

IDENTIFICATION OF NOVEL VIRULENCE FACTORS AND MECHANISMS OF
PATHOGENESIS FROM THE SEXUALLY TRANSMITTED PROTOZOAN

TRITRICHOMONAS FOETUS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

In

Veterinary Molecular Biology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2006

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ACKNOWLEDGEMENTS

I would first like to thank my advisor Dr. Allen Harmsen. Without his faith and support, this work or this degree may have never have been achieved. Although he initially had little background with the project I finished in his lab, he always made a tremendous effort to understand and guide me throughout the entire process. I would also like to thank Allen for his honesty and candor, his valuable advice, and most importantly his patience.

Every research project needs an initial spark in the beginning. I would like to thank my former advisor Dr. Donald Burgess for that spark. His wisdom and guidance helped to build my scientific creativity and perseverance, for which I will always be grateful.

I would also like to thank my committee members Dr. Mark Quinn, Dr. Michele Hardy, and Dr. Jim Burritt. Their guidance and ideas throughout the years have been invaluable.

I have also been fortunate to be surrounded by a tremendous support system. I would like to thank Kirk and Lynnelle for their selflessness and unwavering friendship. I would like to also thank everyone in the Harmsen lab for all of the scientific and moral support, guidance, and technical assistance they gave me. I feel blessed to have worked side by side with such wonderful people. I would like to thank everyone at the ARC, especially Tammy Marcotte. In addition, I would like to thank all of my friends at the VMB, who have not only made six years of graduate school enjoyable but unforgettable.

Finally, I would like to thank my family. Their continual faith in me and their love have helped me to overcome any obstacle. My best friend and husband August Rutkowski has provided me with undying love, support, and understanding. Throughout this all, he has been a continual reserve for my sanity. He has taught me that whatever direction life throws you in don't ever give up.

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ABSTRACT

Trichomonas foetus (*T. foetus*) is the cause of trichomoniasis in cattle. Little is known regarding the basis of virulence in this parasite. Early host-parasite interactions most likely affect the ability of the parasite to colonize the female reproductive tract. These early interactions shape the course of disease and ultimately control the outcome of pathogenesis. The aim of the studies herein is to provide insight into the factors that alter the progression of trichomoniasis. We examined how environmental stress and estradiol treatment affect pathogenesis of trichomoniasis. Acute *T. foetus* infection in normal mice resulted in facile colonization of the reproductive tract with little epithelial damage, inflammation, or cytokine expression. Infection in estradiol-treated or stressed mice resulted in increased tissue damage, inflammation, and inflammatory cytokine expression. However, estradiol-treatment or stress did not result in enhanced *T. foetus* colonization within the reproductive tract. Since *T. foetus* has been associated with heavy neutrophil and macrophage accumulation in severe disease states, an additional goal was to examine the role of the innate immune system in the colonization of *T. foetus* within the murine reproductive tract. Mice depleted of neutrophils were more susceptible to infection than mock-depleted controls. Additionally, mice with deficiencies in RNS production had substantially larger parasite burdens than mice with the ability to generate RNS, whereas mice with the ability to generate ROS were equally able to control dissemination of *T. foetus* throughout the reproductive tract, compared to wild-type controls. Lastly, we examined the relationship between *T. foetus* and epithelial cell interactions to trichomonad virulence. Investigation of host-parasite interactions revealed that *T. foetus* exhibited increased adhesion and cytotoxicity towards host cells. In addition, a secreted cytoactive factor termed CDF was isolated and purified from activated parasites. This 30 kDa cysteine protease caused rounding and detachment of target cells in addition to inducing apoptosis and 100% cell death by 72 hours of exposure to target cells. These results support our hypothesis that initial parasite/host cell interactions affect trichomonad virulence.

TRITRICHOMONAS FOETUS

Introduction

Tritrichomonas foetus (*T. foetus*) is a sexually transmitted parasite and the causative agent of bovine trichomoniasis. Infection with *T. foetus* results in a range of clinical manifestations, with the greatest economical impact occurring during parturition. Inflammation in bulls harboring the disease is minimal, resulting in a persistence of parasite colonization within the urogenital tract. In females, initial infection oftentimes goes undetected until overall calf crop and quality are greatly diminished. Abortion, low birth weight calves, and temporary sterility are common phenotypes of disease.

Biology

Classification

T. foetus is grouped into the phylum *Parabasalia*, the order *Trichomonadidia*, and into the family *Trichomonadidae*. Based on the pathology of disease and virulence mechanisms *Trichomonas vaginalis*, the causative agent of human trichomoniasis, is the most closely related family member to *T. foetus*. Due to their similarity, *T. foetus* has been proposed for use as an animal model to study the human pathogen and disease³⁸. Parasites from the phylum *Parabasalia* are primitive early grouping eukaryotes, with phylogenetic similarities of metabolic enzymes to eubacteria lineages¹⁵⁶.

In addition, *T. foetus* is also closely related to a pig commensal. *T. foetus* is grouped with *Tritrichomonas suis* into the species *tritrichomonadinae* (Figure 1.1).

Based on morphological studies¹⁴⁷, studies in which cattle and bulls were infected with the porcine commensal¹⁴⁷, pathogenicity of each trichomonad¹⁴⁷, fingerprint analysis of the parasite DNA^{147,154} and phylogenetic analysis of PCR products from internal transcribed spacer units of the 5.8S rRNA subunit^{49,51,79}, these protozoan are identical species. These results are intriguing due to the fact *T. suis* is considered a harmless commensal whereas *T. foetus* is a pathogenic parasite.

Structure and Life Cycle

In culture conditions, *T. foetus* is generally spindle to teardrop shaped with approximate body dimensions of 13 µm in length and 4 µm in width. *T. foetus* has three anterior flagella originating from the basal body and one recurrent flagellum forming the undulating membrane along the surface of the cell and exiting through the posterior end^{8,50}. Also protruding out of the posterior end is the axostyle, a single ribbon of highly ordered microtubules spanning the entire length of the parasite. The axostyle of *T. foetus* has two possible functions: 1) structural support for the cell and 2) participation in cellular division by providing constriction of the nucleus⁸. Figure 1.2 depicts the structure of *T. foetus*.

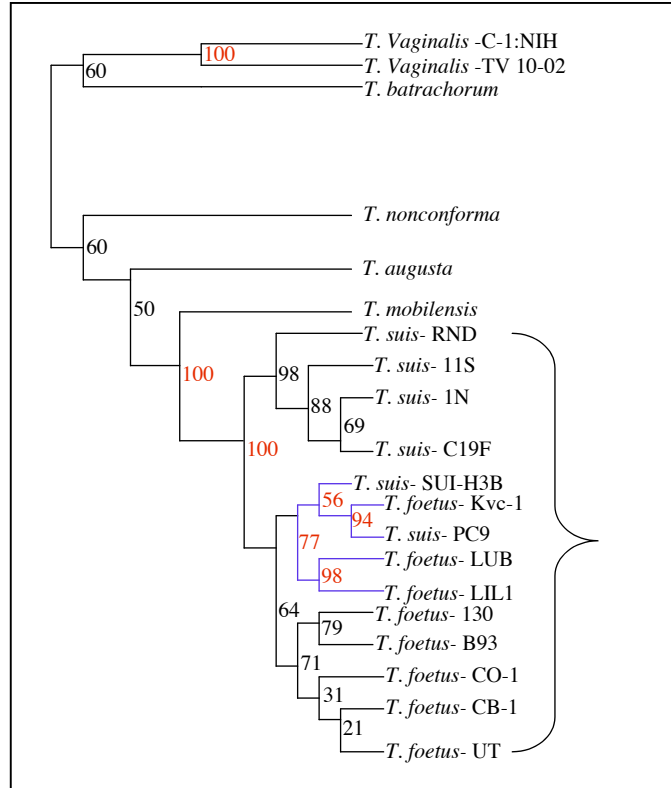


Figure 1.1. Dendrogram analysis of *T. foetus* and *T. suis* DNA using RAPD (random amplification of polymorphic DNA). *T. foetus* and *T. suis* group into two distinct clades from 5 other species of trichomonads. The bracket highlights the similarity between various strains of *T. foetus* and *T. suis*. *T. suis* strains SUI-H3B and PC9 are closely related to three strains of *T. foetus*, indicated by the blue branches. Adapted from reference ¹⁴⁷.

Originally *T. foetus* was described as an extracellular parasite, existing in only a trophozoite form. Recently, studies have observed invagination of the flagella in response to lowered culture temperatures⁶⁰. These non-flagellated and non-motile parasite forms were termed pseudocysts due to the absence of a true cyst wall⁶⁰. Diminished parasite

surface area under various environmental stresses could prevent damage to the flagella and undulating membrane⁶⁰, thus preventing destruction of the cell plasma membrane. Alternatively, it has been proposed that the pseudocyst is a highly viable, yet immotile, life form of trichomonads. It was shown that *T. foetus* pseudocysts could adhere to vaginal epithelial cells better than the trophozoite form¹⁰¹. Thus, the precise function of pseudocyst formation and its role during infection has not yet been fully determined.

T. foetus is an extracellular aerotolerant anaerobe and is easily cultured in axenic culture conditions. *T. foetus* does not have a sexual cycle and divides by mitosis. All skeletal structures of *T. foetus* duplicate, including the flagella, which help power cytokinesis through locomotion⁸. It has not been determined how chromosome segregation occurs within trichomonads and whether parasites can divide in the pseudocyst stage.

Parasite Metabolism

Of particular evolutionary interest is that trichomonads do not possess mitochondria, but instead possess a double membrane organelle termed the hydrogenosome. Similar to mitochondria, the hydrogenosome has been hypothesized to be an endosymbiant within these primitive protozoa. One large difference between mitochondria and hydrogenosomes is that hydrogenosomes do not contain a genome or other molecules indicative of a primitive bacterial endosymbiont³⁴. Currently, the origin of these organelles is intensely debated.

Hydrogenosomes were first reported to be important in anaerobic metabolism. These organelles are responsible for the metabolism of pyruvate to acetate, using protons as terminal electron acceptors for the generation of molecular hydrogen as an end product (Figure 1.3A⁹³). In addition, *T. foetus* hydrogenosomes can undergo pyruvate metabolism using molecular oxygen as terminal electron acceptor during aerobic metabolism (aerobic pathway is depicted in green in Figure 1.3A²⁶). Thus, the hydrogenosome facilitates the ability of *T. foetus* to survive in oxygen for brief periods of time.

