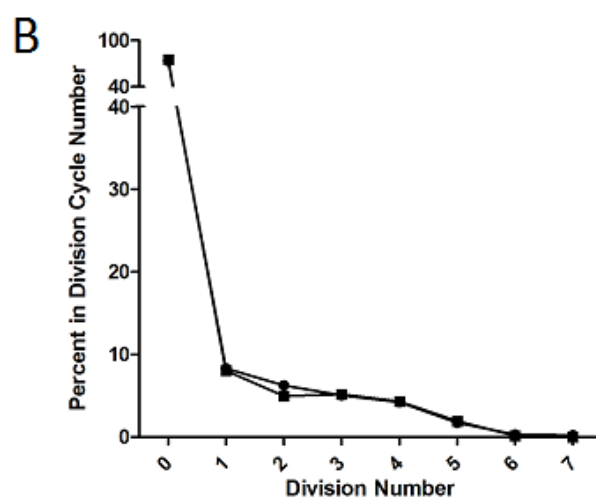
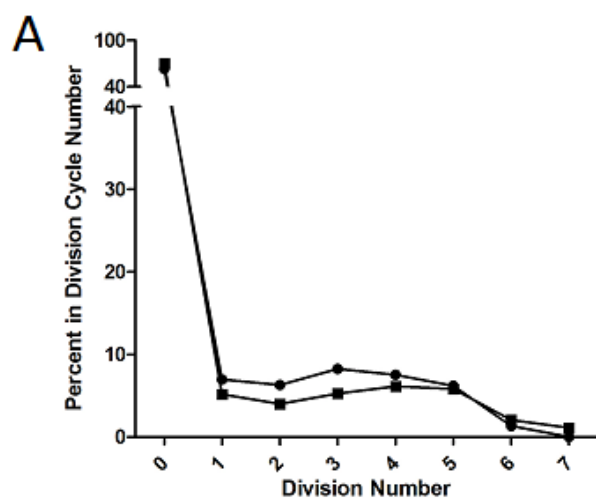


Figure 12

Proliferation of CD4⁺ T cells 7 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). One mouse each from Alum 0.75 and Alum sham had 10% more cells in divisions greater than 0. Furthermore, the one mouse from Alum 0.75 shows the majority of the population of the cells having divided beyond division 3. All other mice show similar division kinetics.

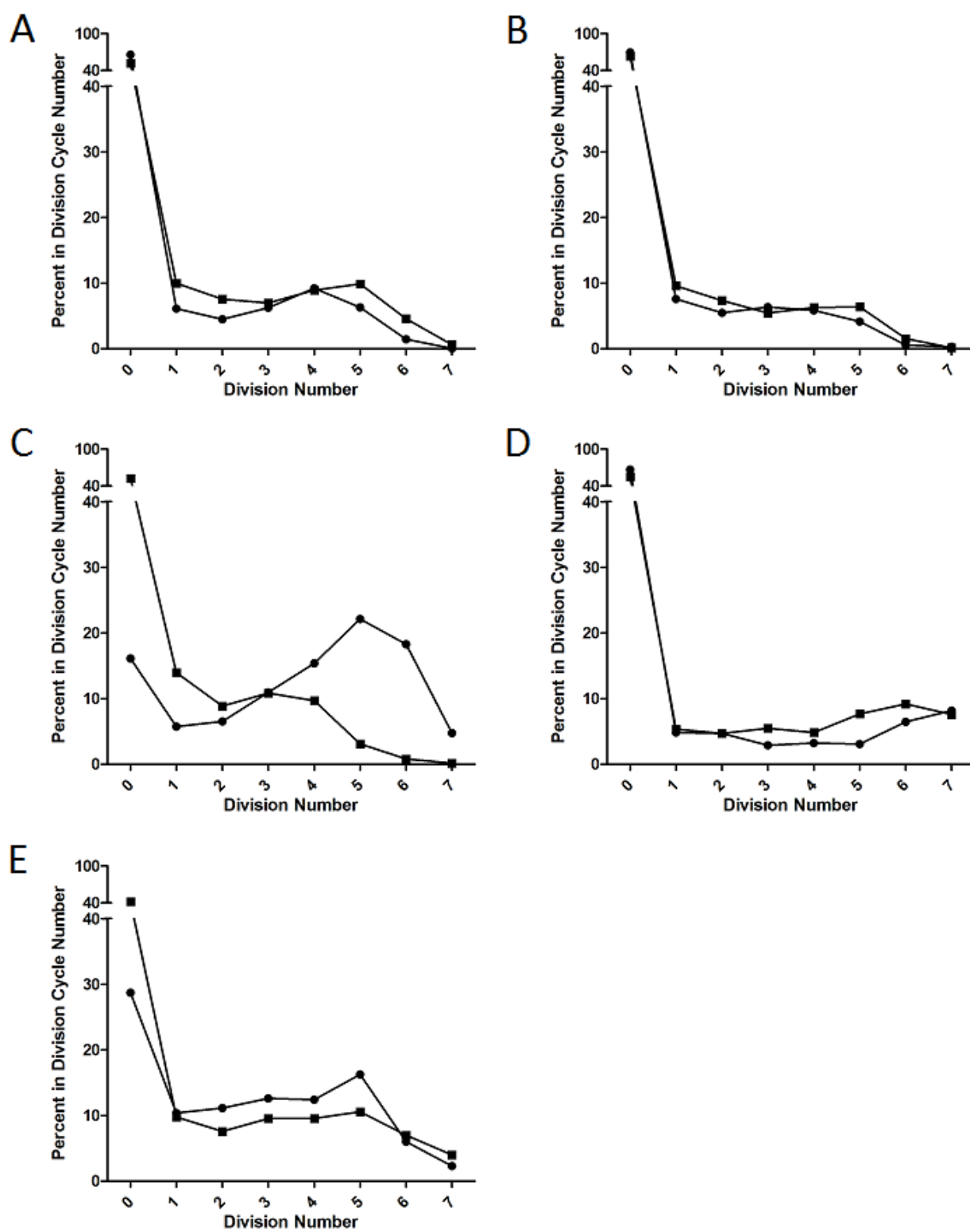


Figure 13

Proliferation of CD4⁺ T cells 14 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). One mouse from Alum 0.75 group and one mouse from unvaccinated group have a high proportion of cells in the sixth and seventh division cycles, respectively, compared to all other mice.

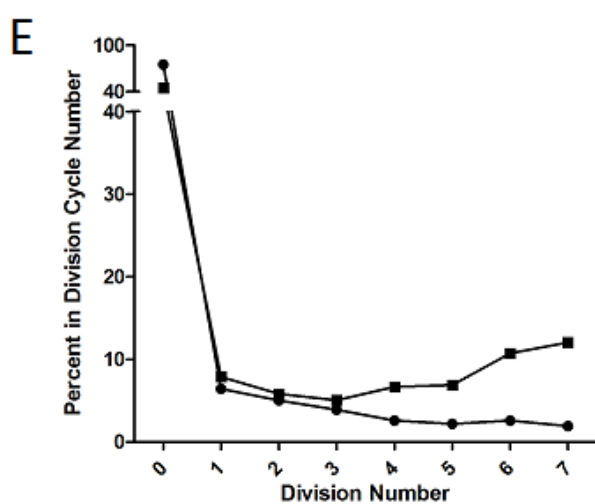
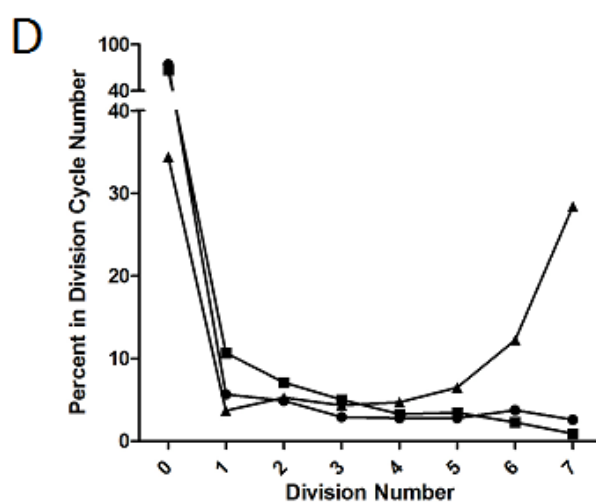
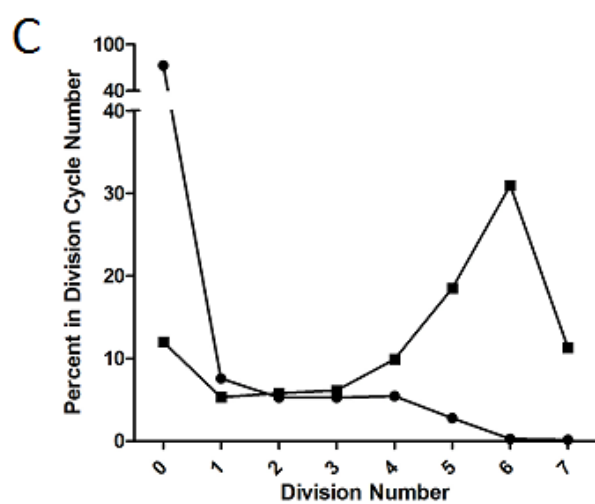
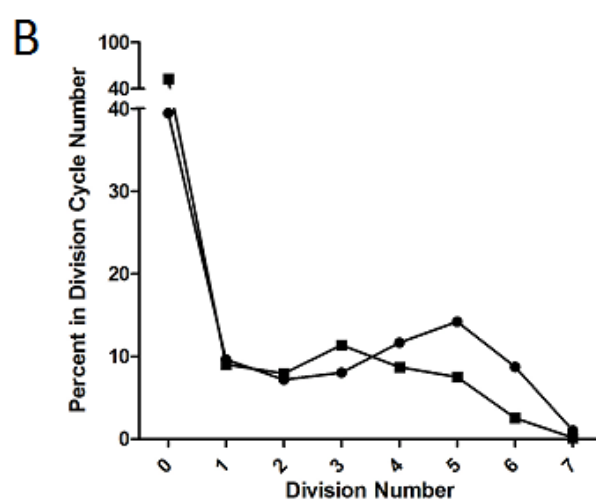
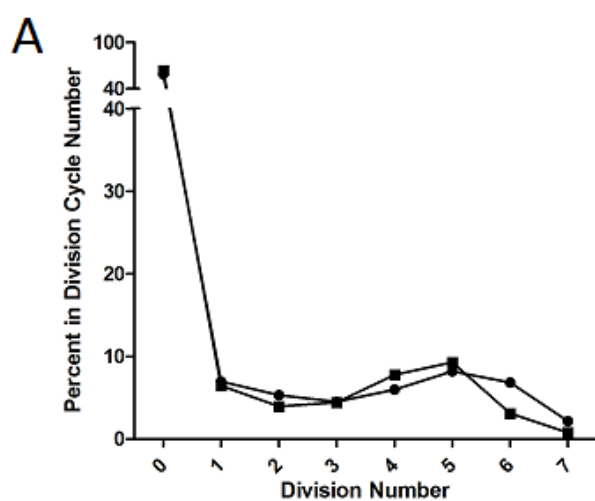


Figure 14

Proliferation of CD4⁺ T cells 21 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). No differences were observed day 21 post-infection.

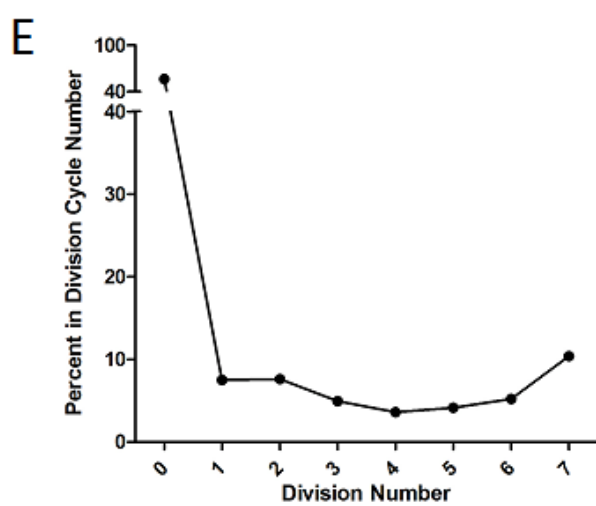
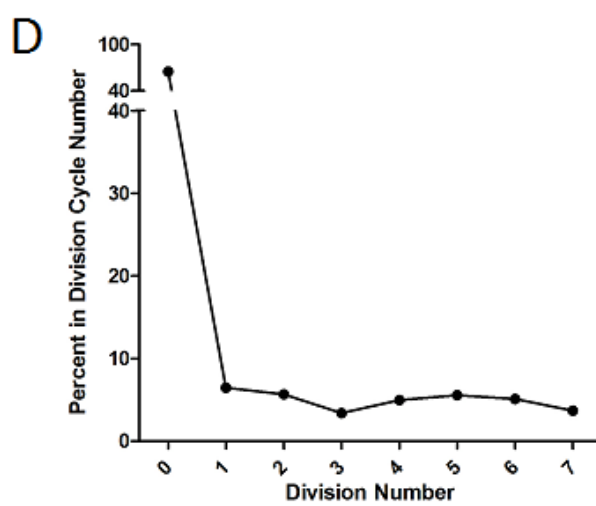
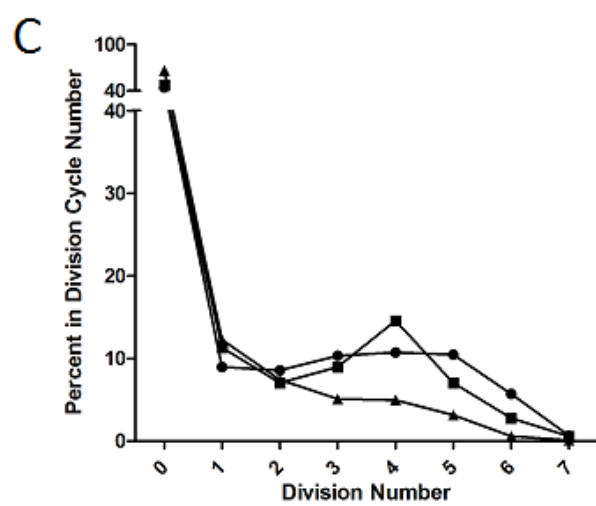
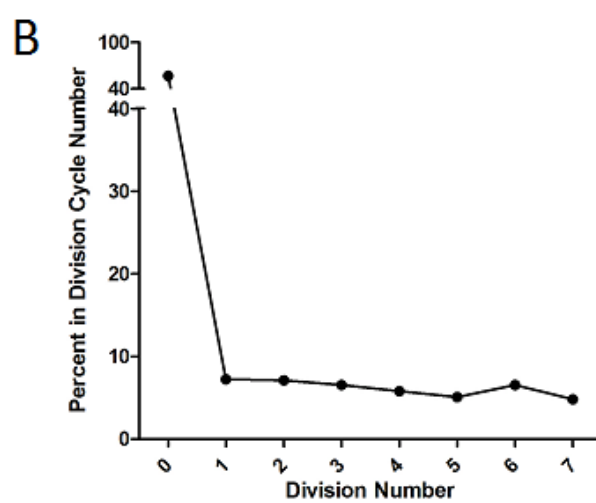
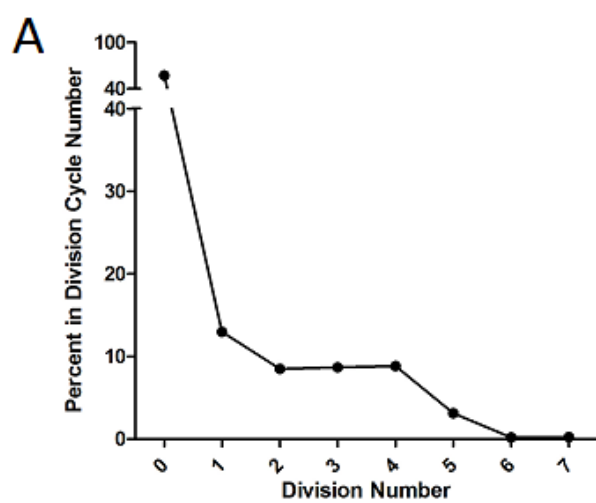


Figure 15

Proliferation of CD4⁺ T cells 28 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Unvaccinated mice had the least percent of undivided cells. Three mice of five in the unvaccinated group increased in percent divided in the sixth and seventh division cycle compared to all other mice of which percent divided declined towards the seventh division cycle. Of the two Alum 0.75 mice, one mouse had a higher proportion of cells in the earlier division cycles, and the other had a higher proportion of cells in the later division cycles, though still declined in the seventh division cycle.