Metabolic abilities of trichomonads have been intensely studied. An important finding was that *T. foetus* was incapable of *de novo* purine and pyrimidine nucleotide synthesis^{159,160}. The unique nucleotide synthesis pathway of *T. foetus* results in a dependence upon external sources for molecules that are essential for purine and pyrimidine synthesis. Parasites can be cultivated in a defined medium containing hypoxanthine, uracil, and thymidine¹⁶¹. In addition to being dependent on environmental sources for nucleotide synthesis, *T. foetus* is also dependent upon uracil for glycogen synthesis¹⁶¹.

Mechanisms of *T. foetus* Cell Death

Since both *T. foetus* and *T. vaginalis* lack mitochondria, it has been accepted that they die by necrosis. However, recent studies have reported a form of programmed cell death resembling apoptosis. A study by Chose et al. described the induction of cell death in *T. vaginalis* using pro-apoptotic drugs²⁷. In addition, they determined *T. vaginalis* treated with pro-apoptotic drugs underwent nuclear fragmentation, chromatin

condensation, and the formation of apoptotic-like bodies. Caspase inhibitors did not prevent this sequence of events, suggesting induction of *T. vaginalis* death with pro-apoptotic drugs occurs through a caspase-independent mechanism.

Caspase-dependent apoptosis is the result of a sequential activation of several caspases. A caspase-3 like protein has been detected in *T. foetus*⁹⁸. When parasites were treated with H₂O₂ and pro-apoptotic drugs, an antibody that binds activated caspase-3 bound to a high molecular weight protein only expressed when the parasites were undergoing cell-death physiologically resembling apoptosis¹⁰². This study was important for two reasons. First, it was demonstrated that oxidative stress induces programmed cell death in *T. foetus* with characteristics similar to those described in the report by Chose et. al. Second, the authors describe a caspase-3-like protein and its activation during programmed cell death. These results suggested amitochondrial parasites possess the machinery to undergo caspase-mediated apoptosis.

It has yet to be determined whether other caspase-like proteases exist in *T. foetus* or *T. vaginalis* through sequence or antibody analysis. In addition to being amitochondriate, *T. foetus* also lacks cytochrome-c⁹⁸. This suggests apoptosis occurs through an undefined and perhaps primitive pathway. An alternate caspase-independent program of cell death has recently been reported for *T. foetus*¹⁰². Parasites were treated with griseofulvin to induce cell death. Cell death with several features resembling apoptosis was observed. Caspase-3 was not detected after treatment with griseofulvin. These results suggested alternate undefined pathways for programmed death do exist in *T. foetus* and possibly other trichomonads. From an evolutionary standpoint, it will be

interesting to determine whether the hydrogenosome is involved in this unique form of programmed cell death and how it differs from mitochondrial driven apoptosis.

Trichomoniasis

Trichomoniasis is a venereal disease of cattle caused by infection with *T. foetus*. Infection occurs during coitus, usually in cattle operations utilizing natural service insemination. Artificial insemination is one option for control or elimination of trichomoniasis from herds, although it is not usually feasible for large cattle operations.

Infection in Bulls

Cows are more susceptible to *T. foetus* infection than bulls, as only 10^3 trichomonads are required to establish infection in females³³. However, up to 10^7 trichomonads are required to establish infection in bulls less than 3 years of age³³. Bulls are the natural carrier of the parasite. They can harbor the organism undetected for years with little overt damage. Unlike females, histopathological changes in bulls are absent. Previous studies have been unable to detect lesions associated with *T. foetus* infection¹¹³. Since infection does not result in significant damage to the host in males, little information exists regarding colonization and pathogenesis of the parasites within the preputial cavity.

In bulls, *T. foetus* colonizes the stratified squamous epithelium of the preputial cavity¹²⁵. The epithelium of the preputial cavity becomes folded as bulls age, producing deep crypts in which parasites thrive¹², resulting in bulls becoming more susceptible to infection³². Deep crypts produce a microaerophilic environment in which parasites remain

for the life of the bull. An early study showed a reduction in herd incidence from 47% to 4% by replacing 8 year old bulls with 2 year old bulls²⁸. This study illustrated that replacement of older bulls with younger virgin bulls was an effective means of decreasing *T. foetus* incidence within a herd.

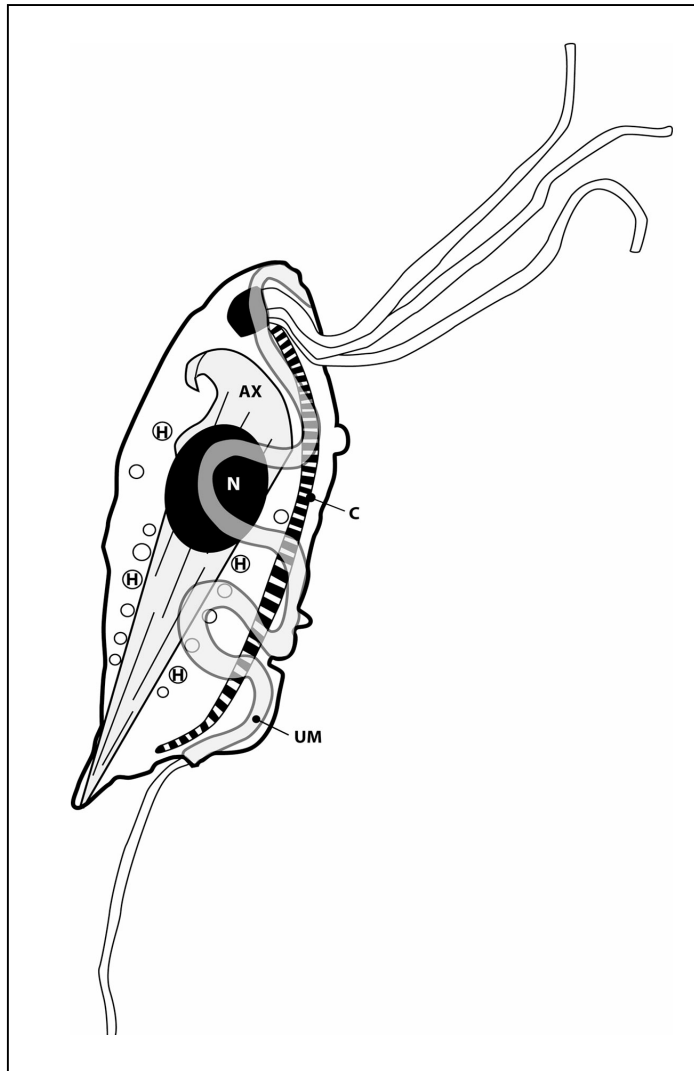


Figure 1.2. Structure of *T. foetus*. Ax: the axostyle running through the body. C: the costa. N: the nucleus. H: represent hydrogenosomes. UM: the undulating membrane that ends in the recurrent flagella protruding from the posterior end. On the anterior end are the three flagella. Adapted from reference⁸.

Systemic vaccination of bulls is protective and has been reported to result in clearance of *T. foetus* from infected animals^{30,31}. Unlike female cattle, bulls do not self-cure without prior vaccination. Parasite-specific IgG1, IgA, IgM, and in low amounts IgG2 have been identified within the preputial cavity of infected bulls¹²⁵, indicating parasite-specific antigen can be taken up by squamous epithelium of the preputial cavity. Bulls have diminished antibody titers to *T. foetus* compared to those commonly observed in females¹⁴¹, supporting the role of bulls as the carrier of *T. foetus* infection.

Infection in Female Cattle

Infection in female cattle is self limiting, resulting in protection against re-infection for up to 1 year^{13,170}. Clearance of the organism from the female reproductive tract usually occurs after 6 and 12 weeks of infection^{13,113}. Although initially disputed, carrier cattle have been identified. Carrier cattle arise due to infection persisting throughout gestation and into the next breeding season without any overt clinical pathology or abortion, when virgin bulls assumed to be free of infection are exposed to carrier females and infected¹³⁸. The carrier state is rare, no more than 1% of infected cattle within a herd will remain infected throughout gestation¹³.

No overt clinical symptoms arise during initial infection, oftentimes resulting in the infection going undetected or misdiagnosed¹³. Mild vaginitis with mucopurulent discharge is common following exposure to the male¹⁷⁰. Histopathologic changes are generally not evident until 60 days post infection, although parasites colonize the entire reproductive tract within two weeks of exposure to an infected male¹¹³. Endometritis and

salpingitis are common sequelae during infection¹¹³. Pyometria can be a consequence of infection, although this is more commonly attributed to post-parturition bacterial infection¹¹.

T. foetus does not prevent conception¹¹³, as it does not directly affect fertilization and implantation of embryos¹⁰. Abortion can occur as early as 17 days after fertilization¹⁷⁰ and preterm delivery has been reported to occur within the first 2-3 months of gestation^{12,113}. Abortion due to *T. foetus* infection has been reported as late as 7 months of gestation¹²². Inflammation within the endometrium and uterine tubes results in a reduction in fertility and oftentimes transient sterility¹¹. It is evident from the varied clinical presentation in female cattle that various factors contribute to disease pathogenesis and outcome. Unfortunately, little information exists regarding pathogenesis of infection.

Early studies suggested trichomonad numbers varied throughout the estrus cycle, with a decline in parasite numbers just before the next estrus¹¹. This is most likely due to variations in estrus cycle affecting host mucosal immunity^{11,163}. Generally considered a lumen dweller, *T. foetus* colonizes the mucosal surface of the entire reproductive tract and is usually harbored within the cervix¹⁴⁰. *T. foetus* invasion of fetal tissues has been reported. Tissue invasion is associated with epithelial degeneration and necrosis, and multifocal hemorrhaging throughout tissues such as the lungs, stomach and intestine, liver, and brain¹²².

Epidemiology and Impact Upon Cattle Operations

Trichomoniasis is most prevalent in cattle operations utilizing natural service insemination and open range management. In the United States alone, 80-95% of commercial beef cattle and 40% of all dairy females are bred by natural service¹³. Although artificial insemination and increased herd management have resulted in lowered incidence of trichomoniasis, it still remains a problem in the United States and other parts of the world¹³.