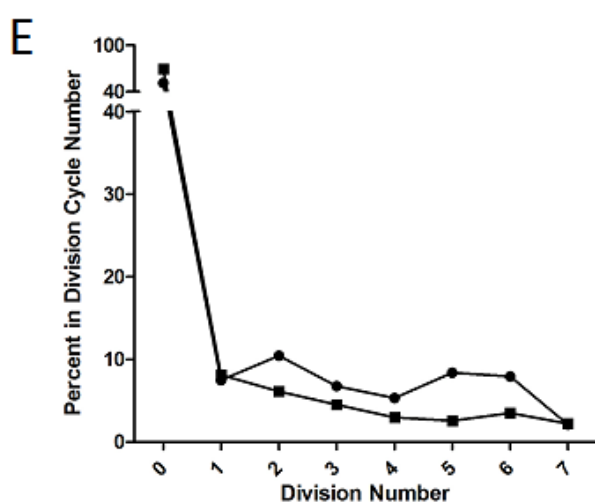
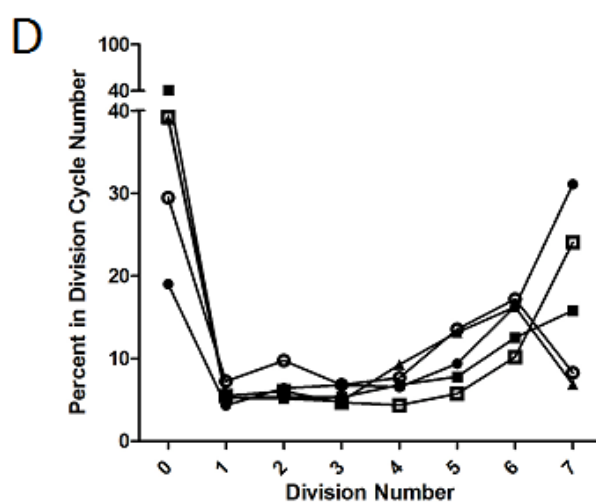
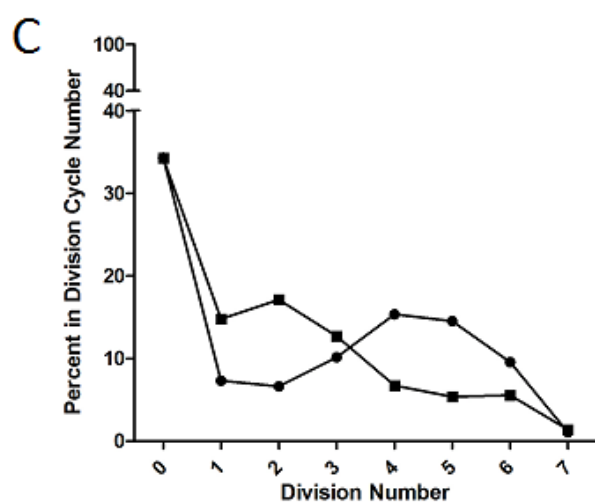
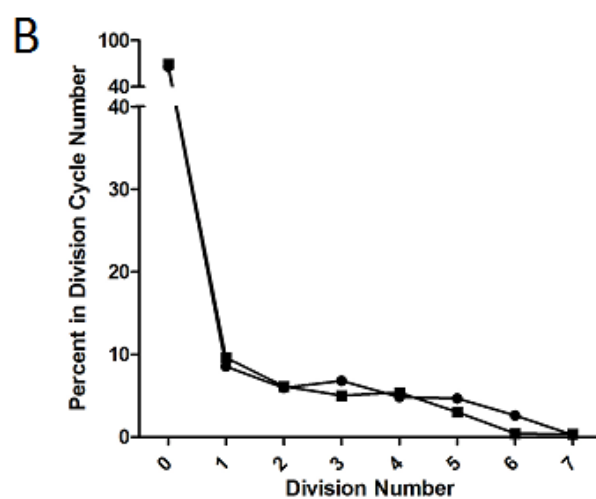
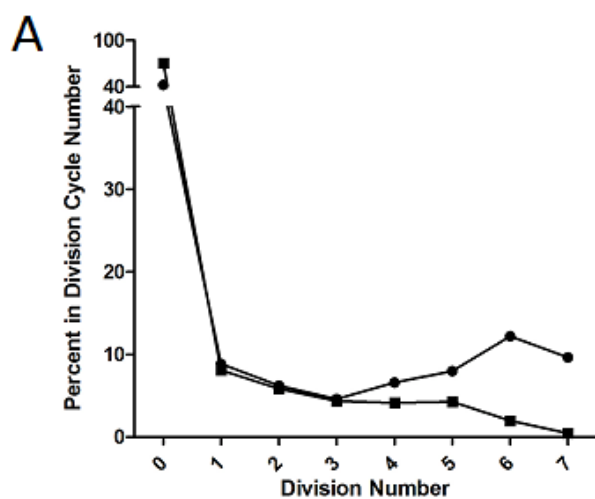


Figure 16

Proliferation of CD8⁺ T cells 1 day post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Alum 0.50 vaccinated mice had very few cells dividing. On the other hand, the remaining groups of mice appear to have a group of cells that have divided into the fourth, fifth, sixth and seventh division cycles.

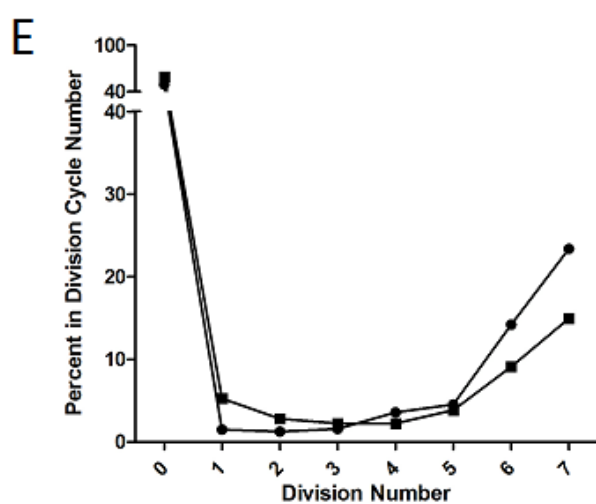
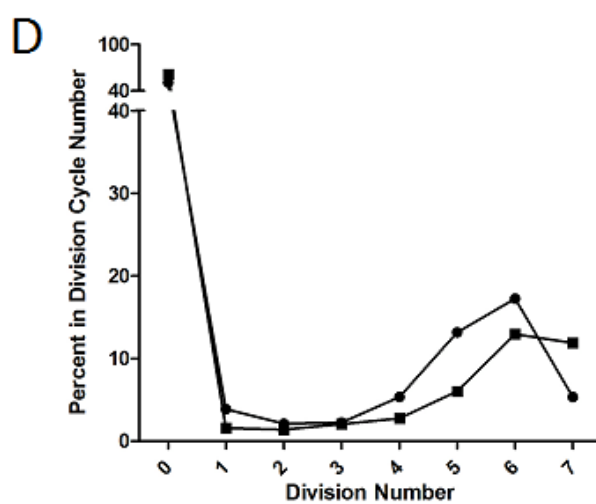
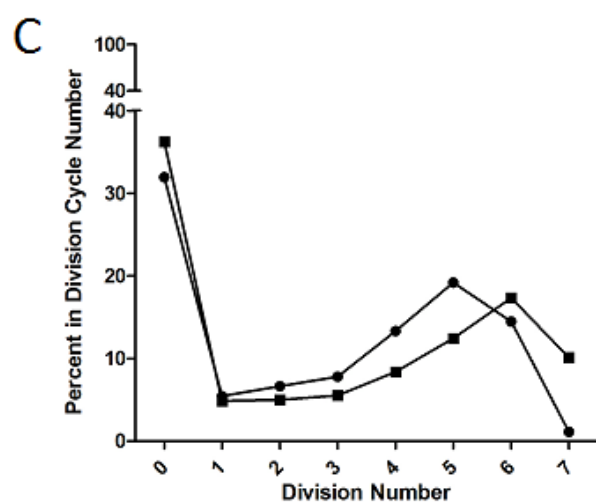
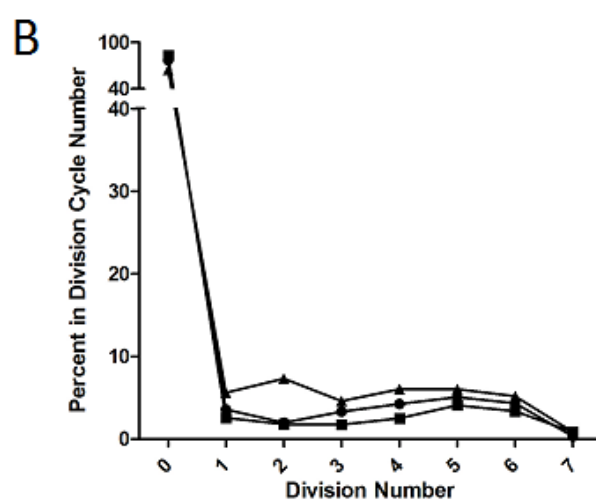
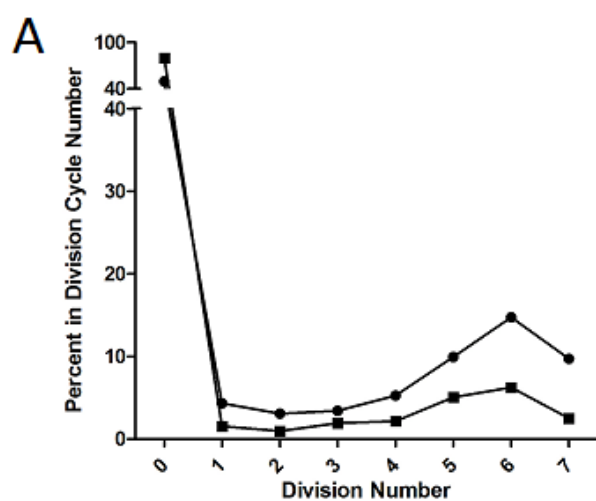


Figure 17

Proliferation of CD8⁺ T cells 3 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Similar to CD8⁺ T cells 1 day post-infection, Alum 0.50 mice had very few cells dividing. Again, the remaining groups of mice appear to have a group of cells that have divided into the fourth, fifth, sixth and seventh division cycles.

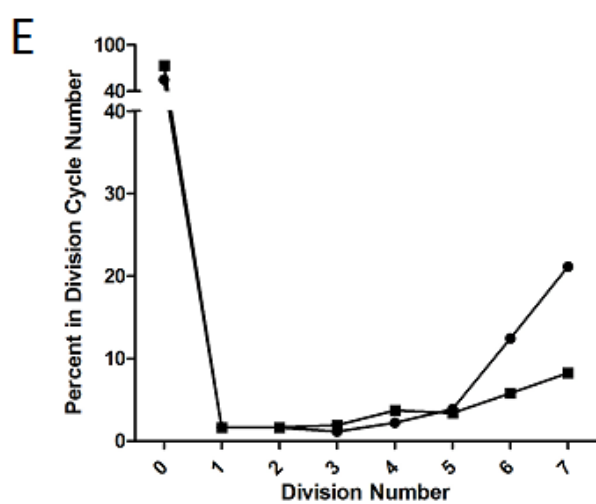
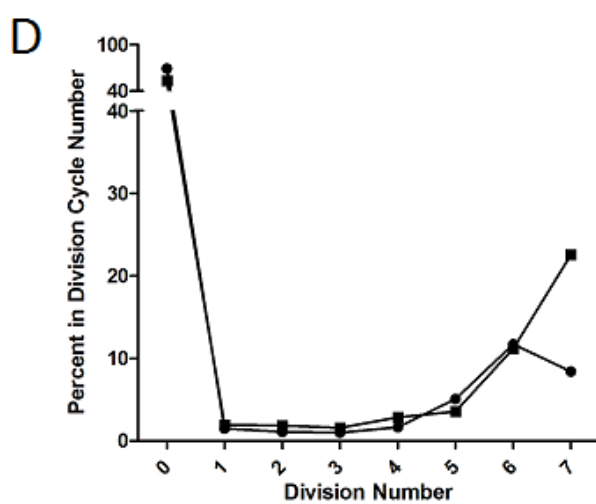
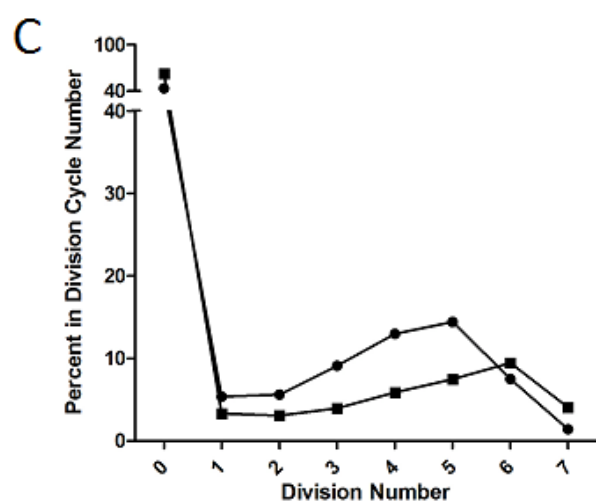
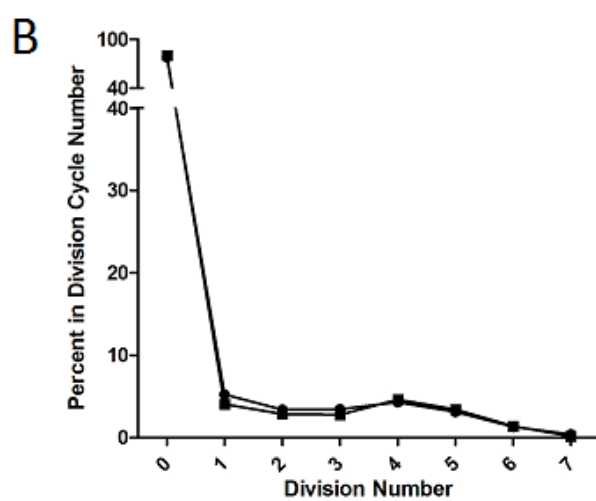
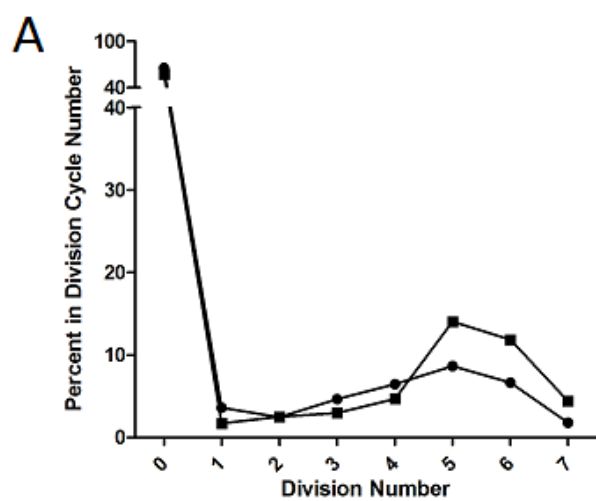


Figure 18

Proliferation of CD8⁺ T cells 7 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Freund's mice have a group of cells in the fourth to sixth division cycles. One Alum 0.50 mice have a small group of cells divided into the fourth and fifth division cycles. One mouse from Alum 0.75 has significant division of the total CD8⁺ T cell population. Unvaccinated mice have groups of cells in the sixth to seventh division cycles. One Alum sham mouse has a small proportion of cells in the sixth to seventh division cycles, while very little division is observed of CD8⁺ T cells in the other Alum sham mouse.

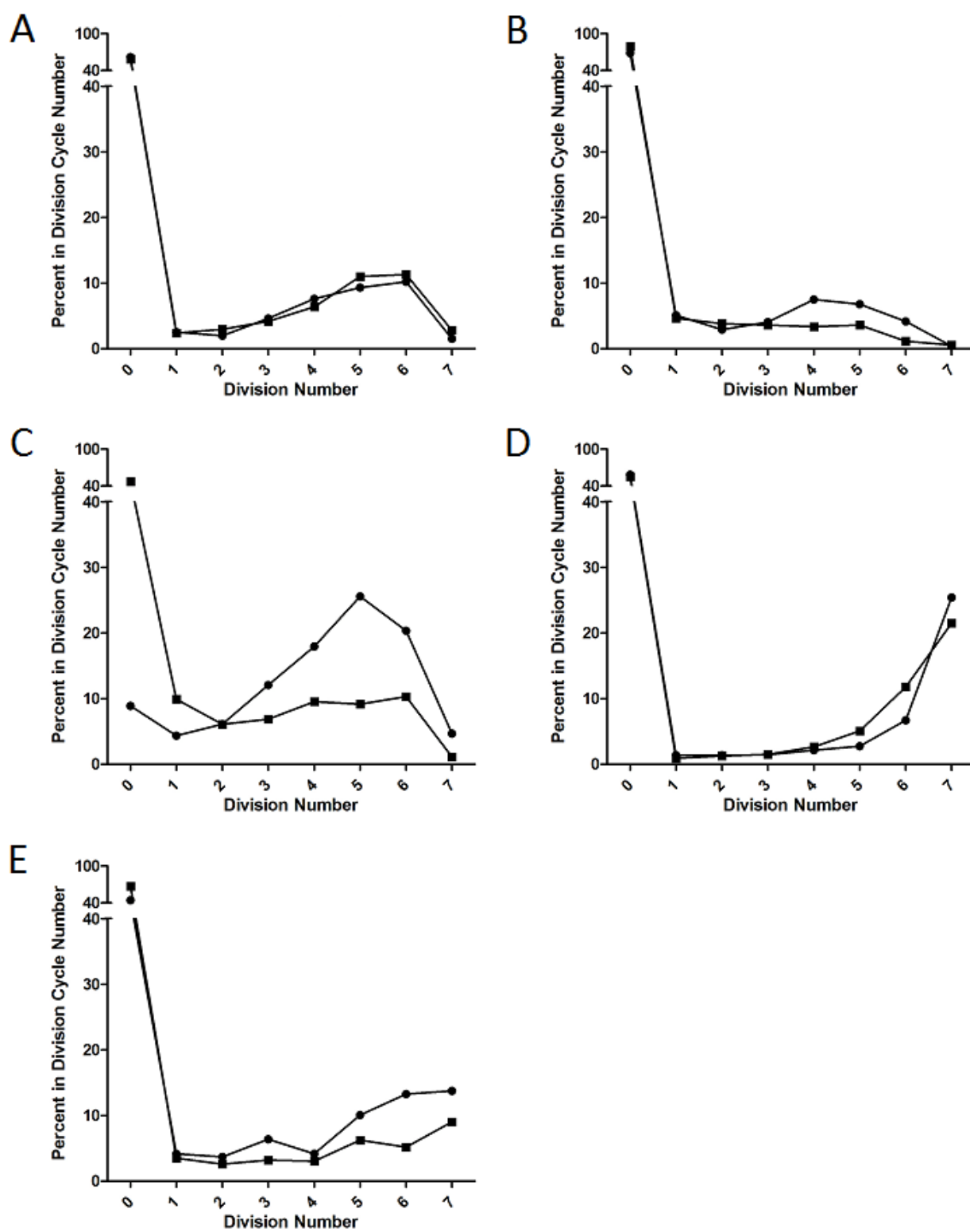


Figure 19

Proliferation of CD8⁺ T cells 14 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). All groups of mice have a peak of divided cells above 10% of the total population beyond the fourth division cycle. A proportion of cells above 30% of the total cell population can be found in a single cell division cycle of Alum 0.75 vaccinated, unvaccinated, and Alum sham groups.