Prevalence of *T. foetus* infection in bulls in northwestern Spain is 2.9%¹⁰³; in Australia, 1.2% bulls at slaughter presented with *T. foetus*¹⁵¹. In Argentina, there was a 10% incidence in female cattle where *T. foetus* was controlled only in bulls compared to a 0% incidence in female cattle in which both infected males and females who failed to become pregnant after the first mating were culled and treated¹⁰⁰. These data indicated the importance of herd management in control of trichomoniasis. In California, 1 out of 7 herds had at least one bull testing positive for *T. foetus*, with an overall bull prevalence of 5% and a herd prevalence of 15.8%¹⁴. In Florida, the bull prevalence was 6% and the herd prevalence ranged from 0-26%¹²⁰. These studies indicate *T. foetus* is a common problem for large cattle operations throughout the United States and other parts of the world.

The mean number of calves produced by cows mated with infected bulls over a period of 3 years was 17.6% lower than the number of calves kept with non-infected bulls³⁰. Economic loss is not only attributed to diminished calf crop. Treatment, culling and replacement of infected cattle are large expenses producers' experience³⁰. In addition, losses are experienced due to a shortened calf season are other expenses calf producers

experience³⁰. Doubling the rate of infection within the bull population leads to an exponential reduction in calf-crop revenue¹¹⁸. In 1989 it was estimated that with a herd incidence of 40%, the annual revenue/cow would drop 35% with a 50% reduction of the annual calf crop¹¹⁸. In 1998, the annual loss in replacement heifers was estimated at \$ 6.1 million/year with each infected bull being responsible for an annual loss of \$ 4,800¹¹⁹. These results illustrate how infection with *T. foetus* results in significant economic losses to the cattle producers.

Treatment

Topical treatments have been approved for treatment of infection, although they remain inconsistently effective¹⁷⁰. Anaerobic 5-nitroimidazole drug compounds, such as metronidazole, were initially attractive drugs due to the fact they inhibit anaerobic metabolism with minimal effects on host cell metabolism and respiration. Metronidazole enters the hydrogenosome and competes for terminal electrons with the hydrogenase, resulting in an inhibition of hydrogen production and the subsequent production of metronidazole anion radicals that are toxic to the parasite⁸³ (Figure 1.3B).

Initially effective, metronidazole compounds are no longer used to treat trichomoniasis. The United States Food and Drug Administration no longer approves the use of metronidazole because it is a suspected carcinogen¹⁷⁰. In addition, both *T. foetus* and *T. vaginalis* have been reported to develop resistance to metronidazole *in vivo*^{81, 82}. Resistance of *T. foetus* can be induced *in vitro* within 3-7 months⁸⁴ whereas *in vitro* resistance of *T. vaginalis* takes 1-2 years to develop⁸⁵. Aerobic and anaerobic resistance

to metranidazole are both due to the gradual loss of pyruvate ferredoxin oxidoreductase (PFOR) enzyme activity⁸³. Aerobic resistance also occurs due to impaired oxygen scavenging resulting in increased levels of oxygen that impair metranidazole activation⁸³. Aerobic metranidazole resistant parasites are sensitive to anaerobic conditions after resistance has developed. Following diminished activity of PFOR, anaerobic resistance results in decreased activity of hydrogenase and lowered levels of the electron carrier ferredoxin (depicted in blue in Figure 1.3⁸³). *T. foetus* also is able to compensate for the loss of PFOR and ferredoxin activity by converting 95% of glucose to ethanol (Figure 1.3B⁸³).

Due to the unique metabolic properties of these early branching eukaryotes, selective chemotherapeutic agents that do not harm host cells have been studied. Since *T. foetus* is incapable of *de novo* synthesis of purines, the parasite preferentially scavenges hypoxanthine from its host and eventually converts it to guanosine monophosphate with the rate limiting enzyme inosine-5'-monophosphate dehydrogenase (IMPDH)¹⁵⁵. The crystal structure of *T. foetus* IMPDH revealed a substrate-binding domain unique from IMPDH binding domains of other species¹⁶². Inhibitors specifically designed to interfere with that domain would thus be attractive drug targets. Vermiculine, an alglycosidic antibiotic, was also found to interfere with nucleic acid synthesis profoundly affecting *T. foetus* viability within 24 hours⁵³.

To date, there is no effective treatment or cure for trichomoniasis. A vaccine is commercially available but only reduces the time it takes for cattle to clear the infection and is completely ineffective in bulls. During a clinical trial for the commercially

available vaccine Trich Guard, heifers that were given the vaccine and exposed to infected bulls in addition to challenge with a high dose of trichomonads (10 million; it only takes 1 million to successfully infect cattle) had nearly a 50% higher calving rate than animals who did not receive the vaccine³¹. However, the calving rate remained low in immunized animals, suggesting only partial protection.

Preventative measures and increased herd management practices are common practices to prevent and control trichomoniasis within a herd. Generally, replacement of infected bulls with virgin bulls less than three years of age in addition to culling cattle who have failed to produce after multiple exposures to the male are employed to increase cattle crop in infected herds^{13,170}. Increased herd surveillance and artificial insemination are two additional means of controlling and eradicating disease.

Diagnosis

Due to the insidious nature of *T. foetus* infection, presence of the parasite within a herd oftentimes goes undetected until substantial loss has already occurred. Infection in females often goes undetected due to early abortion resulting in re-exposure of females to males, increased calving to conception intervals^{13,138}, and smaller, less developed calves due to the shortened weaning season¹¹⁸. Demonstration of live *T. foetus* by culturing scrapings from the preputial smegma in sexually rested bulls is the most common practice to detect infection within a herd^{13,170}. Generally, three negative cultures within a three-week period indicate a true negative result¹⁷⁰. In females, the likelihood of obtaining a positive result from a known infected animal is estimated ~ 60%²⁹. These results could

suggest *T. foetus* has the ability to evade detection by colonizing unaccessible regions of the reproductive tract.

Since other organisms within the reproductive tract of cattle are morphologically similar to *T. foetus*, other detection methods have been examined. In addition, culture methodologies are hampered both by contamination of other microorganisms and the viability and outgrowth of trichomonads within the diagnostic culture pouches. One study demonstrated smears made from media containing *T. foetus* and stained using a modified Wright-Giemsa kit with an iodine solution resulted in distinguishable anatomical features unique to *T. foetus*⁹⁸. This technique was facile, reliable, and relatively inexpensive compared to other strategies utilized in identification of trichomonads. Oftentimes few trichomonads are recovered from the bull or cattle, indicating the need for more rapid and sensitive means of detection than is currently available.

Molecular biology techniques used for the specific detection of various *T. foetus* isolates have been demonstrated. In a study by Felleisen et al.⁵¹, a high stringency PCR that was able to detect as little as 0.03 pg of trichomonad DNA was optimized. To minimize false positive results, the authors utilized a complimentary DNA enzyme immunoassay to easily discriminate between false negative amplification products and *T. foetus* DNA⁵¹.

Virulence factors of *T. foetus*

Little is known regarding the basis of virulence of this parasite, although certain factors (e.g. adhesion, cytotoxicity, protease secretion, immune evasion) are well

characterized with *in vitro* models. With regard to parasite establishment of infection, it is unknown how virulence factors help to circumvent the dynamic defense mechanisms of the reproductive tract.

Adhesion

Studies of *T. foetus* interactions with target cells *in vitro* and host cells *in vivo* indicated an important role for adhesion-mediated cytotoxicity. It has been shown that *T. foetus* initially adheres to target cells through the posterior projection of the axostyle followed by the cell body and flagella^{41,132}. These studies indicated adhesion is the first step involved in colonization and interaction with target cells.

Using antibodies against *T. foetus* surface antigens, Burgess et al.²³ demonstrated *T. foetus*-mediated cytotoxicity was dependent upon adhesion or contact with target cells²³. This indicated that preventing adhesion could be important treatment for trichomoniasis. Inhibition of adhesion and cytotoxicity could have been due to agglutination of the parasites by IgG1 antibodies⁴¹. Although the mechanism that inhibits parasite adhesion is not fully elucidated, a large body of work has focused upon purification of *T. foetus* surface antigens that mediate adhesion.

Characterization of the 190 kDa adhesin (Tf190) molecule described by Burgess²³ demonstrated this molecule was both immunogenic and contained carbohydrate moieties similar to lipophosphoglycan (LPG)¹²⁹. *T. foetus* LPG-mediated adhesion to primary bovine vaginal epithelial cells was shown to be species-specific¹³⁶. Additionally, periodate treatment of parasites diminished adhesion and cytotoxicity¹³⁶ demonstrating the importance of carbohydrate moieties in the adhesion process. Interestingly, pseudocysts express Tf190 and are able to adhere to epithelial cells better than the trophozoite form¹⁰¹. It was shown that immunization of cattle with the Tf190 adhesion resulted in protection against vaginal challenge of *T. foetus* in cattle¹⁵⁷. Antisera from immunized cattle prevented parasite adhesion to monolayers of transformed cell lines¹⁵⁷. This study was important for two reasons; first, it presented *in vivo* evidence supporting the rationale that parasite adhesion was important for establishment of infection, second, it further supported the hypothesis that cytotoxicity and host colonization was a contact-dependent mechanism. Additional adhesins have been identified (TF1.17) that share biochemical and antigenic similarities to TF-LPG¹³⁴.

Parasite adhesion directly to the epithelium of the reproductive tract is often minimized due to the large amounts of mucus covering the vaginal epithelium. *T. foetus* first binds to the mucous and degrades it with enzymes to expose the underlying epithelium⁹⁰. *T. foetus* utilizes lectins with sialic acid specificity for adhesion to the mucosal surfaces of the reproductive tract⁵. Although the reproductive tract has evolved a significant barrier to prevent colonization of foreign microorganisms, *T. foetus* has evolved mechanisms to get past this initial first line of defense.

Parasite adhesion could be a mechanism the parasites use to communicate with the surrounding epithelium and vaginal environment. A 118 kDa laminin-binding protein was detected on the surface of both *T. foetus* and *T. vaginalis*²⁵. Adhesion to polystyrene and an epithelial cell line was significantly reduced by the addition of anti-laminin antibodies²⁵. The authors found that levels of laminin-binding protein on the surface of trichomonads correlated to subsequent cytotoxicity towards host cells. Binding of *T. foetus* to laminin globular domains induces metalloprotease activity¹³³. In addition, it has been shown that parasites cultured in reduced iron media had a diminished ability to bind to and kill target cells¹⁰⁶. These results suggest binding of *T. foetus* to laminin induces signals important for initial colonization and subsequent cytotoxicity within the host.

Adhesion of both *T. foetus* and *T. vaginalis* to epithelial cells has also been shown to alter the host gene expression. Adhesion of *T. vaginalis* to an immortalized vaginal epithelial cell line resulted in parasite-induced upregulation of epithelial genes encoding cell structure maintenance, extracellular matrix components, proinflammatory molecules, and molecules indicative of apoptosis⁸². More importantly, it was shown that both *T. foetus* and *T. vaginalis* induce expression of COX-2 protein⁸², an inflammatory cytokine induced by growth factors and attributed to poor pregnancy outcomes³⁶. Thus, a portrait emerges illustrating a dynamic environment in which both parasite and host genes are regulated after parasite contact with the host epithelium.

Proteases

Throughout the literature describing *T. foetus* and *T. vaginalis*, it has been well established these parasites possess an abundance of proteases. Proteases are important in the life cycle of certain parasites and are involved in cellular destruction and killing, helping parasites to establish infection by invading host tissues. In addition, proteases show a broad adaptability for different substrates and are stable in various biological environments (reviewed in ¹²⁷).

Pathogenic urogenital trichomonads such as *T. foetus* and *T. vaginalis* have more active proteases than the commensal intestinal trichomonads such as *Trichomonas batrachorum* and *Pentatrichomonas hominis* ⁹⁵, suggesting proteases are virulence factors of pathogenic trichomonads. Previous studies of cytoactive factors secreted from *T. vaginalis* have suggested these soluble proteins have the capabilities to detach cells^{54,99} and their protein expression is changed during prolonged culture *in vitro* ¹⁰⁹. Proteases secreted from *T. vaginalis* also showed great immunogenicity¹⁰⁹, indicating strategies that target proteases for vaccines have potential for therapeutic uses.

Initial studies indicated that proteases isolated from conditioned medium or extracts of *T. foetus* and *T. vaginalis* were mainly of the cysteine proteinase type. These studies were based on activation with dithiothreitol and inhibition with the cysteine proteinase inhibitors antipain, leupeptin, *N*- α -*p*-tosyl-*L*-lysine chloromethyl ketone (TLCK), and *L*-3-carboxy-2,3-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64)⁹⁵. Cysteine proteases also show various specificities for hydrolysis of certain amino

acids¹¹⁰. In addition, cysteine proteases have been identified that are involved in cytoadherence of *T. vaginalis*⁹⁶.

Proteases from *T. foetus* have not been as well characterized as the proteases from *T. vaginalis*. In this regard, non-cysteine proteinase virulence factors such as metalloproteases¹⁸ and phospholipase C⁹⁷ have been identified and characterized from *T. vaginalis*, suggesting the possibility of yet unknown virulence factors to be identified from *T. foetus*. In a recent study, hemagglutinating ability and cytotoxic activity was identified in a strain of *T. foetus*. After subsequent purification and characterization, a 40 kDa non-proteinase virulence factor was identified⁷⁷, confirming *T. foetus* does have undefined cytotoxic capabilities.

Proteases isolated from *T. foetus* have recently been proposed to play an important role in colonization and perhaps even immune evasion. Sing et al^{135,137} have recently isolated and purified a 30 kDa cysteine proteinase (CP30) from *T. foetus* that is able to kill primary bovine vaginal and uterine epithelial cells. These were the first studies to examine the effects of secreted proteases upon natural host cells *in vitro*. CP30 initiated cell death by inducing apoptosis of both bovine vaginal and epithelial cells. Apoptosis was species-specific, and appeared to be dependent upon caspase-3 activity. Induction of apoptosis appeared to occur within 6 hours of application of CP30 onto target cells. The authors suggested the ability of *T. foetus* to induce apoptosis was a means to destroy the host epithelium without releasing inflammatory proteases from host cells, thus initiating colonization without inducing a host inflammatory response¹³⁷.

Epithelial apoptosis could be the reason why there is little overt inflammation observed within the bovine reproductive tract until after 60 days of infection¹¹³.

Iron Sequestration

Iron is a critical component for the metabolism of *T. vaginalis* in addition to its virulence *in vivo*. Beta-hemolytic activity of *T. vaginalis* was found to correlate with virulence of the parasite in human and mouse infections⁸¹, demonstrating a potential role for iron-mediated virulence in particular strains of *T. vaginalis*. The role of iron during *T. foetus* infection is not as well established, although it was determined that iron was critical for growth and metabolism of *T. foetus*. Initially, a 20 kDa thiol proteinase capable of proteolytic activity towards denatured hemoglobin was identified and characterized¹⁰⁵.

Binding sites for lactoferrin¹⁴⁷, in addition to lactoferrin-binding receptors⁵⁸, were identified on the surface of *T. foetus*, demonstrating how *T. foetus* acquired iron from its host. *T. foetus* binding to lactoferrin is mediated by hydrophobic and charge-related interactions of specific receptors⁵⁸. In addition to obtaining iron from lactoferrin, it is now known that *T. foetus* obtains iron from transferrin¹⁴⁶ and perhaps from red blood cells. A 100 kDa adhesin was identified on the surface of *T. foetus* that was able to induce erythrocyte agglutination⁴³ and species-specific hemolysis of erythrocytes through adhesion to the surface of the cells⁴⁵.

A study in which *T. foetus* was cultured in iron-rich conditions, or cultured in the presence of the iron chelator 2,2-dipyridyl, showed that parasites maintained in iron-

restricted conditions had a reduction in activities of several hydrogenosomal enzymes, such as hydrogenase, ferredoxin oxidoreductase, malic enzyme, and pyruvate:ferredoxin oxidoreductase¹⁵³. This study illustrated that regulation of hydrogenosomal proteins was iron-dependent. The protein responsible for incorporation of iron into the hydrogenosome was identified as ferredoxin, which was subsequently absent in metronidazole-resistant strains of *T. foetus*¹⁴³. The absence of ferredoxin most likely results in the inactivity of iron-dependent hydrogenosomal enzymes in the presence of metronidazole.

Trichomonad parasitism of host cells for acquisition of iron indicates this metabolic property is indeed a virulence factor. In addition, other studies have suggested iron directly affects the ability of *T. foetus* to establish infection. Administration of ferric ammonium citrate significantly increased replication and infectivity of *T. foetus* in mice⁸³. In addition, cytotoxicity of *T. foetus* to bovine vaginal epithelial cells was diminished by metranidazole treatment, although trichomonads could still adhere to the cells¹³⁶. Since metronidazole treatment results in reduction of hydrogenosomal enzymatic activity due to diminished levels of ferredoxin, it is likely iron is an important regulator of protein activity. Therefore, diminished cytotoxicity in the presence of metronidazole could be a result of the inability to metabolize iron, resulting in reduced expression of cytotoxic proteases. Iron-mediated activity of other non-hydrogenosomal enzymes in *T. foetus* has been documented. Recently, superoxide dismutase has been identified in *T. foetus*⁶¹, which also uses iron as a cofactor.

Immune Evasion

Antigenic heterogeneity and phenotypic variation of *T. foetus* surface markers is a phenomenon resulting in evasion of host responses. *T. foetus* and *T. vaginalis* exist in heterogeneous populations and have the ability to alternate surface expression of at least two classes of surface markers^{1,42}. Antigenic variation of surface molecules could diminish the effectiveness of parasite-specific antibody responses directed against surface antigens. Parasites co-cultured with hybridomas secreting antibodies against certain *T. foetus* surface molecules for long periods of time were able to down-regulate surface expression of the antigens each hybridoma was generated against⁵⁹. This study illustrated the ability of *T. foetus* to alter expression of surface molecules under immunologic pressure.

A hallmark study of secreted proteases from *T. foetus* illustrated the physiological relevance of cysteine proteases as virulence factors that eliminated host defense mechanisms within the reproductive tract¹⁴⁸. Cleavage of protective host proteins such as fibrinogen and fibronectin was shown to occur, which interferes with monocyte⁶⁹ and neutrophil¹⁰⁸ adhesion to reproductive tract surfaces colonized with trichomonads. This study indicated the ability of *T. foetus* to inhibit innate immune mechanisms through proteolytic activity. The innate first lines of defense are most likely critical during establishment of infection and determining the course of the disease. Diminishing the innate host response is a strategy the parasites utilize to colonize the host.

Trichomonads were also shown to have the ability to cleave IgG1 and IgG2 present within reproductive tract secretions¹⁴⁸, which are important for the opsonization

and subsequent phagocytosis of *T. foetus*^{13,39,140}. This study indicated *T. foetus* could effectively inhibit acquired mechanisms of defense within reproductive tract of cattle. *T. foetus* was also shown to rapidly internalize and degrade antibodies bound to the surface of the parasites⁵⁹. Therefore, studies have illustrated *T. foetus* has the ability to evade both innate and acquired mechanisms within the female reproductive tract.

Summary

In summary, it is evident *T. foetus* possesses a barrage of virulence factors critical to bypass the various barriers in order to colonize the female reproductive tract. An interesting strategy to verify trichomonad-specific virulence factors was proposed by Kucknoor AS et al⁸². Transient episomal transfection of *T. vaginalis* with putative virulence factors from *T. foetus* can be utilized to verify or further characterize specific virulence factor/host cell interactions. By transfecting another distinct parasite, *T. foetus*-specific virulence factors can be verified at the single virulence factor level.

Immunity to *T. foetus*

Innate Immunity

Since *T. foetus* is extracellular and infection in females is generally self-curing, a focus on the role of acquired humoral immune responses on resistance is prevalent in the literature. The only study examining histopathologic changes during early infection in virgin heifers indicated inflammation predominately occurred after 60 days of infection¹¹³. Neutrophils and macrophages were the predominant cellular infiltrates within the endometrium, stratum compactum, glandular epithelium, and the uterine lumen.

These inflammatory cellular infiltrates were generally associated with vaginitis, salpingitis, endometritis, and pyometria¹¹³. Animals with impending abortion had severe inflammatory lesions consisting of macrophages and neutrophils in association of large numbers of *T. foetus* within the placentome and within inflammatory material throughout the lumen of the uterus and uterine glands. This study implicated severe inflammation and heavy parasite burden as a cause of abortion¹¹³. This study was important as it indicated the disease-associated pathologic sequela is due to inflammation.