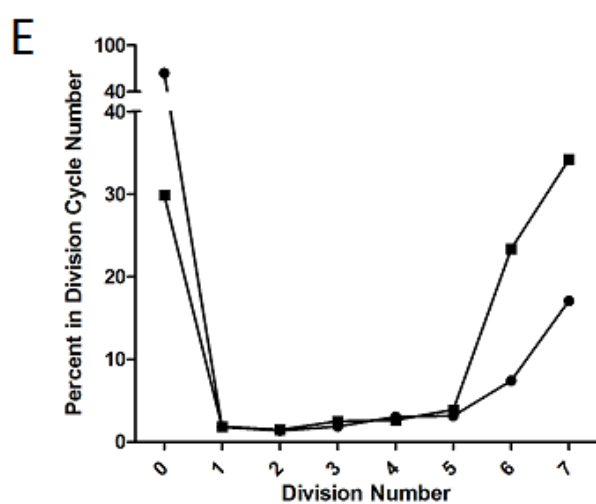
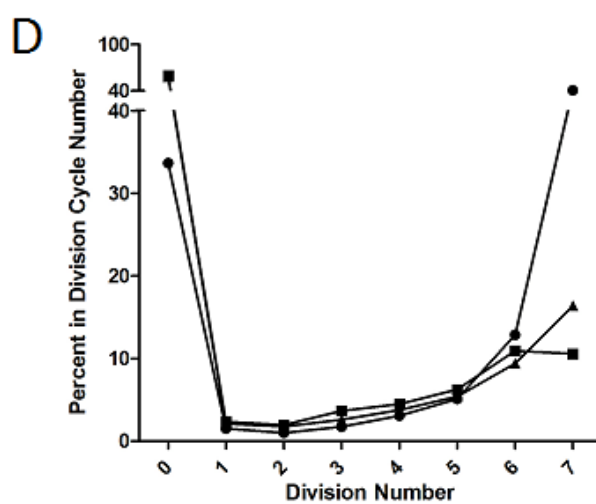
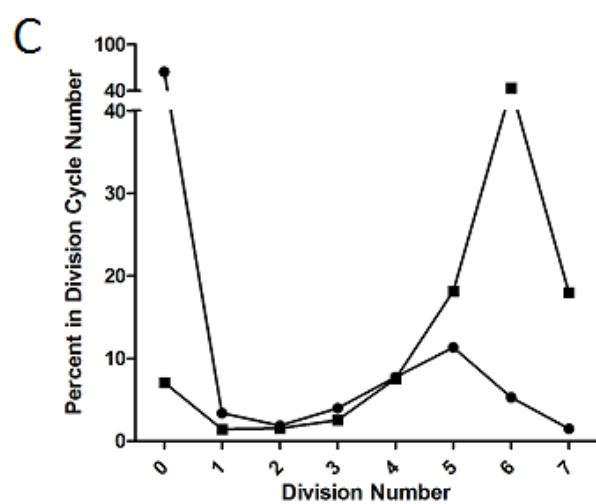
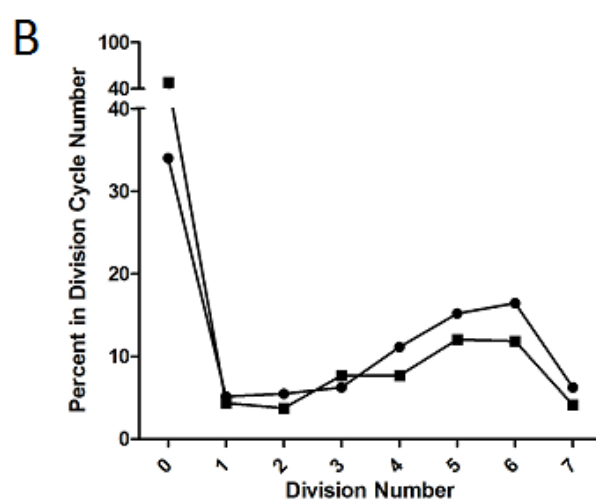
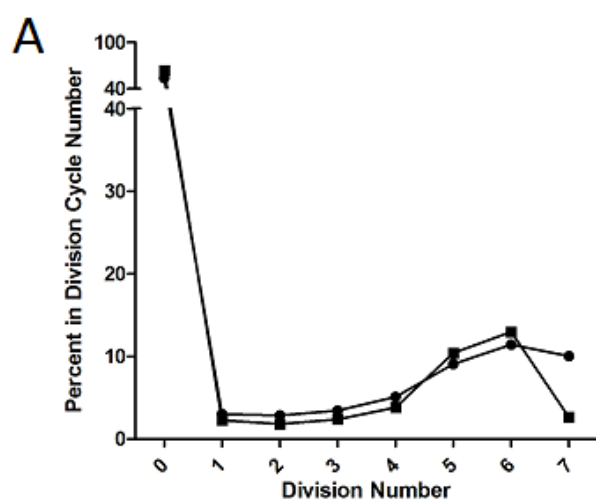


Figure 20

Proliferation of CD8⁺ T cells 21 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). One Freund's mouse has a peak of divided cells in the earlier division cycles. All other groups have peaks of divided cells in the later division cycles.

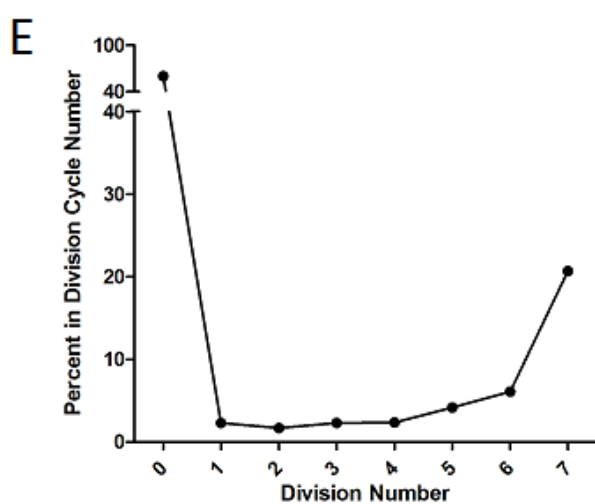
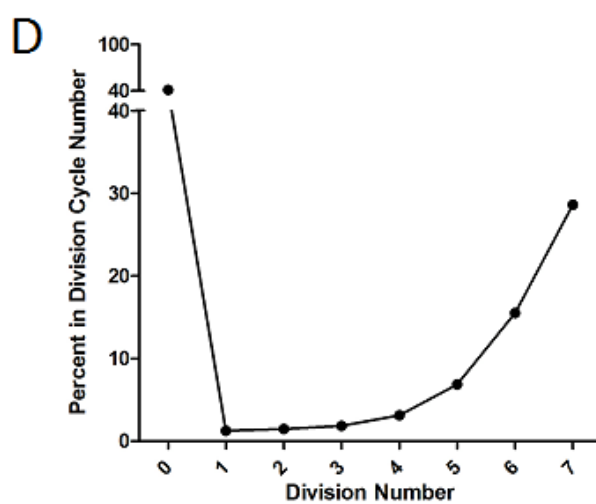
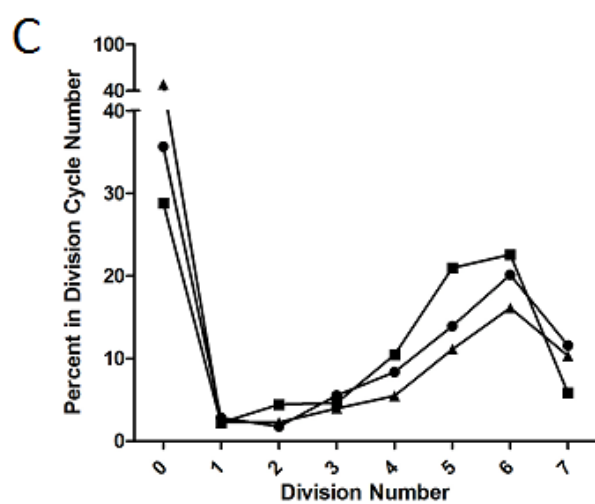
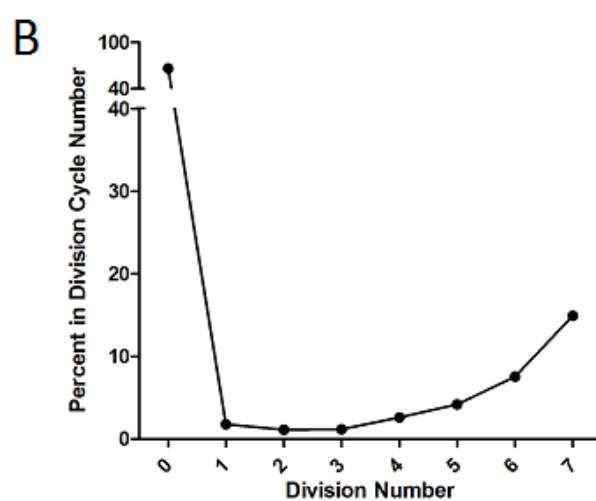
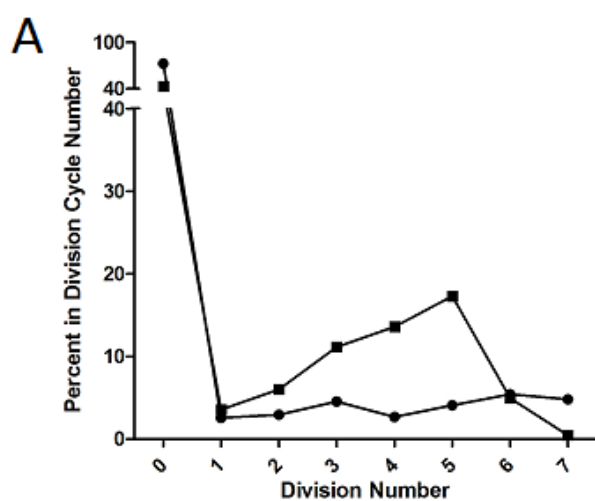
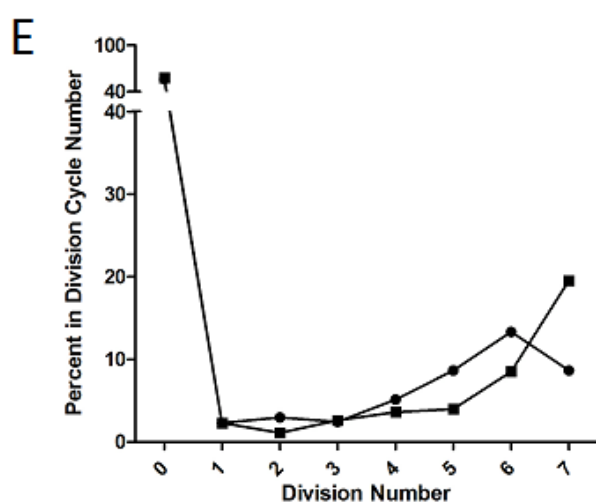
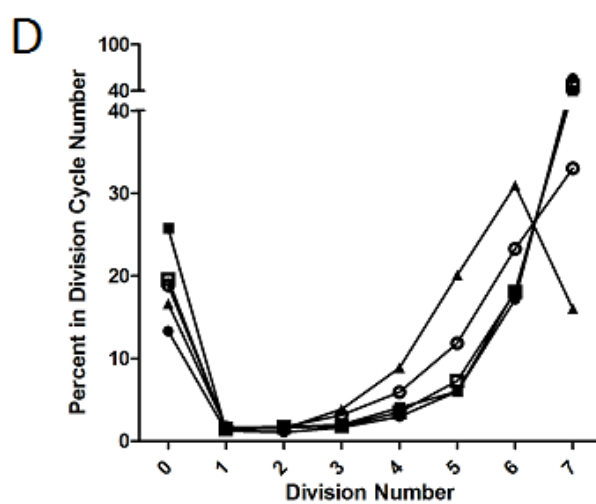
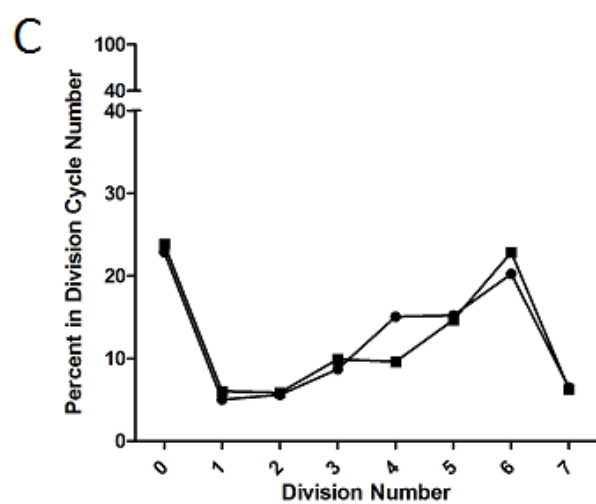
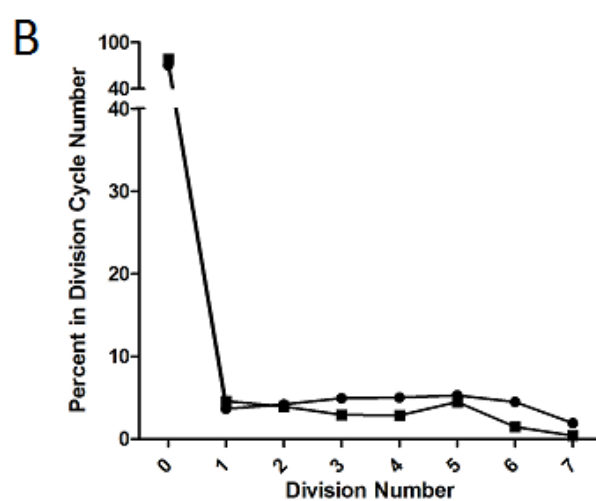
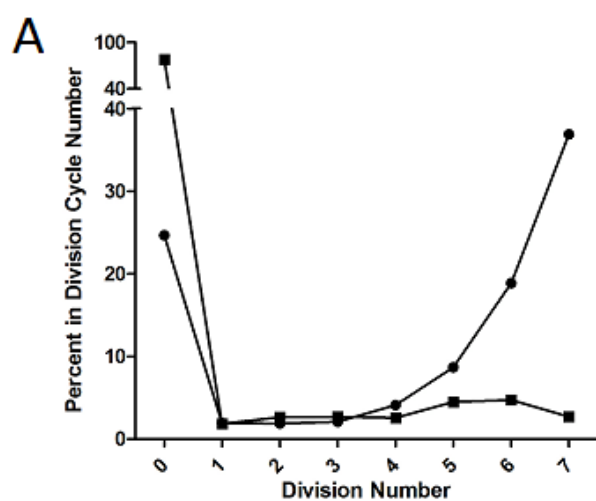


Figure 21

Proliferation of CD8⁺ T cells 28 days post-infection. Each set of points connected by a line represents one mouse sample. Graphs are separated by groups of mice: Freund's (A), Alum 0.50 (B), Alum 0.75 (C), unvaccinated (D), and Alum sham (E). Unvaccinated mice show a high consistency of a large proportion of the CD8⁺ T cell population divided into the sixth and seventh division cycle. Little to no division is observed in the Alum 0.50 group and one of two Freund's vaccinated mice. The other Freund's vaccinated mouse shows similar division to unvaccinated mice. The majority of cells from the Alum 0.75 vaccination group are divided into the fourth to seventh division cycles. Alum sham mice have a peak of divided cells in the sixth and seventh division cycles, but overall have a low total proportion of divided cells to undivided cells.



3.4 Vaginal Histology

To qualitatively investigate adaptive cellular mediated immunity presence in the vaginal tissue of vaccinated and unvaccinated mice, immunohistochemical staining was performed on vaginal tissues. Two serial sections of vaginal tissue for each antibody were examined by light microscope at 40x magnification to count the number of total cells detected on a given tissue sample. Distribution of cells in the vaginal tissue was not uniform. Therefore, viewing only one field of view at 40x magnification would not provide an accurate assessment of the presence of cells in the tissue. The rating of number of cells detected in the vaginal tissue was categorized as none (0 cells), rare (1-5 cells), few (6-15 cells), or some (>15 cells). Cells were labelled using a rat anti-mouse antibody (CD4, CD8a, and CD19 for CD4⁺, CD8⁺, and pan-B cell staining). Secondary goat anti-rat antibodies were added, endogenous peroxidase activity quenched and peroxidase labelling and resolution by an avidin-biotin-peroxidase complex with diaminobenzidine method. Both vaginal and spleen tissues stained without secondary antibody served as negative controls (Figure 22). Spleens stained with varying dilutions of primary antibody were used to determine optimum antibody dilution and were used as positive controls (Figure 23). An untreated mouse was sacrificed as a control. No CD4, CD8, or B cell labelling was detected in this mouse.

3.4.1 CD4 Antibody

The ratings of number of detected CD4⁺ cells are summarized in Table 3. CD4⁺ cells were not detected in any of the uninfected control tissues. In infected mice, CD4⁺ cells were detected in 61.5%, 50.0%, 53.8%, 50.0%, and 31.2% for Freund's, Alum 0.50, Alum 0.75, Alum sham, and unvaccinated, respectively. A rating of few or some CD4⁺ cells was

detected in 30.8%, 21.4%, 38.5%, 33.3%, and 12.5% for Freund's, Alum 0.50, Alum 0.75, Alum sham, and unvaccinated, respectively. Therefore, vaginal tissues from vaccinated and sham vaccinated mice were more likely to have CD4⁺ cells present in the vaginal tissue, and to have more CD4⁺ cells per tissue versus unvaccinated mice. Also, it was more likely to find CD4⁺ cells early post-infection up to day 14 than later in infection (day 21 or day 28). Detection of CD4⁺ cells was not specific to mice that were culture positive for *T. vaginalis*. CD4⁺ cells were also detected in vaginal tissues of mice without a current *T. vaginalis* infection (culture negative). Thus, culture positivity was not a predictor of detection of CD4⁺ cells; however, vaginal challenge lead to CD4⁺ cell presence. Overall, vaccination increases the prevalence of CD4⁺ cells in vaginal tissues following infection compared to unvaccinated controls. Typically, ratings of rare or few CD4⁺ counts (Figure 24) were associated with localization in the adventitia. Ratings of some (Figure 25) were typically clusters of CD4⁺ cells in the lamina propria. CD4⁺ cells were not detected in the vaginal epithelium.

3.4.2 CD8 Antibody

No CD8⁺ labelling beyond background was detected in any of the tissues sampled. Methodological issues are not suspected given the results from positive and negative controls. Representative tissues are presented in Figure 26.

3.4.3 CD19 Antibody

No CD19 labelling beyond background was detected in any of the tissues sampled. Methodological issues are not suspected given the results from positive and negative controls. Representative tissues are presented in Figure 27. Plasma cells were not assessed.

Figure 22

Negative control tissues (primary antibody labelling omitted). No specific labelling of CD4 (A and B), CD8 (C and D), and CD19 (E and F) antibodies in murine spleen (A, C, and E) and vaginal tissue (B, D, and E). Representative vaginal tissues shown from random vaccination groups. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 100x.

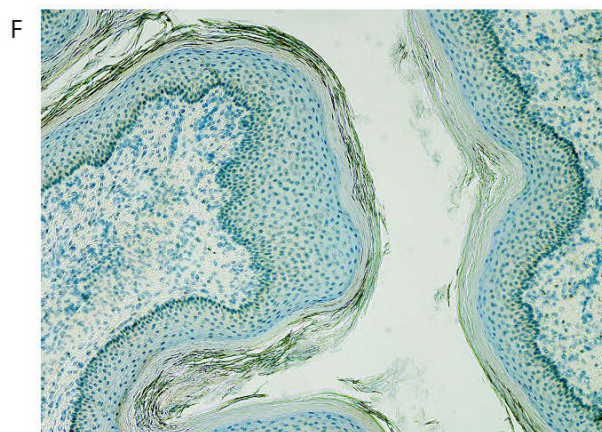
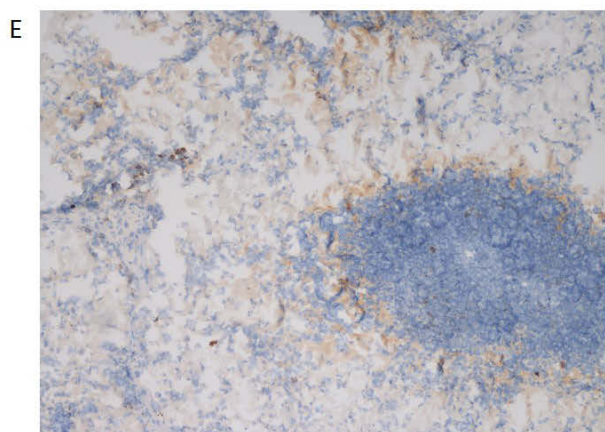
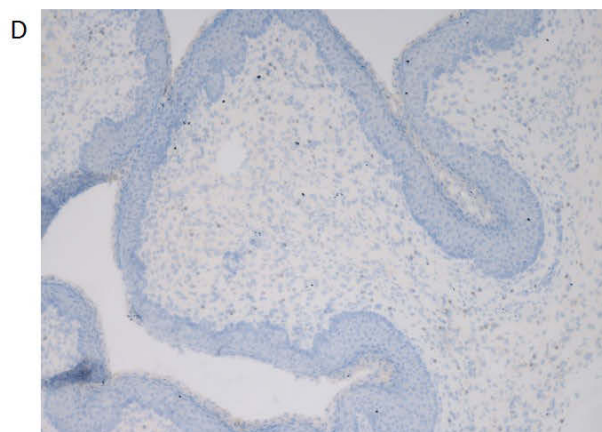
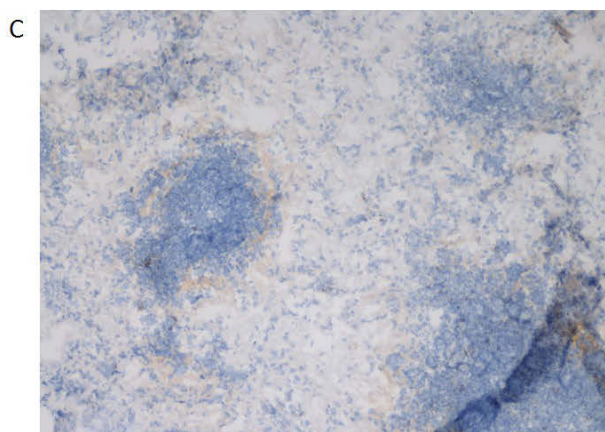
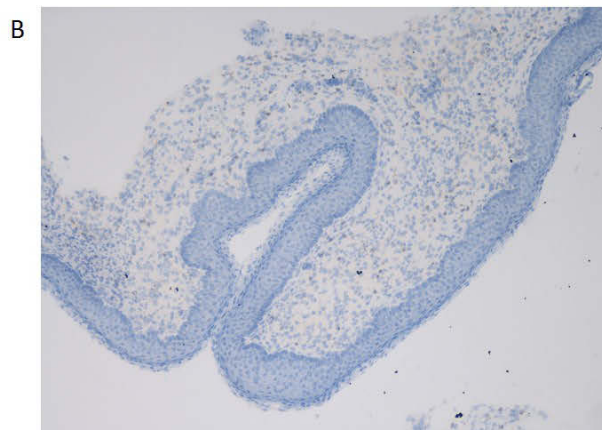
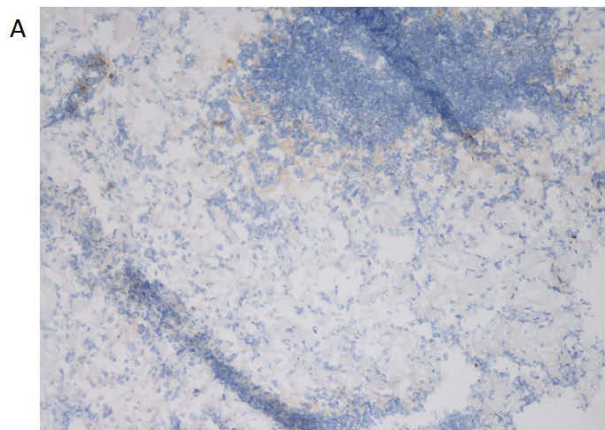
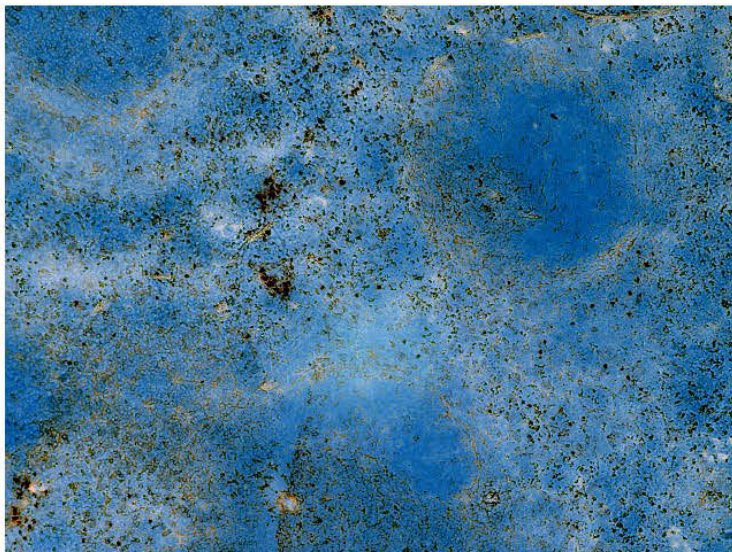


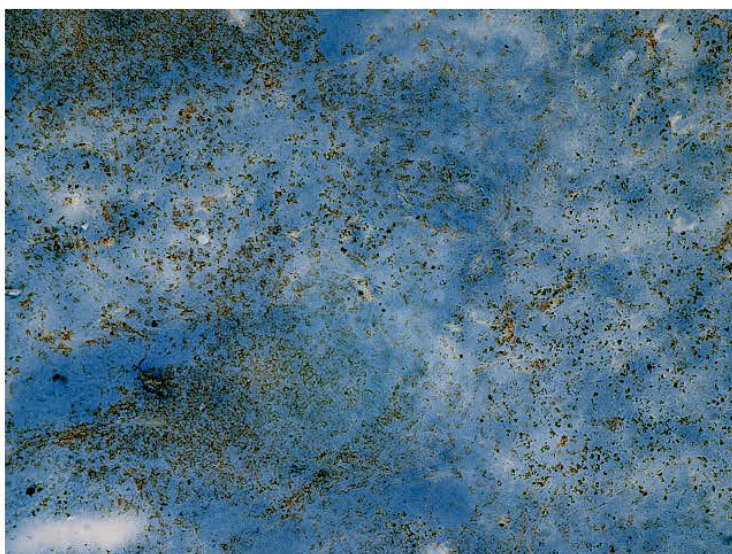
Figure 23

Positive control tissues. Detection of specific labelling using CD4 (A), CD8 (B), and CD19 (B cell) (C) antibodies in murine spleen tissue. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 100x.

A



B



C

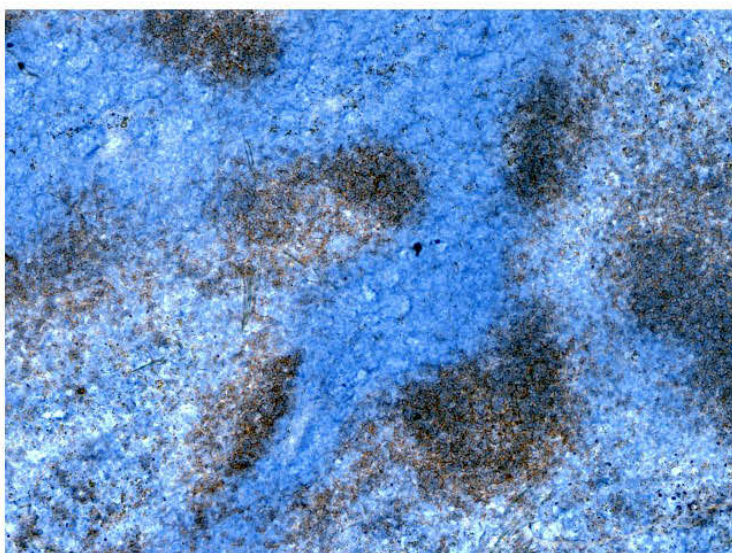


Figure 24

Positive immunoperoxidase staining with CD4 antibodies in murine vaginal tissue. Images are representative of rare (A and B) and few (C and D) cell count ratings for Freund's, Alum 0.50, Alum 0.75, unvaccinated and Alum sham groups. Arrows denote location of CD4 cell. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 200x (A and C) and 400x (B and D).

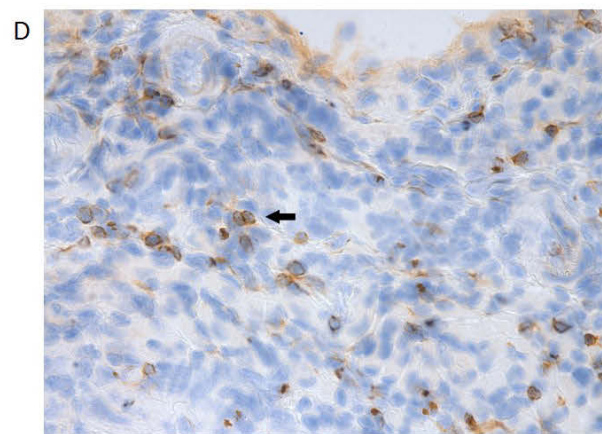
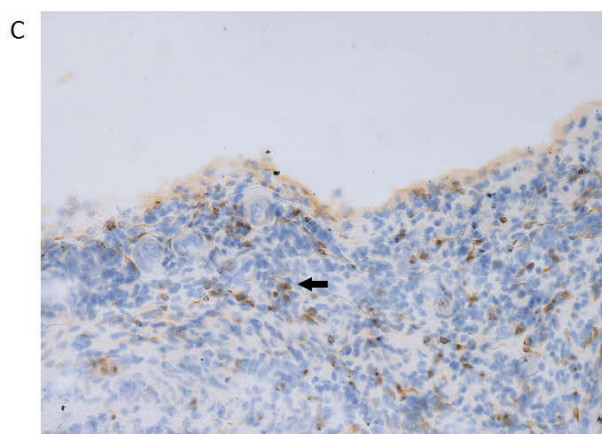
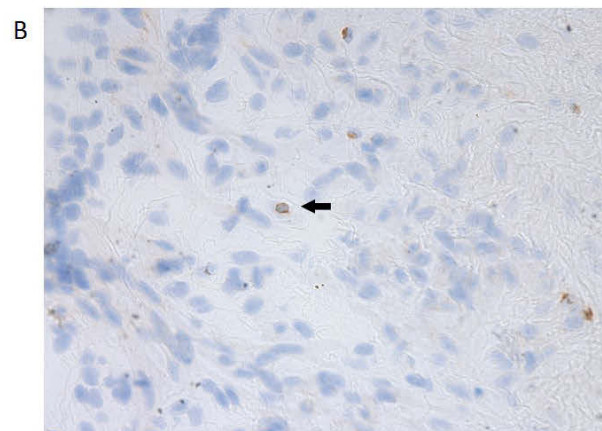
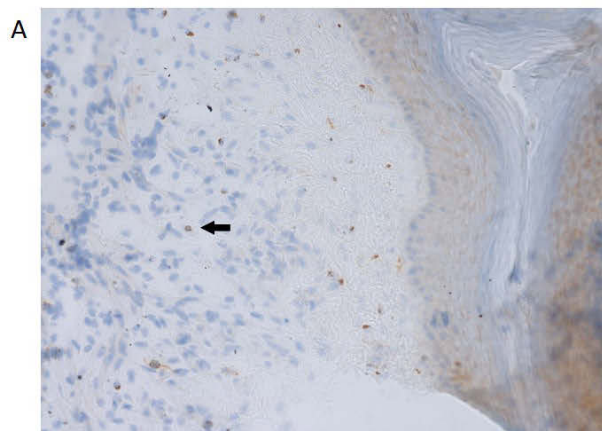


Figure 25

Positive immunoperoxidase staining with CD4 antibodies in murine vaginal tissue. Images are representative of some cell count rating for Freund's, Alum 0.50, Alum 0.75, and unvaccinated groups. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 200x (A) and 400x (B).