Two studies that examined aborted fetuses from infected cattle also indicated macrophages and neutrophils were important during infection^{122,124}. Parasites were stained within the tissues and were usually associated with heavy neutrophil and macrophage infiltration. In addition, phagocytized trichomonads were present within macrophages and giant cells. Although early studies demonstrated trichomonad killing, the mechanisms whereby trichomonads are killed is largely unknown.

Murine peritoneal macrophages have been shown to tightly associate with *T. foetus* and subsequently phagocytize parasites presumably destroying them intracellularly⁴⁴. Parasites pre-treated with the mitogen concanavalin A associated with macrophages in greater numbers than did untreated parasites to unactivated macrophages⁴⁴. This study illustrated the ability of macrophages to phagocytize parasites, although it did not directly demonstrate killing of *T. foetus*. Nitric oxide was shown to be an effector of macrophage-mediated cytotoxicity towards the human pathogen *Trichomonas vaginalis*¹¹². This study was the first to demonstrate reactive nitrogen species (RNS) were able to kill the extracellular venereal parasites. However, this did not

implicate RNS as being a critical molecule in the establishment of trichomonad infection within the reproductive tract. Sodium nitrite (NaNO_2) was found to inhibit *T. foetus* motility and growth, in addition to inducing parasite killing at high concentrations⁹⁴. This suggested the possibility of utilizing compounds such as NaNO_2 as topical anti-trichomonidal reagents.

Neutrophil killing of *T. foetus*⁴ and *T. vaginalis*¹²¹ has been demonstrated. Killing of *T. vaginalis* occurred efficiently without either parasite-specific antibodies or agglutination, although complement derived from non-immune sera was needed for killing to be observed¹²¹. This study indicated that if neutrophils are important during human infection, perhaps the parasites have the ability to activate complement via the alternate pathway, as no antibody was necessary for neutrophil killing. Neutrophil killing of *T. foetus* is most effective when parasite-specific antibody and complement are present⁴. Complement killing via the classic pathway is the most effective means of killing *T. foetus*, suggesting neutrophil responses are important after parasite-specific antibody is present.

Mechanisms that initiate innate responses and inflammation during *T. foetus* infection are currently unknown. Recent studies using the human pathogen *T. vaginalis* suggest inflammation during infection is achieved through stimulation of Toll-like receptor 4 (TLR-4) with unknown substances that are only present during infection with *T. vaginalis*¹⁷¹. Although this study did not directly implicate *T. vaginalis*/TLR-4 engagement, it presented a mechanism whereby inflammation is induced. Additional studies with *T. vaginalis* have demonstrated the parasite has the ability to induce IL-8

production from both human monocytes¹³⁰ and human neutrophils¹²⁶. Studies with *T. vaginalis* indicate there are most likely mechanisms whereby *T. foetus* is also able to initiate inflammation within the bovine host.

Acquired Immunity

Immunity, specifically towards surface antigens of *T. foetus*, has been examined thoroughly. Antibodies against surface antigens of *T. foetus* were shown to mediate complement lysis, agglutinate the parasites, and act as opsonins^{21,64}. Naturally infected animals have been shown to mount strong IgA and IgG1 vaginal and uterine antibody responses¹³⁹, suggesting the presence of these antibodies resulted in protection and clearance of the parasites. Vaccination studies have focused on the local IgA and IgG1 responses, as an initial vaccination trial of virgin heifers with TF 1.17 indicated enhanced local IgA and IgG1 responses in addition to high serum levels of IgG1 and IgG2¹⁵. Vaccination of cattle with purified surface antigen TF 1.17 not only elicited a strong uterine IgA antibody response, but the response was correlated to faster clearance of *T. foetus* and less histological evidence of endometritis². This study indicated potential for the surface antigen TF 1.17 as a therapeutic measure to prevent abortion and significant calf loss.

Since TF 1.17 consistently elicited strong IgA and IgG1 vaginal antibody responses, one study examined the use of this surface antigen as a means of detection for the presence of *T. foetus* during infection. Mucosal vaginal IgA responses within all

infected heifers reacted with TF 1.17 surface antigen⁷⁰. These results indicated TF 1.17 could be utilized as a vaccine in addition to a rapid means of detection for infected cattle.

In an experimental vaccine trial utilizing killed *T. foetus* in an oil-adjuvant, a significant reduction in duration of infection was observed with vaccination, although no curative effects were observed in bulls or cattle⁶². Additionally, specific IgM and IgG1 serum antibodies and mucosal IgA antibodies were detected in heifers infected with heat-killed *T. foetus*⁵⁵. Several other studies have developed vaccination strategies with surface antigens^{35,38,157,158} and various routes of immunization and challenge, although no cure for trichomoniasis has been developed.

An important study demonstrated an effector response after immunization with Tf190 surface antigen. This was the first study to demonstrate antigen-specific cellular responses that may be involved in antibody production or clearance of the parasite from the reproductive tract. Voyich et al.¹⁵⁸ showed that antigen-specific CD4⁺ T cells, when re-stimulated with various antigen preparations of *T. foetus*, upregulated IFN γ expression. This resulted in the activation of macrophages, which are important in controlling *T. foetus* infection. This was an important study that linked effector responses such as CD4⁺ T cell proliferation, memory, and IFN γ production to vaccination, antibody production, and protection against *T. foetus*. It demonstrated that other immune mechanisms could be targeted to result in protection in addition to IgA and IgG responses.

Animal Models

Due to the inherent difficulty and expense of large animal studies, researchers have tried to develop small animal models to study *T. foetus* infection. *T. foetus* intravaginal infections have been attempted in the rabbit, hamsters, and guinea pigs⁶⁵. Hamsters are susceptible to infection with indigenous intestinal trichomonads⁶⁵, infection in rabbits is unpredictable and not easily repeated⁶⁵, and guinea pigs need weekly treatment with high doses of estradiol and triamcinolone acetate⁶⁵. Therefore, other means to elucidate the pathogenesis of infection in a reproductive environment needed to be developed.

Initial mouse models indicated mice needed to be maintained in a constant state of estrus either by subcutaneous injections or estradiol implants to be infected¹⁴². Unfortunately, the high doses of estradiols administered during subcutaneous injections resulted in purulent vaginal discharge, perivulvar abscesses, and even death¹⁴². This mouse model has proven effective and has resulted in valuable insight into vaginal infection with *T. foetus*. These studies have indicated that estradiol-treated mice had similar lesions to that seen in natural bovine infection¹⁵². Estradiol-treated mouse studies illustrated mouse age does not affect susceptibility to genital infection, whereas the strain of mouse does⁶⁶. In addition, most of the reports using mouse models have identified that the development of lesions is most likely due to host-parasite interactions early in infection. This is difficult to determine using the bovine model due to the fact that infection doesn't result in overt pathological lesions until after 60 days of infection.

Estradiol-treated mice have provided researchers with valuable information regarding pathogenesis. Estradiol-treatment should be examined very carefully when studying immune responses, as it is well established that estrogens have a profound effect on host immune responses. Subcutaneous administration of estrogen results in the suppression of T-cell independent inflammatory responses as well as reducing polymorphonuclear cell circulation throughout the body⁷⁵. Local effects of estrogen administration, within the reproductive tract, include increased antigen presentation and secretion of IgA and IgG in the uterus but with opposing effects in the vagina^{165,167}. Therefore, developing a mouse model without the effects of estrogen would be invaluable.

Recently Mutwiri and Corbeil¹⁰⁷ were able to infect mice without the administration of estrogen, and this infection lasted well beyond 24 weeks. In this study, a proven pathogenic strain of *T. foetus* was used to infect mice. This strain of *T. foetus* was used in several studies to infect the natural bovine host^{2,39,139}. The ability to infect mice with a known pathogenic strain of *T. foetus* without the need for estradiol-treatment suggested that parasite-mediated factors are responsible in establishing and maintaining infection within the reproductive tract. Although bovine and murine immune responses differ, using a mouse model could provide valuable information regarding host or parasite factors involved in early infection. Host-parasite interactions that occur early in infection most likely dictate the eventual course or outcome of infection.

Summary

Although a large body of evidence exists regarding *in vitro* interactions of trichomonads with target cells and acquired immune responses after *T. foetus* infection, little is known regarding the early mechanisms of pathogenesis. We hypothesize that initial parasite/host cell interactions affect trichomonad virulence. In addition, we hypothesize that innate host-responses or unknown factors that directly affect the reproductive environment dictate the ability of parasites to colonize and establish infection, thus affecting the outcome of disease.

TRITRICHOMONAS FOETUS: PATHOGENESIS OF ACUTE INFECTION IN
NORMAL, ESTRADIOL-TREATED, AND STRESSED MICE

Introduction

Tritrichomonas foetus (*T. foetus*) is the causative agent of bovine trichomoniasis, a venereal disease in cattle associated with preterm delivery and oftentimes abortion and temporary sterility¹⁷⁰. Trichomoniasis is prevalent in cattle operations utilizing natural service insemination and open range management^{56,138}. Parasites are transmitted during coitus from males harboring the disease to females in which disease will oftentimes progress undetected, resulting in significant economic losses to cattle operations due to decreased calf crop yield and low birth weight calves^{118,119}. In North America alone, 40% of cattle operations practice natural service insemination¹³ and with herd incidence ranging from an average of 6% in Florida¹²⁰ to 15.8% in California¹⁴, trichomoniasis is a disease of veterinary relevance.

In the natural bovine host, *T. foetus* infection results in varying degrees of vaginitis, endometritis, and on rare occasions pyometria^{113,170}. Although animals can have high parasite burdens within the vagina and uterus, infection doesn't result in significant tissue damage and abortion until 60 days post infection¹¹³. Abortion and fetal loss are usually associated with severe salpingitis and cervicitis, with heavy neutrophil and monocytic infiltration observed throughout the stratum compactum and associated tissue damage and erosion between the placentome and endometrial lining¹¹³. Typically considered a lumen dweller, epithelial invasion of fetal tissues has been reported. Heavy

burdens of *T. foetus*, inflammatory cells, and associated tissue damage were observed in lungs, intestine, liver, and brain of aborted fetuses¹²². These studies suggest undefined host or parasite-mediated factors influence the ability of trichomonads to become tissue invasive.