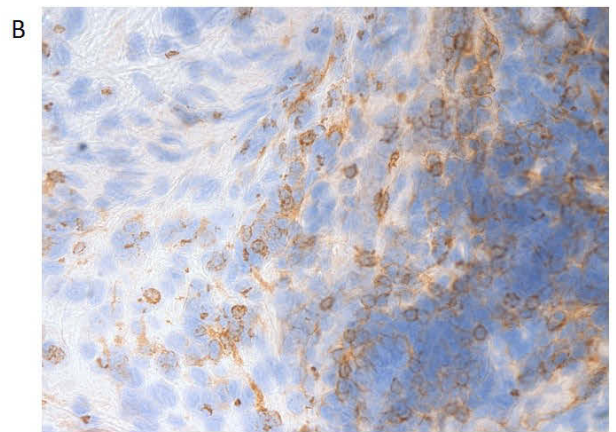
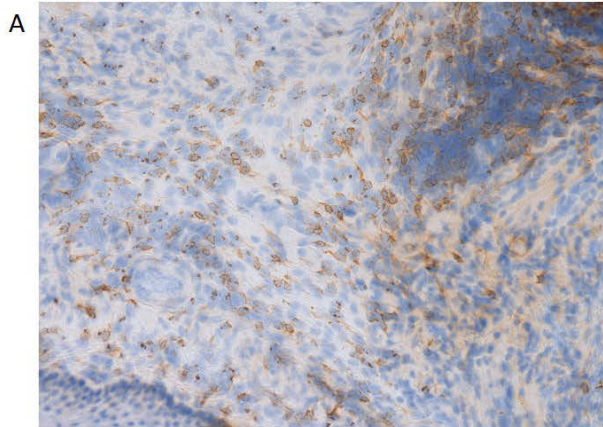


Figure 26

Immunoperoxidase staining was not seen when using CD8 antibodies in murine vaginal tissue day 1, 3, 7, 14, 21, and 28. For brevity, a randomly selected tissue was used as representative of tissues for Freund's, Alum 0.50, Alum 0.75, unvaccinated and Alum sham groups at any time point. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 100x (A) and 200x (B).

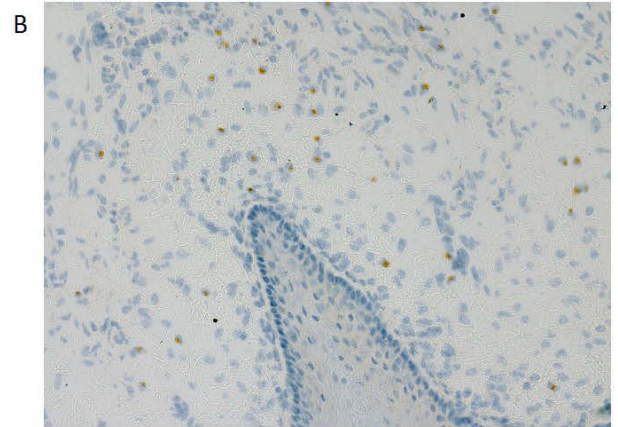
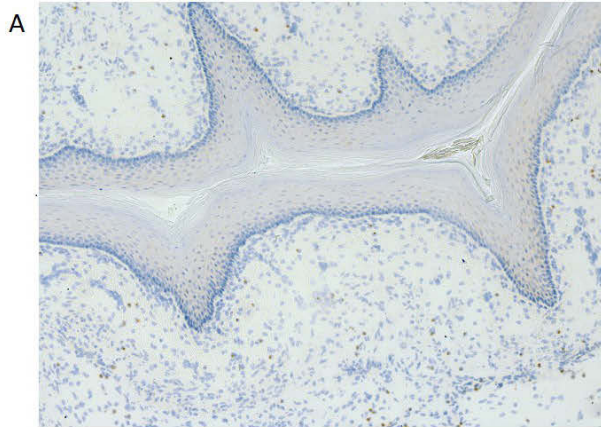


Figure 27

Immunoperoxidase staining was not seen when using CD19 antibodies (B cell) in murine vaginal tissue day 1, 3, 7, 14, 21, and 28. For brevity, a randomly selected tissue was used as representative of tissues for Freund's, Alum 0.50, Alum 0.75, unvaccinated and Alum sham groups at any time point. Frozen sections were cut on cryostat at 7 μ m thickness. Original magnification 100x (A) and 200x (B).

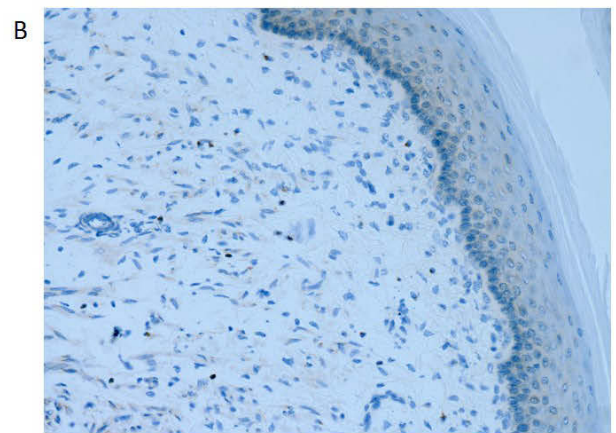
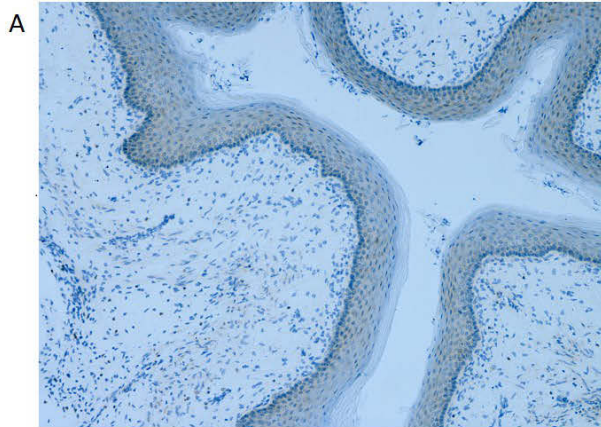


Table 3

Detection of CD4⁺ cells in murine vaginal tissue sections following immunoperoxidase labelling of CD4 antigen. Samples were obtained before infection (day -2) and at various time points after *T. vaginalis* challenge (Day 1, 3, 7, 14, 21 and 28). Representative tissue sections can be found in Figure 24 and Figure 25. Tissues were screened in duplicate from serial sections. Counts are indicative of whole-tissue prevalence.

Tissue Harvest Date	Number of samples with indicated CD4 prevalence				
	Freund	Alum 0.50	Alum 0.75	Alum Sham	Unvaccinated
Pre-infection ^a					
None	1	1	1	1	1
Rare	0	0	0	0	0
Few	0	0	0	0	0
Some	0	0	0	0	0
Day 1					
None	0	1	1	1	2
Rare	1	2	0	0	0
Few	1	0	0	1	0
Some	0	0	1	0	0
Day 3					
None	0	2	1	1	1
Rare	1	0	0	1	1
Few	0	0	0	0	0
Some	1	0	1	0	0
Day 7					
None	3	0	0	1	3
Rare	0	1	0	0	0
Few	0	0	1	0	0
Some	0	2	1	1	0
Day 14					
None	0	1	0	0	0
Rare	1	1	1	0	1
Few	0	0	1	1	0
Some	1	1	0	1	1
Day 21					
None	1	1	2	1	3
Rare	1	0	1	1	1
Few	0	0	0	0	0
Some	0	0	0	0	0
Day 28					
None	1	2	2	2	2
Rare	0	0	0	0	0
Few	1	0	0	0	0
Some	0	0	0	0	1

^a An untreated mouse was sacrificed for immunohistochemistry and consequently categorized as no CD4 presence.

4. Discussion

4.1 *Trichomonas vaginalis* Infection and Immunity

Trichomonas vaginalis infection in humans and experimental animal models do not result in protection against reinfection (Abraham et al., 1996; Petrin et al., 1998). Indeed, reinfection is a problem for at risk demographics including sex workers, people who engage in risky sexual behaviours, and lack of partner directed treatment (Hobbs et al., 2006; Hollman et al., 2010; Shuter et al., 1998; Sutton et al., 2007). Additional infections can be attributed to lack of adherence to treatment as well as treatment failure (Peterman et al., 2009; Gatski and Kissinger, 2010). Repeat infections or untreated infections are particularly adverse to pregnant women with respect to pregnancy outcomes and to the acquisition and transmission of HIV (Cotch et al., 1997; Fichorova, 2009; Mavedzenge et al., 2010).

Metronidazole is an effective drug for treatment of trichomoniasis. However, drug resistant strains of *T. vaginalis* have been reported (Schwebke and Barrientes, 2006; Upcroft and Upcroft, 2001). A vaccine would be better suited to preventing infection especially in preventing asymptomatic carrier states which often are a reservoir of *T. vaginalis* infections. Vaccines for bovine trichomoniasis, *Tritrichomonas foetus*, have been successful in preventing infections in young bulls, reducing duration of infection in heifers resulting in greater pregnancy rates and successful birth, and producing preputial and systemic IgG₁, IgG_{2a} and IgA (Clark et al., 1983; Cobo et al., 2009; Corbeil et al., 2005; Kvasnicka et al., 1989; Kvasnicka et al., 1992). Whole cell *T. foetus* vaccines as well as subunit vaccines were employed in the bovine studies. A murine model of *T. vaginalis* infection using a whole cell vaccine has been successful to reduce the incidence of infection as well as the duration of infection compared to unvaccinated controls (Abraham et al., 1996).

This study used the same animal model and vaccination procedures as Abraham and colleagues (1996) except the use of an adjuvant formulation safe for human vaccination. Since the use of Freund's adjuvant is not safe for human applications we aimed to evaluate a vaccine formulation with an adjuvant that is approved as safe for humans. Using the OC15 *T. vaginalis* isolate, derived from a symptomatic patient, we produced a simple whole cell vaccine with two different concentrations of aluminum from a pre-made Alhydrogel adjuvant (Brenntag Biosector). Both Alhydrogel vaccines (Alum 0.50 and Alum 0.75) and the reference FCA vaccine had significantly fewer initial infections measured day 7 post-intravaginal challenge with *T. vaginalis* compared to unvaccinated mice ($p < 0.05$).

An Alum sham vaccine was not significantly different from either vaccinated or unvaccinated groups. Additional sham vaccinated mice are needed to determine whether the sample number is a factor contributing to the lack of significance or that Alum can have a nonspecific effect to prime the immune system and prevent infection. Alum contact with DC stimulates an increase of ICAM-1 and LFA-1 that are adhesion molecules to CD4 T cells and is an antigen-nonspecific function (Flach et al., 2011). If either the primed DC or Alum crystals can migrate to vaginal tissues then the DC in vaginal tissue could have tighter associations to present *T. vaginalis* antigen for the first time compared to unvaccinated mice. This could explain the discrepancy of the numbers of initial infections and the duration of infection between Alum sham and unvaccinated mice. Nevertheless, it remains that vaccinated mice are significantly protected from infection day 7 post-infection compared to no vaccination.

Four weeks following intravaginal challenge, the majority of unvaccinated mice that were infected day 7 post-infection were still infected on day 28 post-infection. On the other

hand, the majority of vaccinated mice with infections on day 7 had cleared infection by day 28. Again, the effect of sham vaccination is unclear since 5/8 of mice infected on day 7 cleared infection by day 28. No significant differences were observed between vaccinated groups.

The data suggests that vaccination in a murine model with Alhydrogel adjuvant is equally as protective as the previously employed Freund's adjuvant. This corresponds to our first hypothesis that the aluminum hydroxide vaccine would decrease infection rates and increase clearance. The protection afforded by vaccination is significant compared to receiving no vaccination. Since a protective response was attained, we investigated immunological markers of the adaptive immune system to determine differences between vaccinated and unvaccinated mice before and after infection.

4.2 Serum Ig and Vaginal Ig

The Alhydrogel adjuvanted vaccine is effective at reducing infections and increases rate of clearance of infection. We then sought to investigate whether correlates of protection could be found with respect to the adaptive immune system. First, we aimed to assess local and systemic *T. vaginalis*-specific antibody production.

The presence of anti-trichomonal immunoglobulin (Ig) during infection has been documented in humans. Serum IgG, IgM and IgA, and vaginal IgG and IgA have been detected following experimental human infection using symptomatic and asymptomatic isolates (Kaur et al., 2008). IgA was shown to decrease in both serum and vaginal secretions following metronidazole treatment of infection (Sharma et al., 1991). The role of Ig remains undetermined since virulence factors of *T. vaginalis* are capable of degrading Ig (Hernández-

Gutiérrez et al., 2004) and may prevent complement activation or antibody dependent cell-mediated cytotoxicity (Alderete et al., 1995). Although Ig is detected during active vaginal infection, its presence does not seem to afford protection during or after infection. Natural infection treated with metronidazole does not confer protection from reinfection and yet vaccination of mice does prevent infection. Thus there is a potential for a vaccine to prevent acquisition of *T. vaginalis* infection. The success of the HPV vaccine is a demonstration of the ability of a human vaccine to provide protection, and reduction of an active STI infection where the naturally acquired immune response did not confer protection.

Mice infected with *T. vaginalis* isolates obtained from symptomatic and asymptomatic individuals resulted in serum and vaginal secretions of IgG, IgM and IgA that peak at day 14 post-infection and decline towards day 28 post-infection. IgG₁ and IgM were detected in greater quantities from serum and vaginal secretions in response to infection with symptomatic *T. vaginalis* isolates (Yadav et al., 2005). However, higher parasite load also was detected in mice infected with symptomatic isolates, which could explain the higher titer of IgG₁ and IgM. In another study, higher Ig levels were detected in mice infected with asymptomatic isolates (Paintlia et al., 2002). Perhaps Ig levels in natural infection are ineffective, but Ig levels achieved after vaccination could be required for clearance.

Differences in antigen presentation may also explain this discrepancy in response.

Adaptive immune response priming is necessary to ensure anamnestic response to *T. vaginalis* infection. Mice vaccinated with whole cell *T. vaginalis* using a symptomatic isolate and infected with the same isolate had significantly higher serum and vaginal IgG, and detectable vaginal IgA compared to unvaccinated controls (Abraham et al., 1996). Mice in the study vaccinated with a lower dosage of *T. vaginalis* organisms had much lower levels

of serum IgG compared to higher dosage vaccinations, and similar vaginal IgG levels compared to control mice. Despite these differences of Ig in local and systemic environments the lower dosage vaccination still provided significant protection from *T. vaginalis* challenge compared to unvaccinated mice (Abraham et al., 1996).

In our study we detected IgG, IgG₁ and IgG_{2a} 3 weeks following prime vaccination and a higher response 3 weeks following boost vaccination in vaccinated groups, but not in controls. Significantly higher levels of IgG and IgG₁ in serum of vaccinated mice were found at days 7, 14, 21 and 28 post-infection ($p < 0.05$) compared to controls that had nearly absent responses. IgG_{2a} was detected from 3 weeks following prime vaccination until the final serum collection on day 28 post-infection in only vaccinated mice and not in controls. Mice with vaginal IgG values above a cutoff were detected in Alum vaccinated groups on days 1, 3, 7, 14, and 21 following infection. Positive values were detected for Freund's vaccinated groups on day 1, 3, 7, 14, 21 and 28 post-infection. Vaginal IgG was only detected in four of forty-four control samples. Only six of thirty-nine Alum vaccinated mice, one of twenty-one Freund's vaccinated mice, three of thirty-six unvaccinated, and two of twenty-one sham vaccinated mice had positive IgA values. Limitation of sensitivity for IgA detection is likely due to an over dilution in our procedure to acquire Ig from mucus in vaginal washes. The same procedure could be followed if a concentration step was taken after the final mucus extraction step. Moreover, active *T. vaginalis* infections secrete proteases that can cleave Ig making detection of the actual level of Ig production more difficult, let alone the small quantities normally obtained from a mouse vagina without the pooling of vaginal washes from multiple mice. We did not pool the vaginal washes because we were trying to correlate individual mouse infection and vaccination to the immune responses.

Overall the results coincide with that reported by Abraham and colleagues (1996) that Ig responses are detected at higher levels in serum following vaccination compared to controls particularly post-vaginal infection. Also, vaginal IgG is more likely to be detected in vaccinated mice compared to controls following infection. Alum vaccination was equally as effective at producing serum and vaginal Ig responses as Freund's vaccination. This shows that the viability of a human adapted *T. vaginalis* vaccine is a possibility for future protection studies.

4.3 *Trichomonas vaginalis* Antigen Stimulation of CD4⁺ T Cells and CD8⁺ T Cells

Proliferation of peripheral blood lymphocytes (PBL) from patients infected with *T. vaginalis* was demonstrated using thymidine incorporation into PBL proliferating in response to a 5 day culture with soluble *T. vaginalis* antigen. Healthy controls did not show incorporation in response to *T. vaginalis* antigen (Yano et al., 1983). This experiment had been repeated using symptomatic and asymptomatic *T. vaginalis* isolates with similar results. Antigen from both types of isolates, whether secretory or cellular antigen preparations, stimulated proliferation of PBL from *T. vaginalis* infected, seropositive patients, but not uninfected, *T. vaginalis* seronegative patients (Mason and Patterson, 1985). A later experiment by Mason and colleagues (1990) shows a significant reduction of proliferation of PBL from women with active trichomonal infections compared to women with no infection or evidence of a past infection that has been treated in response to mitogens such as PHA and Con A. This is evidence for an immunosuppressive effect of *T. vaginalis* infection on PBL proliferation in response to mitogens.