Inherent difficulties and expense of large animal studies have prevented researchers from elucidating how *T. foetus* establishes infection and what factors (host or parasite-mediated) determine the course of infection. Estradiol-treated mice have been used to study host immune and inflammatory responses to *T. foetus*. Establishing infection in mice with a laboratory adapted strain of *T. foetus* required induction and maintenance of a persistent state of estrus^{66,67,142,152} a period in which neutrophils are absent from the lower reproductive tract. A study by Mutwiri and Corbeil¹⁰⁷ demonstrated no correlation between stage of estrus and establishment of infection in mice with a proven pathogenic strain of *T. foetus*, indicating both host and parasite-mediated factors are responsible for establishment and maintenance of infection. However, these studies focused on the host response after 2 weeks of infection and did not examine early time points during which *T. foetus* establishes infection.

There is a lack of understanding regarding the role of the female reproductive tract in establishing and maintaining varying degrees of *T. foetus* infection. Invasion of the mucous layer by trichomonads, an important initial step in colonization of the reproductive tract, requires parasite adhesion, secretion of mucinases, and motility⁹⁰. Estrogens can increase production of mucous and oviductal secretions¹¹. Additionally, estrogens can inhibit the growth and adherence of *Trichomonas vaginalis*¹⁴⁴, the human

pathogen closely related to *T. foetus*. These studies demonstrate mucous and reproductive hormones are important physiological barriers against trichomonad colonization. Additionally, reproductive hormones^{17,20,163-165,167} and stress^{13,52} directly affect the immune response within the female reproductive tract. These studies indicate factors such as reproductive hormones and environment may affect *T. foetus* establishment of infection, pathogenesis, and clearance from the reproductive tract.

The goal of this study was two fold: 1) to compare how estradiol-treatment or stress affect the ability of *T. foetus* to establish infection in mice 2) to determine whether estradiol-treatment or stress alter or change the pathologic sequelae of early *T. foetus* infection. We compared infection, associated innate and cellular immune responses, and histological changes during initial colonization (4 days) to three weeks post *T. foetus* infection between untreated BALB/c mice (normal mice), estradiol-treated BALB/c mice, and stressed BALB/c mice exposed to constant bright illumination (LL). Mice exposed to LL begin to enter into a state of persistent estrus, characterized by continual cornification of the epithelial layer of the reproductive tract and continual receptiveness to males for breeding¹²⁸, after approximately two estrus cycles (7-8 days). Previous studies in mice have suggested longer periods of darkness, and thus more secretion of melatonin, result in higher numbers of circulating B and T cells in addition to enhancing natural killer cell cytotoxicity, whereas mice housed in LL have lower numbers of circulating B and T cells in addition to diminished NK cell cytotoxicity¹⁶⁹.

The present study illustrates how estradiol-treatment or stress alters the ability of *T. foetus* to establish infection and cause associated tissue damage. Specifically, estradiol-

treatment diminished the ability of *T. foetus* to colonize the reproductive tract, resulting in a significantly lower parasite burden by 21 days of infection. Conversely, estradiol-treatment accelerated infection-induced tissue damage and inflammation. Stressed mice had similar parasite burdens to normal mice, although inflammation and tissue damage were enhanced in comparison to normal mice. Normal mice were colonized with comparatively higher numbers of parasites, although increased parasite burden did not result in extensive tissue damage or inflammation, resembling the normally insidious bovine infection. Therefore, it can be concluded that various treatments such as estradiol and stress directly affect early parasite colonization in addition to enhancing inflammation and associated tissue damage during infection.

Materials and Methods

Mice

Female BALB/c mice 6-8 weeks of age were purchased from National Cancer Institute or bred in the animal facility at Montana State University. Mice were housed in microisolator cages and provided with sterilized food and water *ad libitum* in accordance with the *Guide for the Care and Use of Laboratory Animals*. Fluorescent lighting was provided on a 12 hour light/dark cycle unless otherwise indicated. All experimental procedures and protocols were approved by and followed the guidelines set forth by the Institutional Animal Care and Use Committee.

Parasites and Inoculums

T. foetus strain D1, a low passage isolate originally isolated from a cow with pyometria that has been previously utilized to successfully infect cattle^{2,39,140} was a kind gift from Lynnette B. Corbeil (University of California, San Diego). *T. foetus* strain 99 (TF99) is a clone isolated from the lung of an aborted fetus in 1999. Parasites were maintained in axenic culture at 37°C in modified Diamonds TYM media⁴⁶ supplemented with 10% fetal bovine serum (Atlas Biologicals; Ft Collins, CO) and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco, Carlsbad CA). Parasite inocula were prepared by incubating an aliquot of D1 as previously mentioned until logarithmic growth was achieved. Parasites were counted on a Neubauer hemocytometer, washed three times in sterile HBSS and resuspended in HBSS. Quantification of parasite burden was achieved by incubating reproductive tract homogenates for 48 hours in Diamonds media. 40 µl of incubated homogenate was applied to a slide and examined for motile parasites. 0-20 parasites per microscopic field was assigned a value of 1, 20-40 parasites/field was assigned a value of 2, 40-60 parasites/field was assigned a value of 3, and greater than 60 parasites/field was assigned a value of 4. An average of three to five fields/slide were examined. Too many parasites to count (> 60) were assigned a value of 4.

Normal Infection

On two consecutive days, untreated mice housed in normal conditions (absence of hormonal and environmental stress) were inoculated intravaginally with 1×10^6 trichomonads in 20 μ l of sterile HBSS. Controls were infected with HBSS only.

Estradiol-Treatment

Two days prior to infection all mice were given a subcutaneous injection containing 125 μ g of Estradiol 17- β cypionate (Sigma, St. Louis MO) dissolved in sterile sesame oil. Estradiol injections were continued throughout the course of the experiment, and given to the mice every 4 days. All mice were then checked for stage of estrus by lavaging the vagina with 50 μ l sterile HBSS. Lavage fluids were diluted 1:2 in HBSS and applied to a slide using a cytospin centrifuge. After drying, the slides were fixed and stained using Diff-Quick solution (Dade Behring; Newark, DE) by immersing slides for 2 minutes in each solution. Slides were then microscopically examined for cellular morphology. Estrus was determined as previously described⁴⁷. Mice were then infected with *T. foetus* as described above.

Stressed Infection

Approximately one week prior to infection until termination of the experiment mice were housed in LL. After mice were confirmed to be in persistent estrus (defined by constant cornification of the vaginal epithelium for at least two consecutive estrus cycles) mice were infected with *T. foetus* as described above.

Tissue Removal

Infection in all groups of mice was allowed to persist for 4, 14, or 21 days, after which mice were sacrificed by a lethal injection of sodium pentobarbital (100 μ g/ml) and exsanguinated. Whole reproductive tracts were removed and gently homogenized through a mesh screen in 2 ml HBSS containing 3 mM EDTA. To detect infected animals and assess the severity of trichomonad burden within the entire reproductive tract, 200 μ l of homogenate was removed and added to 3 ml Diamonds medium and cultured 48 hours. Homogenate cell suspensions were filtered and total leukocytes were enumerated. Total macrophage, neutrophil, and lymphocyte numbers were determined with a differential leukocyte analysis. Homogenates were diluted 1:5 and applied to a slide with a cytospin centrifuge and stained with Diff-Quick. Total numbers of leukocyte subsets were calculated by multiplying the percentage of subsets obtained in the differential analysis by the total number of cells in the single-cell suspension. Homogenates were pelleted by centrifugation at 250 x g for 10 minutes and prepared for flow cytometry. Supernatants were retained and frozen at -80°C for future cytokine analysis.

Flow Cytometry

Vaginal cell suspensions were centrifuged at 250 x g and resuspended in RBC lysis buffer (150 mM NH_4CL , 1.0 mM KHCO_3 , and 0.1 mM $\text{Na}_2\text{-EDTA}$). Samples were then centrifuged and cells were resuspended in PBS containing 2% calf serum and anti-mouse FC receptor antibody (Trudeau Institute) to prevent nonspecific binding of antibodies. T lymphocytes were stained with CyChrome-conjugated anti-mouse CD4,

allophycocyanin-conjugated anti-mouse CD8, fluorescein-conjugated anti-mouse CD62 or CD44, and phycoerythrin-conjugated anti-mouse CD25 or CD43 (BD PharMingen). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). CellQuest software (BD Pharmingen) was used to analyze lymphocyte sub-populations. Lymphocytes were gated based on size and CD4 or CD8 expression. Total lymphocyte subsets were calculated by multiplying the total cell count from the recovered tissue to the percent of cells expressing surface phenotypes of interest.

Cytokine Assays

Supernatants from the reproductive tract homogenates were analyzed for inflammatory cytokine expression using BD Cytometric Bead Array mouse inflammation kit (BD Biosciences). Samples were thawed on ice and assayed according to manufacturer's instructions. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). Reproductive tract cytokine protein levels were measured by comparing the mean FL2 intensity for each cytokine to standard curves generated for each of the analytes using BD CBA software (BD Biosciences).

Histology

Vaginal tracts were removed from all groups of infected animals 4, 14, or 21 days post infection. To examine tissue morphology subsequent to infection, tissues were fixed in phosphate buffered formalin for at least 24 hours, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin by using standard histological techniques.

Whole vaginal tracts were also cryopreserved in OCT compound, sectioned at 5 μm , and stained for apoptosis or to assess parasite burden.

TUNEL Stain

Apoptosis was detected by fluorescently labeling DNA single strand breaks in high molecular weight DNA using TdT-mediated dUTP nick end labeling (TUNEL) with an *in situ* cell death detection kit (Roche) according to manufacturer specifications. Positive control slides were treated with DNA polymerase I (Sigma, St. Louis MO) for 10 minutes at room temperature. Negative control slides were fluorescently labeled without the terminal transferase enzyme.

Tritrichomonas foetus Stain

T. foetus parasites were fluorescently labeled using antibody 1417-4, a rabbit polyclonal antibody generated against whole heat killed TF99 (QED Bioscience Inc.). Briefly, tissues were fixed in 75%/25% ethanol/acetone for 5 minutes and washed in Dulbeccos phosphate buffered saline with 0.2% bovine serum albumin (0.2%-DPBS) 3 times for 5 minutes each. Polyclonal antibody 1417-4 or an isotype matched control rabbit IgG was titrated and diluted 1:800 and 1:1000 respectively in PBS with normal 2.5% donkey serum (DS-PBS), incubated in a humidified chamber for 1 hour at room temperature, and washed thoroughly with 0.2%-DPBS. Texas Red conjugated donkey anti-rabbit IgG diluted 1:250 in DS-PBS was added and slides were incubated for 1 hour in a dark humidified chamber, washed well with pure DPBS and coverslipped with

ProLong Gold antifade reagent (Invitrogen). Slides were allowed to dry over night and examined the next day.

Statistical Analysis

Prism (GraphPad) was used for all statistical tests of significance (p values of ≤ 0.05). For data that had a normal (Gaussian) distribution, unpaired two-tailed t tests were used to compare two groups of data. A nonparametric test (Mann-Whitney test) was used to analyze pairs of data that were assumed to have abnormal distribution. In most cases non-parametric analysis of normally distributed populations gave similar P values to two-tailed t test.

Results

Parasite Burden and Incidence is Diminished Due to Estradiol-treatment

Compared to normal and stressed mice, estradiol-treated mice had both lower parasite burden within the reproductive tract and a lower frequency of infection. Estradiol treatment of mice resulted in similar percentages of infected animals compared to normal and stressed mice on day 4 (Figure 2.1A) although this diminished significantly by 21 days of infection compared to normal and stressed mice ($P = .0008$ and $.0053$ respectively). Additionally, numbers of parasite within the reproductive tract of estradiol-treated mice after 21 days of infection were significantly lower than the numbers that were found in normal and stressed mice (Figure 2.1B, $P = .0088$ and $.0038$ respectively).

These data indicate that estradiol-treatment, but not stress, reduced the ability of *T. foetus* to establish a fastidious infection within the murine reproductive tract.

Estradiol-treatment and Stress Enhance Tissue Damage, and Cell Death

We next wanted to examine how infection in estradiol-treated and stressed mice affected the associated sequelae during the course of infection. Each group of mice exhibited unique histological lesions throughout the course of infection. Normal and estradiol-treated mice both had mild cellular inflammation consisting of PMNs within the uterine endometrium after 4 days (Figures 2.2A and 2.2B) and no detectable inflammation or damage within the vagina. No lesions were observed within the reproductive tract of stressed mice after 4 days of infection.

Both estradiol-treated and stressed mice infected with *T. foetus* for 14 days had notable damage and cellular inflammation within the vagina, including epithelial damage and sloughing of damaged epithelium into the vaginal lumen (Figures 2.3A and 2.3B). There was intense PMN accumulation throughout the lamina propria of the vagina, with migration towards the epithelium and vaginal lumen in estradiol-treated and infected mice. In stressed mice, large vacuoles containing inflammatory cells and debris and large sections of epithelium sloughing into the lumen were noted (Figure 2.3B). Unlike estradiol-treated mice, stressed mice infected for 14 days also had notable endometrial inflammation consisting primarily of PMNs (Figure 2.3C). There was no observable tissue damage within the vagina or uteri of normal mice infected with *T. foetus* for 14 days.

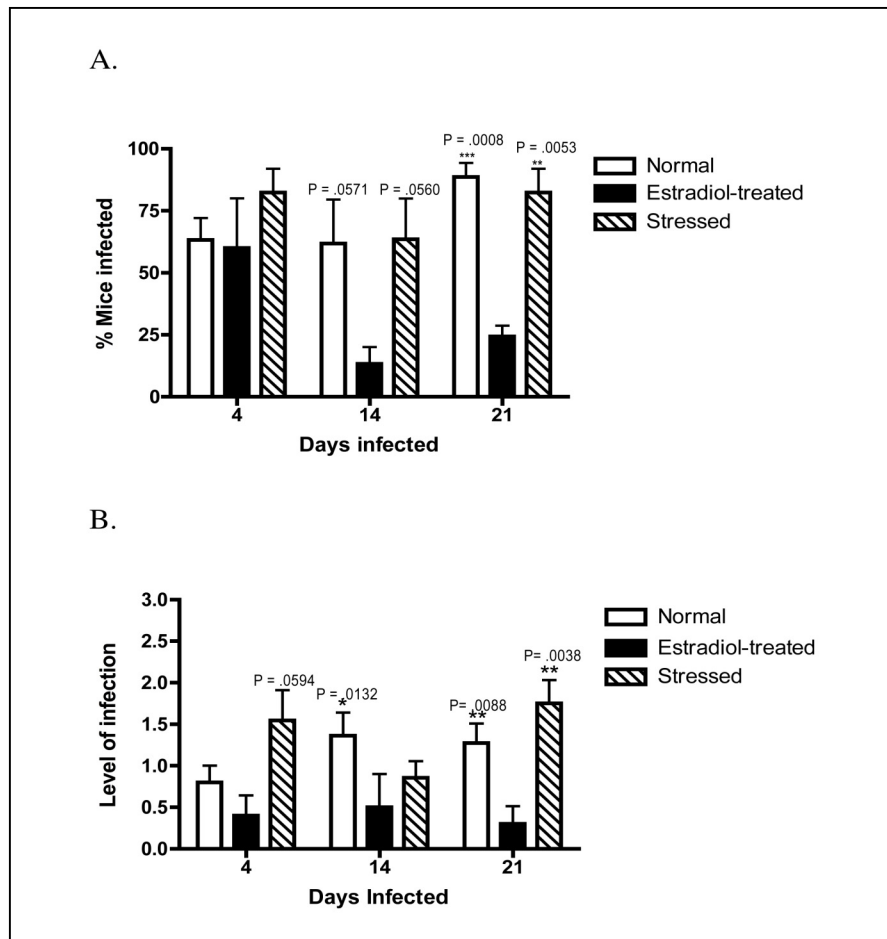


Figure 2.1: Estradiol-treatment results in diminished incidence of infection and lower levels of *T. foetus* within the reproductive tract. Data was obtained from mice with positive *T. foetus* cultures after 48 hours. A: Percentage of mice from each time-point with positive trichomonad cultures. B: Level of *T. foetus* infection within the reproductive tracts of each group of mice. Parasites were enumerated from reproductive tract homogenates as previously described in Materials and Methods. Significance

By 21 days of infection, notable inflammation was observed in the vagina of normal infected mice whereas severe inflammation and tissue damage was observed within the vagina of estradiol-treated mice. Normal mice had moderate PMN migration into the vaginal lamina propria (Figure 2.4A) after 21 days of infection. Estradiol-treated

mice infected for 21 days had desquamation of keratinized epithelium in addition to gross epithelial damage and PMN infiltration into the vaginal epithelium and lumen, which was not observed in uninfected controls (Figure 2.4B). In addition, at 21 days the estradiol-treated mice had inflammation within the uterus, consisting primarily of abscessed uterine glands containing neutrophils and adjacent inflammatory foci within the endometrium (Figure 2.4C). Stressed mice infected for 21 days appeared to have large cellular exudates within the lumen of the vagina with what appeared to be *T. foetus* organisms (Figure 2.4D).

We next used fluorescent microscopy to locate *T. foetus* parasites during the course of infection in each group of animals to determine if tissue damage could be attributed to parasite colonization and invasion of the lamina propria. Generally, parasites were not detected within the lamina propria of the vagina in any of the groups, although parasites were detected within the columnar vaginal epithelium of normal mice (Figure 2.5A). In addition, *T. foetus* was detected mainly in the uterine lumen and within the surrounding epithelium of estradiol-treated and stressed mice infected after 14 days (Figure 2.5B). Estradiol-treated mice had the greatest amount of *T. foetus* antigen within the uterine glands after 14 and 21 days of infection (Figure 2.5C). The location of *T. foetus* staining within the vagina and uterus seemed to correlate to the observed tissue damage of the lower reproductive tract in estradiol-treated and stressed mice. Therefore, cellular mediators of inflammation may be responsible for observed damage within the vaginal epithelium in estradiol-treated and stressed mice infected with *T. foetus*.

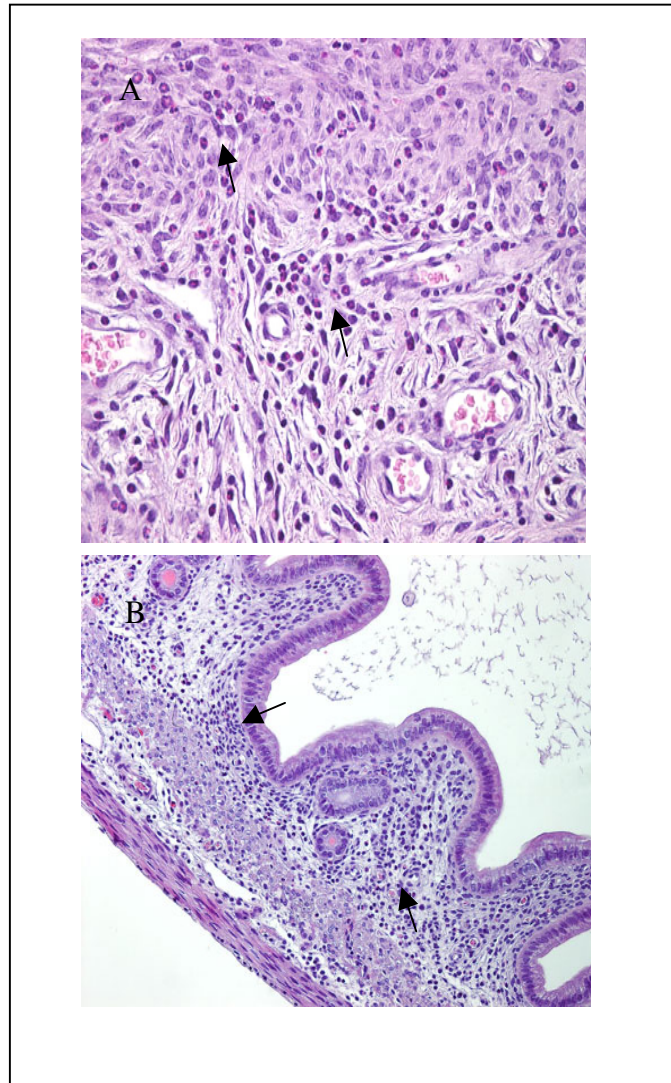


Figure 2.2. Mild inflammation in the uteri of normal and estradiol-treated mice after 4 days of infection. Normal A: and estradiol-treated. B: mice infected with *T. foetus* for 4 days both had inflammatory cellular infiltration within the uterine endometrium (arrows) consisting mainly of eosinophils and neutrophils. Figure A is 400X magnification, figure B is 200X magnification.