In experimental *T. vaginalis* infections of mice, splenomegaly was observed for

infected mice versus uninfected controls (Mason and Gwanzura, 1988). Additionally, the mice were infected in two groups. One group received *T. vaginalis* isolates that were isolated from acute trichomoniasis patients and caused larger lesions following subcutaneous inoculation in mice. The other group received *T. vaginalis* isolates that were isolated from asymptomatic patients and caused small lesions following subcutaneous inoculation in mice. Infection with asymptomatic isolates caused significantly greater splenomegaly. Regardless of the isolate used for infection the proliferative response by thymidine incorporation was similar following four days of *T. vaginalis* antigen stimulation. However, significantly greater proliferation was measured six days following *T. vaginalis* stimulation in mice infected with symptomatic isolates of *T. vaginalis*. Additionally, more proliferation was measured using *T. vaginalis* antigen derived from symptomatic isolates (Mason and Gwanzura, 1988). Since these observations were not seen using human PBL the difference may be due to differences of pathology in humans versus the murine model of infection. Yet, it may suggest differences in pathology between *T. vaginalis* isolates. As described above for human PBL, active *T. vaginalis* infections in mice result in reduced proliferation of splenocytes compared to mice without infections in response to mitogens (Mason and Gwanzura, 1990). As stated by the authors, the clinical significance of these findings in humans and mice are not known. Nevertheless, *T. vaginalis* appears capable of immunosuppressive effects through a still unknown mechanism.

To evaluate whether priming of the adaptive immune response following vaccination and infection or infection alone would result in proliferative responses with regards to CD4⁺ T cells and CD8⁺ T cells we used a fluorescent CFSE labelling method to measure division of T cells in response to four days of incubation with *T. vaginalis* antigen. The division

kinetics of the majority of CD4⁺ and CD8⁺ T cell populations were the same following stimulation with *T. vaginalis* antigen regardless of whether the mouse was vaccinated or not. A shortcoming of proliferation is noted by Mason and Gwanzura (1988) wherein thymidine incorporation counts per minute varied considerably between mouse samples.

The objective to evaluate proliferation would be better suited and more successful to determine differences, if any, between vaccinated and unvaccinated mice by a CFC assay (Karlsson et al., 2003; Maecker et al., 2005). This procedure is faster than the more lengthy proliferation assay. In this method, lymphocytes are treated with antigen as well as necessary costimulators. Following a pre-determined incubation period (on the scale of a few hours) the lymphocytes are labelled with necessary fluorescent antibodies for proper detection of CD4⁺ and CD8⁺ T cells and internal cytokine production. The cytokines of interest vary depending on the type of T cell being stimulated. Th2 CD4⁺ T cell antigen-specific proliferation responses can be measured by detection of internal IL-4. Th1 CD4⁺ T cell and CD8⁺ T cell antigen-specific proliferation responses can be measured by detection of internal IFN- γ .

4.4 Lymphocytes in the Local Vaginal Tissue

The detection of local humoral immune response could suggest locally produced antibodies or antibodies arising from systemic circulation. The production of antibodies would need to be mediated by APC presentation of *T. vaginalis* antigen to respective CD4⁺ T helper cells. Other adaptive immune mediators may be at play to influence clearance of infection since not all mice who cleared infection had vaginal Ig above the cutoff. Therefore, CD8⁺ T cells or B cells may be of importance.

Paintlia and colleagues (2002) isolated lymphocytes from vaginal and cervical tissue of mice infected with either a symptomatic isolate, an asymptomatic isolate, or no infection. Flow cytometry analysis revealed a lower CD4⁺ T cell percentage from symptomatic isolates versus asymptomatic isolates ($p < 0.001$). Although the percentage of CD4⁺ T cells from symptomatic isolate infections was higher than uninfected it was not statistically significant. No differences existed between percentages of CD8⁺ T cells between isolates and controls. Using an immunohistochemical approach Reighard and colleagues (2011) found significantly higher CD4⁺ cells, B cells, and plasma cells in human endometrial tissue compared to uninfected controls ($p < 0.05$; plasma $p < 0.01$). No differences were observed with respect to CD8⁺ cells. These findings are supportive of the data from mice by Paintlia and colleagues (2002).

One shortfall of the Paintlia and colleagues' (2002) study is the presence of blood vessels in the tissue that contain PBL. The proportion of lymphocytes could be skewed. A histological approach would give a more definite idea of where the lymphocytes are located within the tissue. Also, an immunohistochemical assessment with respect to the presence of lymphocytes following *T. vaginalis* infection in mice that have been vaccinated has not yet been reported. We used an immunohistochemical staining procedure on frozen vaginal samples obtained at various time points post-infection from vaccinated and unvaccinated mice. The lack of detection of CD4⁺ cells in pre-infection groups that include both vaccinated and unvaccinated mice could indicate a lack of local recruitment to the vagina. Local recruitment following infection would increase the probability of detected CD4⁺ cells in a given tissue section. We found CD4⁺ cells in at least half of vaccinated group tissues and the Alum sham tissues, but less in the unvaccinated tissues post-infection. The percentage of

tissues for each group having at least one CD4⁺ cell was 61.5%, 50.0%, 53.8%, 50.0%, and 31.2% for Freund's, Alum 0.50, Alum 0.75, Alum sham, and unvaccinated, respectively. The percentage of tissues for each group having at least six CD4⁺ cells was 30.8%, 21.4%, 38.5%, 33.3%, and 12.5% for Freund's, Alum 0.50, Alum 0.75, Alum sham, and unvaccinated, respectively. With the small number of samples tested at various time points we could not determine the kinetics of CD4⁺ cell presence post-infection. Also, we could not predict the presence of CD4⁺ cells based on positive *T. vaginalis* culture or date of clearance of infection. The local environment of the vagina can have its own microenvironments so it is possible to miss areas of T cell infiltration or presence. Additional serial sections in random areas of the vaginal tissue may provide a more representative view of the presence of cells in a given tissue sample. Clearly *T. vaginalis* induces CD4 presence in the vaginal tissue.

CD4⁺ cells were more likely to be detected in vaccinated mice and in greater numbers within the vaginal tissue. This effect has potentially profound implications on the transmission and acquisition of HIV. While greater numbers of CD4⁺ cells were detected and this may increase the probability of HIV transmission and acquisition it is only for a finite period of time. Clearance of over 50% of initial *T. vaginalis* infections in vaccinated mice occurred by day 28. This is compared to unvaccinated mice wherein less than 25% of initial *T. vaginalis* infections were resolved by day 28. The long-term presence CD4⁺ cells due to chronic *T. vaginalis* infection of unvaccinated mice would be much more hazardous with respect to the chances of HIV transmission and susceptibility versus a brief period of an influx of CD4⁺ cells in the vaginal tissues. It is speculative but a very plausible explanation of the increased risk of increased transmission or susceptibility of HIV associated with *T.*

vaginalis infection is due to the presence of CD4⁺ cells in the vaginal tissue which is induced by the *T. vaginalis* infection. We can still conclude that vaccination, which reduces initial infection and duration of infection, will still have a positive effect on the reduction of HIV transmission or acquisition due to the initial or eventual clearance of infection afforded by vaccination. However the transient CD4 cell increase must be considered with caution.

The lack of detection of B cells or CD8⁺ cells could be due to their low numbers in the vaginal tissue coinciding with the lack of increased CD8⁺ reported by Paintlia and colleagues (2002). However, B cells were increased in human tissue samples (Reighard et al., 2011) and yet were not detected in our murine samples.

4.5 Conclusions

Herein we prepared a simple formulation of whole cell *T. vaginalis* antigen with Alhydrogel (Alum), a common, FDA approved adjuvant safe for use in humans and applied it to a murine model of *T. vaginalis* infection.

Previous experiments of inducible immunity in a murine model used Freund's adjuvant, which is not acceptable for human use. The Alum based adjuvant overcomes this obstacle and is equally effective in decreasing the number of initial infections following intravaginal challenge of female BALB/c mice. The Alum vaccine is also capable of increasing clearance of infection compared to unvaccinated controls. The mechanism of how protection is afforded or correlates of protection remain unknown. Regardless, protection is significant compared to controls and is evidence of a viable vaccine against *T. vaginalis*.

The systemic humoral response is markedly Th2 biased due to the nature of aluminum hydroxide and its function to prime DC for antigen presentation to CD4⁺ T cells.

As a result the primary antibody produced is an IgG₁ response. Low levels of IgG_{2a} were detected in serum for Alum vaccinated mice, and were lower than Freund's vaccinated mice. Unvaccinated mice had zero to negligible production of antigen specific total IgG, IgG₁ and IgG_{2a}. The only positive IgG levels as determined by a set cutoff for local vaginal IgG response were almost exclusively from vaccinated mice. Vaginal IgA absorbances were rarely above cutoff values. A greater propensity to observe a local, specific immune response was observed for vaccinated mice versus controls. Thus, vaccination enables an anamnestic response at the site of infection following a subcutaneous injection of adjuvant with whole cell *T. vaginalis* in the regio costalis.

Although proliferation experiments were not fruitful in terms of expected results compared to past thymidine incorporation proliferation experiments it does not rule out that CD4⁺ and CD8⁺ T cells are responding to antigen. Cytokines need to be assessed in future experiments by CFC.

The lack of B cells or CD8⁺ cells detected could be simply due to a scarcity of cells in the vaginal tissue. More than one set of serial sections should be assessed. In conjunction with previous data we report that infection with *T. vaginalis* stimulates CD4⁺ infiltration into the vaginal epithelium. CD4⁺ cells were detected in the vaginal tissue of both vaccinated and unvaccinated mice. Moreover, our results showed that vaginal tissues from vaccinated mice were more likely to contain CD4⁺ cells and in greater numbers. These cells could be partially responsible for the protection and early clearance of *T. vaginalis* infections. CD4⁺ cells are host cells for HIV infection and replication. The presence of CD4⁺ cells in the vaginal mucosa induced by a *T. vaginalis* infection has important implications with regard to the increased relative risk to acquire or transmit HIV infection.

Future research should focus on subunit vaccine production using any of the many identified antigens that play a role in pathogenesis (lactoferrin-binding protein, cysteine proteases, CDF, or adhesion proteins). A *T. foetus* vaccination experiment demonstrated that priming with a specific Tf190 antigen and stimulation with this antigen resulted in a skew of CD4⁺ peripheral blood mononuclear cells (PBMC) (from 19% to 83% of the PBMC population) and less CD8⁺ and $\gamma\delta$ T cells (Voyich et al., 2001). Priming with whole cell *T. foetus* and stimulation with Tf190 showed only a 1-3% difference day 14. Therefore, subunit vaccines are potentially viable for stimulating a strong response and can skew to a predominantly CD4⁺ population. Since CD4⁺ cells may play a role in the protective response against *T. vaginalis* infection, the skew of CD4⁺ cells could offer increased protection against *T. vaginalis* challenge.

With little literature reported on the success of vaccines for *T. vaginalis*, we hope our data will reinvigorate vaccine studies using the murine model, but also pave way to support the next step of study in non-human primate models as well as phase 1 clinical trials. Difficulties of vaginal Ig sampling would be overcome by a primate model. For example, the pigtailed macaque has a similar vaginal environment to the human vaginal environment including lactobacilli, a vaginal pH of 5.5-8.0, similar menstrual cycles, susceptibility to *T. vaginalis* infection, can be treated with metronidazole, and has exhibited signs of erythema following infection (Patton et al., 2006). The drawbacks of this model are its cost and need for special animal facilities to perform primate studies.

References

- Abraham, M.C., Desjardins, M., Filion, L., and Garber, G. (1996). Inducible immunity to *Trichomonas vaginalis* in a mouse model of vaginal infection. *Infect. Immun.* 64, 3571-3575.
- Alderete, J. (1999). Iron modulates phenotypic variation and phosphorylation of P270 in double-stranded RNA virus-infected *Trichomonas vaginalis*. *Infect. Immun.* 67, 4298-4302.
- Alderete, J.F., Millsap, K.W., Lehker, M.W., and Benchimol, M. (2001). Enzymes on microbial pathogens and *Trichomonas vaginalis*: molecular mimicry and functional diversity. *Cell. Microbiol.* 3, 359-370.
- Alderete, J.F., Provenzano, D., and Lehker, M.W. (1995). Iron mediates *Trichomonas vaginalis* resistance to complement lysis. *Microb. Pathog.* 19, 93-103.
- Al-Salihi, F.L., Curran, J.P., and Wang, J. (1974). Neonatal *Trichomonas vaginalis*: Report of three cases and review of the literature. *Pediatrics* 53, 196-200.
- Alvarez-Sánchez, M.E., Solano-González, E., Yañez-Gómez, C., and Arroyo, R. (2007). Negative iron regulation of the CP65 cysteine proteinase cytotoxicity in *Trichomonas vaginalis*. *Microb. Infect.* 9, 1597-1605.
- Anderson, B.L., Firnhaber, C., Liu, T., Swarts, A., Siminya, M., Ingersoll, J., Caliendo, A.M., and Cu-Uvin, S. (2012). Effect of trichomoniasis therapy on genital HIV viral burden among African women. *Sex. Transm. Dis.* 39, 638-642.
- Ardalan, S., Craig Lee, B., and Garber, G.E. (2009). *Trichomonas vaginalis*: The adhesins AP51 and AP65 bind heme and hemoglobin. *Exp. Parasitol.* 121, 300-306.
- Arroyo, R., and Alderete, J. (1989). *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infect. Immun.* 57, 2991-2997.
- Arroyo, R., González-Robles, A., Martínez-Palomo, A., and Alderete, J. (1993). Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesin synthesis follows cytoadherence. *Mol. Microbiol.* 7, 299-309.
- Benchimol, M. (2004). Trichomonads under microscopy. *Microsc. Microanal.* 10, 528-550.
- Benchimol, M., Monteiro, S.P., Chang, T., and Alderete, J.F. (2002). Virus in *Trichomonas*—an ultrastructural study. *Parasitol. Int.* 51, 293-298.
- Brandtzaeg, P. (1997). Mucosal immunity in the female genital tract. *J. Reprod. Immunol.* 36, 23-50.

- Brewer, J.M., Conacher, M., Satoskar, A., Bluethmann, H., and Alexander, J. (1996). In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur. J. Immunol.* 26, 2062-2066.
- Brill, J.R. (2010). Diagnosis and treatment of urethritis in men. *Am. Fam. Physician* 81, 873-878.
- Brotman, R.M., Bradford, L.L., Conrad, M., Gajer, P., Ault, K., Peralta, L., Forney, L.J., Carlton, J.M., Abdo, Z., and Ravel, J. (2012). Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sex. Transm. Dis.* 39, 807-812.
- Bruins, M.J., van Straaten, I.H., and Ruijs, G.J. (2013). Respiratory disease and *Trichomonas vaginalis* in premature newborn twins. *Pediatr. Infect. Dis. J.* 32, 1029-1030.
- Cappuccinelli, P., Lattes, C., Cagliani, I., and Negro Ponzi, A. (1974). Features of intravaginal *Trichomonas vaginalis* infection in the mouse and the effect of oestrogen treatment and immunodepression. *G. Batteriol. Virol. Immunol.* 67, 31-40.
- Chang, J., Kim, S., Choi, I., Lee, S., Morio, T., and Chang, E. (2006). Apoptosis of macrophages induced by *Trichomonas vaginalis* through the phosphorylation of p38 mitogen-activated protein kinase that locates at downstream of mitochondria-dependent caspase activation. *Int. J. Biochem. Cell Biol.* 38, 638-647.
- Chang, J., Ryang, Y., Morio, T., Lee, S., and Chang, E. (2004). *Trichomonas vaginalis* inhibits proinflammatory cytokine production in macrophages by suppressing NF-kappaB activation. *Mol. Cells* 18, 177-185.
- Clark, B., Dufty, J., and Parsonson, I. (1983). Immunisation of bulls against trichomoniasis. *Aust. Vet. J.* 60, 178-179.
- Cobo, E.R., Corbeil, L.B., Gershwin, L.J., and BonDurant, R.H. (2009). Preputial cellular and antibody responses of bulls vaccinated and/or challenged with *Tritrichomonas foetus*. *Vaccine* 28, 361-370.
- Corbeil, L. (1994). Vaccination strategies against *Tritrichomonas foetus*. *Parasitol. Today* 10, 103-106.
- Corbeil, L.B., Campero, C.M., Rhyan, J., Anderson, M., Gershwin, L., Agnew, D., Munson, L., and BonDurant, R.H. (2005). Uterine mast cells and immunoglobulin-E antibody responses during clearance of *Tritrichomonas foetus*. *Vet. Pathol. Online* 42, 282-290.
- Corbeil, L.B., Chatterjee, A., Foresman, L., and Westfall, J.A. (1985). Ultrastructure of cyclic changes in the murine uterus, cervix, and vagina. *Tissue Cell* 17, 53-68.

Corbeil, L.B., Munson, L., Campero, C., and BonDurant, R.H. (2001). Bovine Trichomoniasis as a model for development of vaccines against sexually-transmitted disease. *Am. J. Reprod. Immunol.* 45, 310-319.

Cotch, M.F., Pastorek II, J.G., Nugent, R.P., Hillier, S.L., Gibbs, R.S., Martin, D.H., Eschenbach, D.A., Edelman, R., Carey, C.J., and Regan, J.A. (1997). *Trichomonas vaginalis* associated with low birth weight and preterm delivery. *Sex. Transm. Dis.* 24, 353-360.

Crucitti, T., Van Dyck, E., Tehe, A., Abdellati, S., Vuylsteke, B., Buve, A., and Laga, M. (2003). Comparison of culture and different PCR assays for detection of *Trichomonas vaginalis* in self collected vaginal swab specimens. *Sex. Transm. Infect.* 79, 393-398.

Crucitti, T., Jespers, V., Mulenga, C., Khondowe, S., Vandepitte, J., and Buvé, A. (2011). Non-sexual transmission of *Trichomonas vaginalis* in adolescent girls attending school in Ndola, Zambia. *PloS One* 6, e16310.

de Lemos, P.A.P., and Garcia-Zapata, M.T.A. (2010). The prevalence of *Trichomonas vaginalis* in HIV-positive and negative patients in referral hospitals in Goiania, Goias, Brazil. *Int. J. Trop. Med.* 5, 24-27.

de Miguel, N., Riestra, A., and Johnson, P.J. (2012). Reversible association of tetraspanin with *Trichomonas vaginalis* flagella upon adherence to host cells. *Cell. Microbiol.* 14, 1797-1807.

Diamond, L.S. (1978). A new medium for axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans. R. Soc. Trop. Med. Hyg.* 72, 431-432.

Draper, D., Donohoe, W., Mortimer, L., and Heine, R.P. (1998). Cysteine proteases of *Trichomonas vaginalis* degrade secretory leukocyte protease inhibitor. *J. Infect. Dis.* 178, 815-819.

Draper, D., Jones, W., Heine, R.P., Beutz, M., French, J.I., c Gregor, M., and James, A. (1995a). *Trichomonas vaginalis* weakens human amniochorion in an in vitro model of premature membrane rupture. *Infect. Dis. Obstet. Gynecol.* 2, 267-274.

Draper, D., McGregor, J., Hall, J., Jones, W., Beutz, M., Phillip Heine, R., and Porreco, R. (1995b). Elevated protease activities in human amnion and chorion correlate with preterm premature rupture of membranes. *Am. J. Obstet. Gynecol.* 173, 1506-1512.

Dunne, R., Dunn, L., Upcroft, P., O'Donoghue, P., and Upcroft, J. (2003). Drug resistance in the sexually transmitted protozoan *Trichomonas vaginalis*. *Cell Res.* 13, 239-249.

Escario, J., Barrio, A.G., and Fernández, A. (1995). The relationship of experimental pathogenicity in vivo with in vitro cytoadherence and cytotoxicity of 6 different isolates of *Trichomonas vaginalis*. *Int. J. Parasitol.* 25, 999-1000.

Fichorova, R.N. (2009). Impact of *T. vaginalis* infection on innate immune responses and reproductive outcome. *J. Reprod. Immunol.* 83, 185-189.

Fichorova, R.N., Buck, O.R., Yamamoto, H.S., Fashemi, T., Dawood, H.Y., Fashemi, B., Hayes, G.R., Beach, D.H., Takagi, Y., Delaney, M.L., *et al.* (2013). The Villain Team-Up or how *Trichomonas vaginalis* and bacterial vaginosis alter innate immunity in concert. *Sex. Transm. Infect.* 89, 460-466.

Figuerola-Angulo, E.E., Rendón-Gandarilla, F.J., Puente-Rivera, J., Calla-Choque, J.S., Cárdenas-Guerra, R.E., Ortega-López, J., Quintas-Granados, L.I., Elizabeth Alvarez-Sánchez, M., and Arroyo, R. (2012). The effects of environmental factors on the virulence of *Trichomonas vaginalis*. *Microb. Infect.* 14, 1411-1427.

Fiori, P.L., Rappelli, P., Addis, M.F., Sechi, A., and Cappuccinelli, P. (1996). *Trichomonas vaginalis* haemolysis: pH regulates a contact-independent mechanism based on pore-forming proteins. *Microb. Pathog.* 20, 109-118.

Flach, T.L., Ng, G., Hari, A., Desrosiers, M.D., Zhang, P., Ward, S.M., Seamone, M.E., Vilaysane, A., Mucsi, A.D., and Fong, Y. (2011). Alum interaction with dendritic cell membrane lipids is essential for its adjuvant activity. *Nat. Med.* 17, 479-487.

Franchi, L., and Núñez, G. (2008). The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1 β secretion but dispensable for adjuvant activity. *Eur. J. Immunol.* 38, 2085-2089.

Francioli, P., Shio, H., Roberts, R.B., and Müller, M. (1983). Phagocytosis and killing of *Neisseria gonorrhoeae* by *Trichomonas vaginalis*. *J. Infect. Dis.* 147, 87-94.

Freeman, A.H., Katz, K.A., Pandori, M.W., Rauch, L.M., Kohn, R.P., Liska, S., Bernstein, K.T., and Klausner, J.D. (2010). Prevalence and correlates of *Trichomonas vaginalis* among incarcerated persons assessed using a highly sensitive molecular assay. *Sex. Transm. Dis.* 37, 165-168.

Garber, G.E., Lemchuk-Favel, L.T., and Rousseau, G. (1991). Effect of beta-estradiol on production of the cell-detaching factor of *Trichomonas vaginalis*. *J. Clin. Microbiol.* 29, 1847-1849.

Garcia, A.F., Chang, T., Benchimol, M., Klumpp, D.J., Lehker, M.W., and Alderete, J.F. (2003). Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. *Mol. Microbiol.* 47, 1207-1224.

Garcia, P.J., Holmes, K.K., Carcamo, C.P., Garnett, G.P., Hughes, J.P., Campos, P.E., Whittington, W.L., and Peru PREVEN Study Team. (2012). Prevention of sexually transmitted infections in urban communities (Peru PREVEN): a multicomponent community-randomised controlled trial. *Lancet* 379, 1120-1128.

- Gatski, M., and Kissinger, P. (2010). Observation of probable persistent, undetected *Trichomonas vaginalis* infection among HIV-positive women. *Clin. Infect. Dis.* *51*, 114-115.
- Gilbert, R., Elia, G., Beach, D., Klaessig, S., and Singh, B. (2000). Cytopathogenic effect of *Trichomonas vaginalis* on human vaginal epithelial cells cultured in vitro. *Infect. Immun.* *68*, 4200-4206.
- Goodman, R.P., Ghabrial, S.A., Fichorova, R.N., and Nibert, M.L. (2011). *Trichomonasvirus*: a new genus of protozoan viruses in the family Totiviridae. *Arch. Virol.* *156*, 171-179.
- Guenthner, P.C., Secor, W.E., and Dezzutti, C.S. (2005). *Trichomonas vaginalis*-induced epithelial monolayer disruption and human immunodeficiency virus type 1 (HIV-1) replication: implications for the sexual transmission of HIV-1. *Infect. Immun.* *73*, 4155-4160.
- Hernández, H.M., Sariego, I., Alvarez, A.B., Marcet, R., Vancol, E., Alvarez, A., Figueredo, M., and Sarracent, J. (2011). *Trichomonas vaginalis* 62 kDa proteinase as a possible virulence factor. *Parasitol. Res.* *108*, 241-245.
- Hernández-Gutiérrez, R., Avila-González, L., Ortega-López, J., Cruz-Talonia, F., Gómez-Gutierrez, G., and Arroyo, R. (2004). *Trichomonas vaginalis*: characterization of a 39-kDa cysteine proteinase found in patient vaginal secretions. *Exp. Parasitol.* *107*, 125-135.
- Hesseltine, H.C., Wolters, S., and Campbell, A. (1942). Experimental human vaginal trichomoniasis. *J. Infect. Dis.* *71*, 127-130.
- Hirt, R.P., de Miguel, N., Nakjang, S., Dessi, D., Liu, Y., Diaz, N., Rappelli, P., Acosta-Serrano, A., Fiori, P., and Mottram, J.C. (2011). *Trichomonas vaginalis* Pathobiology: New Insights from the Genome Sequence. *Adv. Parasitol.* *77*, 87-140.
- Hobbs, M.M., Lapple, D.M., Lawing, L.F., Schwebke, J.R., Cohen, M.S., Swygard, H., Atashili, J., Leone, P.A., Miller, W.C., and Seña, A.C. (2006). Methods for detection of *Trichomonas vaginalis* in the male partners of infected women: implications for control of trichomoniasis. *J. Clin. Microbiol.* *44*, 3994-3999.
- Hollman, D., Coupey, S.M., Fox, A.S., and Herold, B.C. (2010). Screening for *Trichomonas vaginalis* in High-risk Adolescent Females with a New Transcription-mediated Nucleic Acid Amplification Test (NAAT): Associations with Ethnicity, Symptoms, and Prior and Current STIs. *J. Pediatr. Adolesc. Gynecol.* *23*, 312-316.
- Horváthová, L., Šafaříková, L., Basler, M., Hrdý, I., Campo, N.B., Shin, J., Huang, K., Huang, P., Lin, R., and Tang, P. (2012). Transcriptomic Identification of Iron-Regulated and Iron-Independent Gene Copies within the Heavily Duplicated *Trichomonas vaginalis* Genome. *Genome Biol. Evol.* *4*, 905-917.

Huppert, J.S., Mortensen, J.E., Reed, J.L., Kahn, J.A., Rich, K.D., Miller, W.C., and Hobbs, M.M. (2007). Rapid antigen testing compares favorably with transcription-mediated amplification assay for the detection of *Trichomonas vaginalis* in young women. *Clin. Infect. Dis.* 45, 194-198.

Huppert, J.S., Huang, B., Chen, C., Dawood, H.Y., and Fichorova, R.N. (2013). Clinical evidence for the role of *Trichomonas vaginalis* in regulation of secretory leukocyte protease inhibitor in the female genital tract. *J. Infect. Dis.* 207, 1462-1470.

Huston, W.M., Harvie, M., Mittal, A., Timms, P., and Beagley, K.W. (2012). Vaccination to protect against infection of the female reproductive tract. *Expert Rev. Clin. Immunol.* 8, 81-94.

Johansson, E.L., Rudin, A., Wassen, L., and Holmgren, J. (1999). Distribution of lymphocytes and adhesion molecules in human cervix and vagina. *Immunol.* 96, 272-277.

Johansson, M., and Lycke, N.Y. (2003). Immunology of the human genital tract. *Curr. Opin. Infect. Dis.* 16, 43-49.

Johnston, V.J., and Mabey, D.C. (2008). Global epidemiology and control of *Trichomonas vaginalis*. *Curr. Opin. Infect. Dis.* 21, 56-64.

Jones, H.E., Lippman, S.A., Caiaffa-Filho, H.H., Young, T., and van de Wijgert, J.H. (2013). Performance of a rapid self-test for detection of *Trichomonas vaginalis* in South Africa and Brazil. *J. Clin. Microbiol.* 51, 1037-1039.

Karlsson, A.C., Martin, J.N., Younger, S.R., Bredt, B.M., Epling, L., Ronquillo, R., Varma, A., Deeks, S.G., McCune, J.M., Nixon, D.F., and Sinclair, E. (2003). Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J. Immunol. Methods* 283, 141-153.

Kaur, S., Khurana, S., Bagga, R., Wanchu, A., and Malla, N. (2008). Antitrichomonas IgG, IgM, IgA, and IgG subclass responses in human intravaginal trichomoniasis. *Parasitol. Res.* 103, 305-312.

Kirkcaldy, R.D., Augostini, P., Asbel, L.E., Bernstein, K.T., Kerani, R.P., Mettenbrink, C.J., Pathela, P., Schwebke, J.R., Secor, W.E., and Workowski, K.A. (2012). *Trichomonas vaginalis* antimicrobial drug resistance in 6 US cities, STD Surveillance Network, 2009–2010. *Emerg. Infect. Dis.* 18, 939-943.

Kissinger, P., Amedee, A., Clark, R.A., Dumestre, J., Theall, K.P., Myers, L., Hagensee, M.E., Farley, T.A., and Martin, D.H. (2009). *Trichomonas vaginalis* treatment reduces vaginal HIV-1 shedding. *Sex. Transm. Dis.* 36, 11-16.

Kiviat, N.B., Peterson, M., Kinney-Thomas, E., Tam, M., Stamm, W.E., and Holmes, K.K. (1985). Cytologic manifestations of cervical and vaginal infections. *JAMA* 253, 997-1000.

Klebanoff, M.A., Carey, J.C., Hauth, J.C., Hillier, S.L., Nugent, R.P., Thom, E.A., Ernest, J., Heine, R.P., Wapner, R.J., and Trout, W. (2001). Failure of metronidazole to prevent preterm delivery among pregnant women with asymptomatic *Trichomonas vaginalis* infection. *N. Engl. J. Med.* 345, 487-493.

Kool, M., Pétrilli, V., De Smedt, T., Rolaz, A., Hammad, H., van Nimwegen, M., Bergen, I.M., Castillo, R., Lambrecht, B.N., and Tschopp, J. (2008). Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* 181, 3755-3759.

Kozlowski, P.A., Williams, S.B., Lynch, R.M., Flanigan, T.P., Patterson, R.R., Cu-Uvin, S., and Neutra, M.R. (2002). Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. *J. Immunol.* 169, 566-574.

Krieger, J.N. (1995). Trichomoniasis in men: old issues and new data. *Sex. Transm. Dis.* 22, 83-96.

Krieger, J.N., Jenny, C., Verdon, M., Siegel, N., Springwater, R., Critchlow, C.W., and Holmes, K.K. (1993). Clinical manifestations of trichomoniasis in men. *Ann. Intern. Med.* 118, 844-849.

Kucknoor, A.S., Mundodi, V., and Alderete, J. (2005). Adherence to human vaginal epithelial cells signals for increased expression of *Trichomonas vaginalis* genes. *Infect. Immun.* 73, 6472-6478.

Kummer, S., Hayes, G.R., Gilbert, R.O., Beach, D.H., Lucas, J.J., and Singh, B.N. (2008). Induction of human host cell apoptosis by *Trichomonas vaginalis* cysteine proteases is modulated by parasite exposure to iron. *Microb. Pathog.* 44, 197-203.

Kusdian, G., Woehle, C., Martin, W.F., and Gould, S.B. (2013). The actin-based machinery of *Trichomonas vaginalis* mediates flagellate-amoeboid transition and migration across host tissue. *Cell. Microbiol.* doi: 10.1111/cmi.12144.

Kutteh, W., Prince, S., Hammond, K., Kutteh, C., and Mestecky, J. (1996). Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation. *Clin. Exp. Immunol.* 104, 538-542.

Kvasnicka, W., Hanks, D., Huang, J., Hall, M., Sandblom, D., Chu, H., Chavez, L., and Acree, W. (1992). Clinical evaluation of the efficacy of inoculating cattle with a vaccine containing *Trichomonas foetus*. *Am. J. Vet. Res.* 53, 2023-2027.

Kvasnicka, W., Taylor, R., Huang, J., Hanks, D., Tronstad, R., Bosomworth, A., and Hall, M. (1989). Investigations of the incidence of bovine trichomoniasis in Nevada and of the

efficacy of immunizing cattle with vaccines containing *Tritrichomonas foetus*. *Theriogenology* 31, 963-971.

Lehker, M.W., and Alderete, J.F. (2000). Biology of trichomonosis. *Curr. Opin. Infect. Dis.* 13, 37-45.

Lehker, M.W., Arroyo, R., and Alderete, J.F. (1991). The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *Trichomonas vaginalis*. *J. Exp. Med.* 174, 311-318.

Lehker, M.W., and Sweeney, D. (1999). Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. *Sex. Transm. Infect.* 75, 231-238.

Lehker, M., and Alderete, J. (1992). Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins. *Mol. Microbiol.* 6, 123-132.

Lehker, M., Chang, T., Dailey, D., and Alderete, J. (1990). Specific erythrocyte binding is an additional nutrient acquisition system for *Trichomonas vaginalis*. *J. Exp. Med.* 171, 2165-2170.

Lehner, T., Wang, Y., Ping, L., Bergmeier, L., Mitchell, E., Cranage, M., Hall, G., Dennis, M., Cook, N., and Doyle, C. (1999). The effect of route of immunization on mucosal immunity and protection. *J. Infect. Dis.* 179, S489-S492.

Leitsch, D., Drinić, M., Kolarich, D., and Duchêne, M. (2012). Down-regulation of flavin reductase and alcohol dehydrogenase-1 (ADH1) in metronidazole-resistant isolates of *Trichomonas vaginalis*. *Mol. Biochem. Parasitol.* 183, 177-183.

Lockwood, B.C., North, M.J., Scott, K.I., Bremner, A.F., and Coombs, G.H. (1987). The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Mol. Biochem. Parasitol.* 24, 89-95.

Ma, L., Meng, Q., Cheng, W., Sung, Y., Tang, P., Hu, S., and Yu, J. (2011). Involvement of the GP63 protease in infection of *Trichomonas vaginalis*. *Parasitol. Res.* 109, 71-79.

Maecker, H.T., Rinfret, A., D'Souza, P., Darden, J., Roig, E., Landry, C., Hayes, P., Birungi, J., Anzala, O., Garcia, M., *et al.* (2005). Standardization of cytokine flow cytometry assays. *BMC Immunol.* 6, 13.

Makinde, H.M., Zariffard, R., Mirmonsef, P., Novak, R.M., Jarrett, O., Landay, A.L., and Spear, G.T. (2013). IL-22 Levels are Associated with *Trichomonas vaginalis* Infection in the Lower Genital Tract. *Am. J. Reprod. Immunol.* 70, 38-44.

Malla, N., Yadav, M., and Gupta, I. (2007). Kinetics of serum and local cytokine profile in experimental intravaginal trichomoniasis induced with *Trichomonas vaginalis* isolates from symptomatic and asymptomatic women. *Parasite Immunol.* 29, 101-105.

- Malla, N., Valadkhani, Z., Harjai, K., Sharma, S., and Gupta, I. (2004). Reactive nitrogen intermediates in experimental trichomoniasis induced with isolates from symptomatic and asymptomatic women. *Parasitol. Res.* *94*, 101-105.
- Marrack, P., McKee, A.S., and Munks, M.W. (2009). Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* *9*, 287-293.
- Mason, P.R., and Patterson, B.A. (1985). Proliferative response of human lymphocytes to secretory and cellular antigens of *Trichomonas vaginalis*. *J. Parasitol.* *71*, 265-268.
- Mason, P.R., and Gwanzura, L. (1988). Mouse spleen cell responses to trichomonal antigens in experimental *Trichomonas vaginalis* infection. *J. Parasitol.* *74*, 93-97.
- Mason, P.R., and Gwanzura, L. (1990). Reduced lymphocyte responses to mitogens in natural and experimental trichomoniasis. *Infect. Immun.* *58*, 3553-3557.
- Mavedzenge, S.N., Van Der Pol, B., Cheng, H., Montgomery, E.T., Blanchard, K., de Bruyn, G., Ramjee, G., and van der Straten, A. (2010). Epidemiological synergy of *Trichomonas vaginalis* and HIV in Zimbabwean and South African women. *Sex. Transm. Dis.* *37*, 460-466.
- McClelland, R.S., Sangaré, L., Hassan, W.M., Lavreys, L., Mandaliya, K., Kiarie, J., Ndinya-Achola, J., Jaoko, W., and Baeten, J.M. (2007). Infection with *Trichomonas vaginalis* increases the risk of HIV-1 acquisition. *J. Infect. Dis.* *195*, 698-702.
- McGrory, T., and Garber, G. (1992). Mouse intravaginal infection with *Trichomonas vaginalis* and role of *Lactobacillus acidophilus* in sustaining infection. *Infect. Immun.* *60*, 2375-2379.
- Meysick, K.C., and Garber, G.E. (1992). Interactions between *Trichomonas vaginalis* and vaginal flora in a mouse model. *J. Parasitol.* *78*, 157-160.
- Mhlongo, S., Magooa, P., Müller, E.E., Nel, N., Radebe, F., Wasserman, E., and Lewis, D.A. (2010). Etiology and STI/HIV coinfections among patients with urethral and vaginal discharge syndromes in South Africa. *Sex. Transm. Dis.* *37*, 566-570.
- Midlej, V., and Benchimol, M. (2010). *Trichomonas vaginalis* kills and eats—evidence for phagocytic activity as a cytopathic effect. *Parasitology* *137*, 65.
- Mitteregger, D., Aberle, S.W., Makristathis, A., Walochnik, J., Brozek, W., Marberger, M., and Kramer, G. (2012). High detection rate of *Trichomonas vaginalis* in benign hyperplastic prostatic tissue. *Med. Microbiol. Immunol. (Berl.)* *201*, 113-116.

- Moodley, P., Wilkinson, D., Connolly, C., Moodley, J., and Sturm, A.W. (2002). *Trichomonas vaginalis* is associated with pelvic inflammatory disease in women infected with human immunodeficiency virus. *Clinical Infectious Diseases* 34, 519-522.
- Muzny, C.A., and Schwebke, J.R. (2013). The clinical spectrum of *Trichomonas vaginalis* infection and challenges to management. *Sex. Transm. Infect.* 89, 423-425.
- Nam, Y.H., Min, D., Kim, H., Song, K., Kim, K.A., Lee, Y.A., Kim, S.H., and Shin, M.H. (2011a). Leukotriene B₄ receptor BLT-mediated phosphorylation of NF- κ B and CREB is involved in IL-8 production in human mast cells induced by *Trichomonas vaginalis*-derived secretory products. *Microb. Infect.* 13, 1211-1220.
- Nam, Y.H., Min, D., Park, S., Kim, K.A., Lee, Y.A., and Shin, M.H. (2011b). NF- κ B and CREB are involved in IL-8 production of human neutrophils induced by *Trichomonas vaginalis*-derived secretory products. *Korean J. Parasitol.* 49, 291-294.
- Neutra, M.R., Pringault, E., and Kraehenbuhl, J. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14, 275-300.
- Nweze, E., and Mouneke, G. (2011). *Trichomonas vaginalis* in HIV/AIDS subjects in Nigeria. *Asian Pac. J. Trop. Dis.* 1, 282-286.
- Nye, M.B., Schwebke, J.R., and Body, B.A. (2009). Comparison of APTIMA *Trichomonas vaginalis* transcription-mediated amplification to wet mount microscopy, culture, and polymerase chain reaction for diagnosis of trichomoniasis in men and women. *Am. J. Obstet. Gynecol.* 200, 188. e1-188. e7.
- Okumura, C.Y., Baum, L.G., and Johnson, P.J. (2008). Galectin-1 on cervical epithelial cells is a receptor for the sexually transmitted human parasite *Trichomonas vaginalis*. *Cell. Microbiol.* 10, 2078-2090.
- Paintlia, M., Kaur, S., Gupta, I., Ganguly, N., Mahajan, R., and Malla, N. (2002). Specific IgA response, T-cell subtype and cytokine profile in experimental intravaginal trichomoniasis. *Parasitol. Res.* 88, 338-343.
- Parr, E.L., and Parr, M.B. (1997). Immunoglobulin G is the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated *Herpes simplex virus* type 2. *J. Virol.* 71, 8109-8115.
- Pastorek II, J.G., Cotch, M.F., Martin, D.H., and Eschenbach, D.A. (1996). Clinical and microbiological correlates of vaginal trichomoniasis during pregnancy. *Clin. Infect. Dis.* 23, 1075-1080.
- Patil, M.J., Nagamoti, J.M., and Metgud, S.C. (2012). Diagnosis of *Trichomonas vaginalis* from vaginal specimens by wet mount microscopy, in pouch TV culture system, and PCR. *J. Glob. Infect. Dis.* 4, 22-25.

Patton, D.L., Sweeney, Y.T.C., Agnew, K.J., Balkus, J.E., Rabe, L.K., and Hillier, S.L. (2006). Development of a nonhuman primate model for *Trichomonas vaginalis* infection. *Sex. Transm. Dis.* 33, 743-746.

Paul, H., Peter, D., Pulimood, S.A., Abraham, O.C., Mathai, E., Prasad, J.H., and Kannangai, R. (2012). Role of polymerase chain reaction in the diagnosis of *Trichomonas vaginalis* infection in human immunodeficiency virus-infected individuals from India (South). *Indian J. Dermatol. Venereol. Leprol.* 78, 323-327.

Paz-Bailey, G., Sternberg, M., Puren, A.J., Steele, L., and Lewis, D.A. (2010). Determinants of HIV type 1 shedding from genital ulcers among men in South Africa. *Clin. Infect. Dis.* 50, 1060-1067.

Peterman, T.A., Tian, L.H., Metcalf, C.A., Malotte, C.K., Paul, S.M., and Douglas, J.M. (2009). Persistent, undetected *Trichomonas vaginalis* infections? *Clin. Infect. Dis.* 48, 259-260.

Petrin, D., Delgaty, K., Bhatt, R., and Garber, G. (1998). Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clin. Microbiol. Rev.* 11, 300-317.

Phukan, N., Parsamand, T., Brooks, A.E., Nguyen, T.N., and Simoes-Barbosa, A. (2013). The adherence of *Trichomonas vaginalis* to host ectocervical cells is influenced by lactobacilli. *Sex. Transm. Infect.* 89, 455-459.

Provenzano, D., and Alderete, J. (1995). Analysis of human immunoglobulin-degrading cysteine proteinases of *Trichomonas vaginalis*. *Infect. Immun.* 63, 3388-3395.

Quinlivan, E.B., Patel, S.N., Grodensky, C.A., Golin, C.E., Tien, H., and Hobbs, M.M. (2012). Modeling the Impact of *Trichomonas vaginalis* Infection on HIV Transmission in HIV-Infected Individuals in Medical Care. *Sex. Transm. Dis.* 39, 671-677.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., *et al.* (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl 1, 4680-4687.

Reighard, S.D., Sweet, R.L., Vicetti Miguel, C., Vicetti Miguel, R.D., Chivukula, M., Krishnamurti, U., and Cherpes, T.L. (2011). Endometrial leukocyte subpopulations associated with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* genital tract infection. *Am. J. Obstet. Gynecol.* 205, 324. e1-324. e7.

Rendón-Maldonado, J.G., Espinosa-Cantellano, M., González-Robles, A., and Martínez-Palomo, A. (1998). *Trichomonas vaginalis*: In vitro phagocytosis of lactobacilli, vaginal epithelial cells, leukocytes, and erythrocytes. *Exp. Parasitol.* 89, 241-250.

- Roth, A.M., Williams, J.A., Ly, R., Curd, K., Brooks, D., Arno, J., and Van Der Pol, B. (2011). Changing sexually transmitted infection screening protocol will result in improved case finding for *Trichomonas vaginalis* among high-risk female populations. *Sex. Transm. Dis.* 38, 398-400.
- Ryu, J., Choi, H., Min, D., Ha, S., and Ahn, M. (2001). Effect of Iron on the Virulence of *Trichomonas vaginalis*. *J. Parasitol.* 87, 457-460.
- Schwebke, J.R., and Barrientes, F.J. (2006). Prevalence of *Trichomonas vaginalis* isolates with resistance to metronidazole and tinidazole. *Antimicrobial Agents Chemother.* 50, 4209-4210.
- Schwebke, J.R., and Hook, E.W. (2003). High rates of *Trichomonas vaginalis* among men attending a sexually transmitted diseases clinic: implications for screening and urethritis management. *J. Infect. Dis.* 188, 465-468.
- Sehgal, R., Goyal, K., and Sehgal, A. (2012). Trichomoniasis and Lactoferrin: Future Prospects. *Infect. Dis. Obstet. Gynecol.* 2012: 536037.
- Seña, A.C., Miller, W.C., Hobbs, M.M., Schwebke, J.R., Leone, P.A., Swygard, H., Atashili, J., and Cohen, M.S. (2007). *Trichomonas vaginalis* infection in male sexual partners: implications for diagnosis, treatment, and prevention. *Clin. Infect. Dis.* 44, 13-22.
- Sharma, P., Malla, N., Gupta, I., Ganguly, N., and Mahajan, R. (1991). Anti-trichomonal IgA antibodies in trichomoniasis before and after treatment. *Folia Microbiol. (Praha)* 36, 302-304.
- Shuter, J., Bell, D., Graham, D., Holbrook, K.A., and Bellin, E.Y. (1998). Rates of and risk factors for trichomoniasis among pregnant inmates in New York City. *Sex. Transm. Dis.* 25, 303-307.
- Singh, B.N., Hayes, G.R., Lucas, J.J., Sommer, U., Viseux, N., Mirgorodskaya, E., Trifonova, R.T., Sassi, R.R.S., Costello, C.E., and Fichorova, R.N. (2009). Structural details and composition of *Trichomonas vaginalis* lipophosphoglycan in relevance to the epithelial immune function. *Glycoconj. J.* 26, 3-17.
- Smith, L.M., Wang, M., Zangwill, K., and Yeh, S. (2002). *Trichomonas vaginalis* infection in a premature newborn. *J. Perinatol.* 22, 502-503.
- Song, H., Shin, M., Ahn, M., Min, D., Kim, Y., and Ryu, J. (2008). *Trichomonas vaginalis*: Reactive oxygen species mediates caspase-3 dependent apoptosis of human neutrophils. *Exp. Parasitol.* 118, 59-65.
- Sutton, M., Sternberg, M., Koumans, E.H., McQuillan, G., Berman, S., and Markowitz, L. (2007). The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001–2004. *Clin. Infect. Dis.* 45, 1319-1326.

- Thapar, M., Parr, E., and Parr, M. (1990a). Secretory immune responses in mouse vaginal fluid after pelvic, parenteral or vaginal immunization. *Immunology* 70, 121.
- Thapar, M.A., Parr, E.L., and Parr, M.B. (1990b). The effect of adjuvants on antibody titers in mouse vaginal fluid after intravaginal immunization. *J. Reprod. Immunol.* 17, 207-216.
- Thurman, A.R., and Doncel, G.F. (2011). Innate immunity and inflammatory response to *Trichomonas vaginalis* and bacterial vaginosis: relationship to HIV acquisition. *Am. J. Reprod. Immunol.* 65, 89-98.
- Upcroft, P., and Upcroft, J.A. (2001). Drug targets and mechanisms of resistance in the anaerobic protozoa. *Clin. Microbiol. Rev.* 14, 150-164.
- Van Der Pol, B., Kraft, C.S., and Williams, J.A. (2006). Use of an adaptation of a commercially available PCR assay aimed at diagnosis of chlamydia and gonorrhea to detect *Trichomonas vaginalis* in urogenital specimens. *J. Clin. Microbiol.* 44, 366-373.
- Van Der Pol, B., Kwok, C., Pierre-Louis, B., Rinaldi, A., Salata, R.A., Chen, P., van de Wijgert, J., Mmiro, F., Mugerwa, R., and Chipato, T. (2008). *Trichomonas vaginalis* infection and human immunodeficiency virus acquisition in African women. *J. Infect. Dis.* 197, 548-554.
- Vilela, R.C., and Benchimol, M. (2012). *Trichomonas vaginalis* and *Tritrichomonas foetus*: interaction with fibroblasts and muscle cells-new insights into parasite-mediated host cell cytotoxicity. *Mem. I. Oswaldo Cruz* 107, 720-727.
- Vilela, R.C., and Benchimol, M. (2011). Interaction of *Trichomonas vaginalis* and *Tritrichomonas foetus* with keratin: an important role in parasite infection. *Mem. I. Oswaldo Cruz* 106, 701-704.
- Voyich, J.M., Palecanda, A., and Burgess, D.E. (2001). Antigen-specific T-cell responses in cattle immunized with antigens of *Tritrichomonas foetus*. *J. Parasitol.* 87, 1040-1048.
- Wang, A.L., and Wang, C.C. (1986). The double-stranded RNA in *Trichomonas vaginalis* may originate from virus-like particles. *PNAS* 83, 7956-7960.
- Wang, C.C., McClelland, R.S., Reilly, M., Overbaugh, J., Emery, S.R., Mandaliya, K., Chohan, B., Ndinya-Achola, J., Bwayo, J., and Kreiss, J.K. (2001). The effect of treatment of vaginal infections on shedding of human immunodeficiency virus type 1. *J. Infect. Dis.* 183, 1017-1022.
- Wendel, K.A., Erbeling, E.J., Gaydos, C.A., and Rompalo, A.M. (2002). *Trichomonas vaginalis* polymerase chain reaction compared with standard diagnostic and therapeutic protocols for detection and treatment of vaginal trichomoniasis. *Clin. Infect. Dis.* 35, 576-580.

Weston, T., and Nicol, C. (1963). Natural history of trichomonal infection in males. *Br. J. Vener. Dis.* 39, 251-257.

Wølner-Hanssen, P., Krieger, J.N., Stevens, C.E., Kiviat, N.B., Koutsky, L., Critchlow, C., DeRouen, T., Hillier, S., and Holmes, K.K. (1989). Clinical manifestations of vaginal trichomoniasis. *JAMA* 261, 571-576.

Workowski, K.A., Berman, S., and Centers for Disease Control and Prevention (CDC). (2010). Sexually transmitted diseases treatment guidelines, 2010. *MMWR Recomm Rep.* 59, 1-110.

World Health Organization. Global incidence and prevalence of selected curable sexually transmitted infections - 2008. Geneva, Switzerland: WHO; 2012.

World Health Organization. Prevalence and incidence of selected sexually transmitted infections. Chlamydia trachomatis, Neisseria gonorrhoeae, syphilis and Trichomonas vaginalis: methods and results used by WHO to generate 2005 estimates. Geneva, Switzerland: WHO; 2011.

Wu, H., Abdu, S., Stinson, D., and Russell, M.W. (2000). Generation of female genital tract antibody responses by local or central (common) mucosal immunization. *Infect. Immun.* 68, 5539-5545.

Yadav, M., Gupta, I., and Malla, N. (2005). Kinetics of immunoglobulin G, M, A and IgG subclass responses in experimental intravaginal trichomoniasis: prominence of IgG1 response. *Parasite Immunol.* 27, 461-467.

Yano, A., Aosai, F., Yui, K., Kojima, S., and Kawana, T. (1983). Antigen-specific proliferation responses of peripheral blood lymphocytes to Trichomonas vaginalis antigen in patients with Trichomonas vaginitis. *J. Clin. Microbiol.* 17, 175-180.

Yin, Y., Wu, Z., Lin, C., Guan, J., Wen, Y., Li, L., Detels, R., and Rotheram-Borus, M.J. (2008). Syndromic and laboratory diagnosis of sexually transmitted infection: a comparative study in China. *Int. J. STD AIDS* 19, 381-384.

Zariffard, M.R., Harwani, S., Novak, R.M., Graham, P.J., Ji, X., and Spear, G.T. (2004). Trichomonas vaginalis infection activates cells through toll-like receptor 4. *Clin. Immunol.* 111, 103-107.

Zhang, Z., Graham, S., Yu, S., Marshall, J., Zielezny, M., Chen, Y., Sun, M., Tang, S., Liao, C., and Xu, J. (1995). Trichomonas vaginalis and cervical cancer: A prospective study in China. *Ann. Epidemiol.* 5, 325-332.