Since soluble cytoactive factors from *T. foetus* induce apoptosis on bovine vaginal and uterine epithelial cells^{135,137}, we wanted to examine whether estradiol-treatment and stress during early infection of mice affected the ability of parasites to induce apoptosis. Estradiol-treatment and stress during infection resulted in early apoptosis compared to a normal infection. Normal mice infected for 4 days had no observable apoptosis within the vagina or uterus (Figures 2.6A and 2.6B), but apoptosis progressively increased in the vagina after 14 and 21 days of infection. There was no detectable apoptosis within the uterus of normal mice throughout the course of infection. Conversely, vaginal and uterine epithelium from estradiol-treated and stressed mice was undergoing apoptosis as early as 4 days after infection (Figures 2.6A and 2.6B) and peaked in the uterus by 14 days of infection, especially in the stressed mice (Figure 2.6B). These results indicate estradiol-treatment and especially stress during *T. foetus* infection accelerate apoptosis of the vaginal and uterine epithelium and that it occurs before tissue damage is observed. Thus, infection with *T. foetus* must result in the induction of apoptosis during early infection and colonization in order to establish infection.

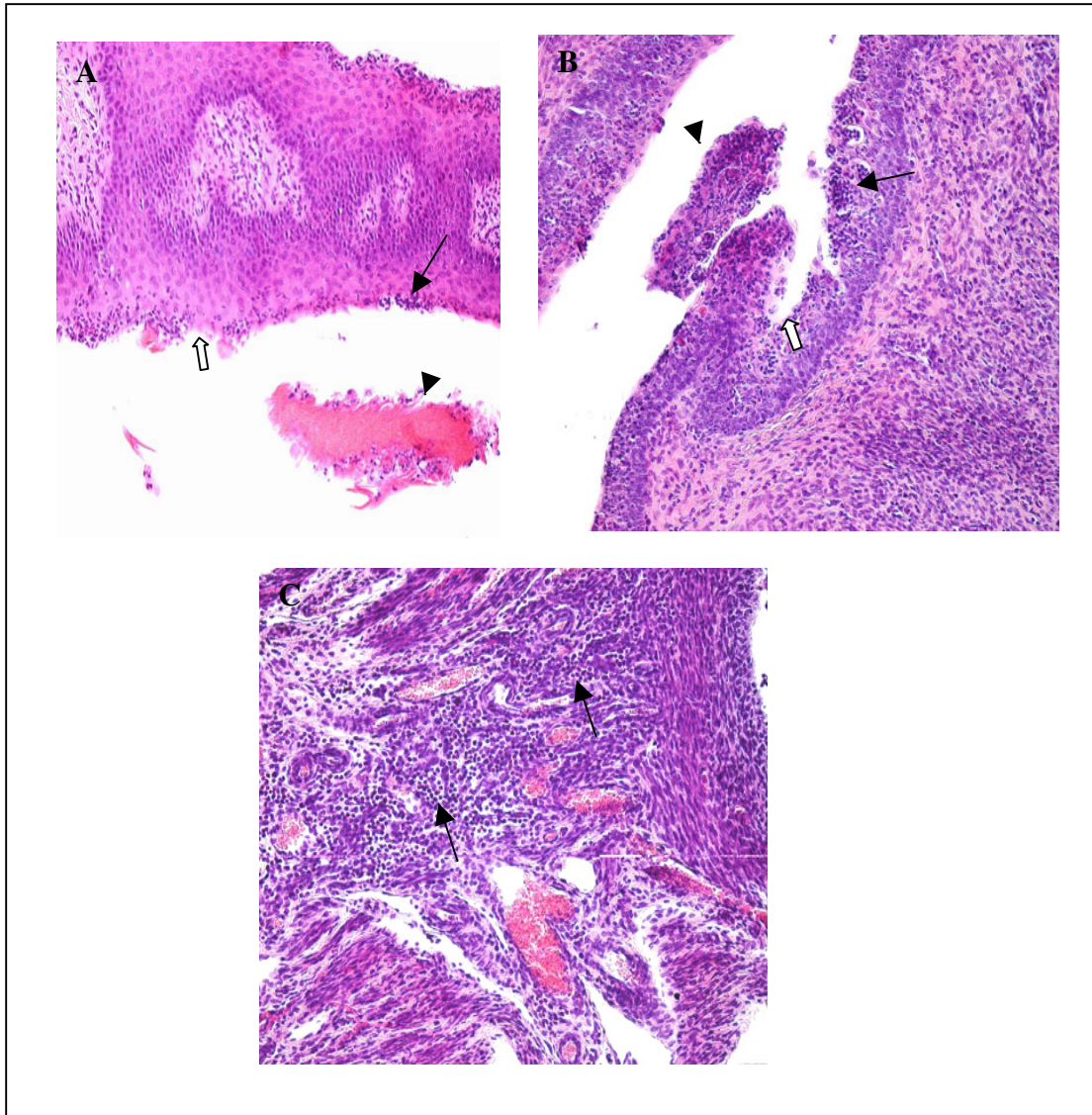


Figure 2.3: Inflammation and damage in the reproductive tracts of estradiol-treated and stressed mice after 14 days of *T. foetus* infection. A: vagina of estradiol-treated mice. B - C represent tissues from stressed mice. B: vagina. C: uterus. Arrows indicate neutrophil accumulation. White arrows indicate areas of tissue damage. Black arrow-head depicts sloughing epithelium. All images 200X magnification.

Neutrophil Responses to *T. foetus* Infection
Enhanced Due to Estradiol-treatment or Stress

Since damage and inflammation was observed in tissues of both estradiol-treated and stressed mice infected with *T. foetus*, we wanted to determine what cellular factors may be responsible for the observed pathology. *T. foetus* infection in both estradiol-treated and stressed mice, independent of the magnitude of parasite burden, resulted in increased neutrophil accumulation within the reproductive tract during later points of infection.

T. foetus infection in normal mice did not result in significant neutrophil increases within the reproductive tract throughout the course of infection (Figure 2.7). Estradiol treatment of mice resulted in large increases of neutrophils in both infected and uninfected mice, although mice infected for 14 and 21 days had significantly higher numbers of neutrophils accumulating within the reproductive tract compared to uninfected controls ($P = .0349$ and 0.0216 respectively, Figure 2.7). Stress also resulted in significant increases of neutrophils within the reproductive tract in mice infected with *T. foetus* for 14 and 21 days ($P = .0378$ and $.0246$ respectively, Figure 2.7). Infection with *T. foetus* did not result in a significant influx of macrophages in any group of mice. However, estradiol treatment of mice resulted in increased macrophage numbers within the reproductive tract, compared to macrophage numbers in normal and stressed mice (data not shown). These data demonstrate that either estradiol-treatment or stress resulted in increased neutrophil accumulation within the reproductive tract during *T. foetus* infection.

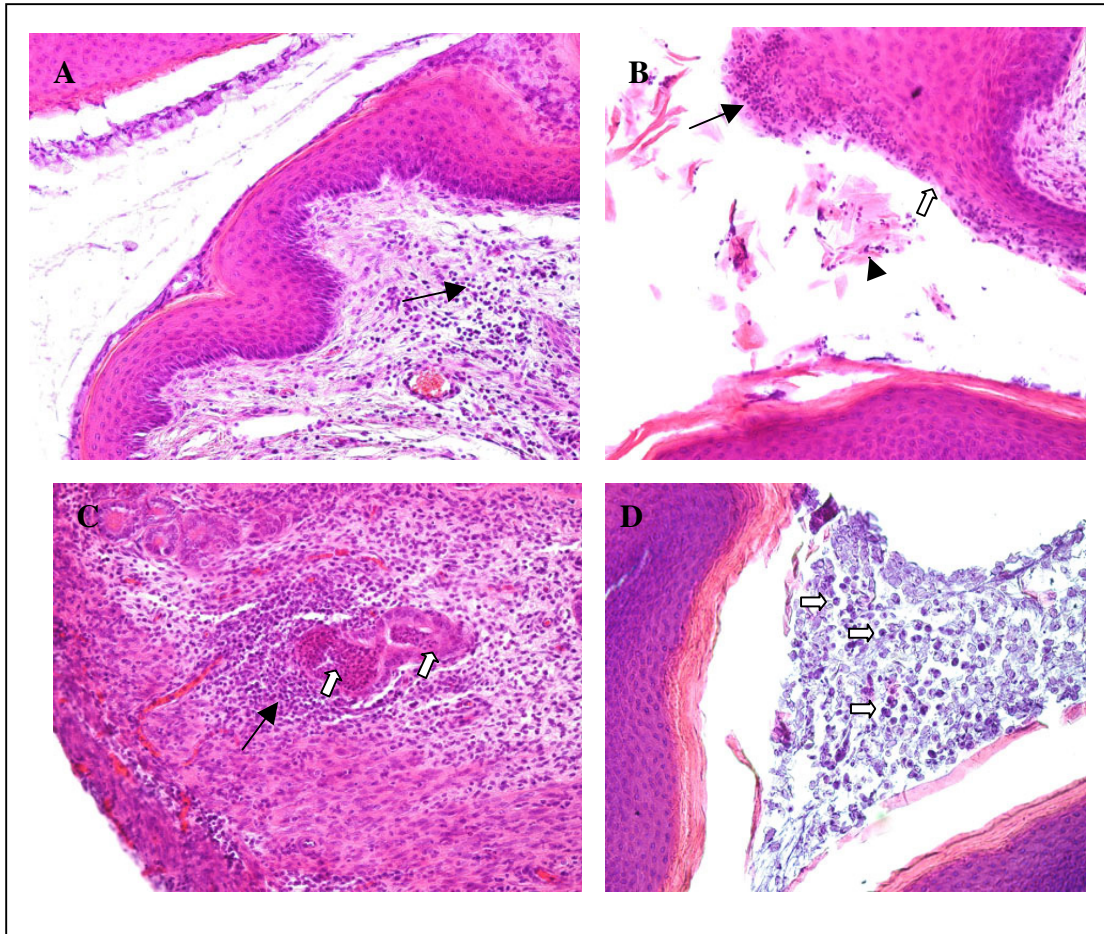


Figure 2.4: Inflammation in the vagina of all mice detected after 21 days of infection, although only damage is detected in estradiol-treated mice A: Vagina of normal mice. B: Vagina of estradiol treated mice. White arrows indicate damaged epithelium. Black arrowhead indicates sloughing epithelium within the vaginal lumen. Arrow indicates neutrophil inflammation is evident. C: Uterus of estradiol-treated mice. White arrow shows inflammation within the glands of the uterus. Black arrow indicates inflammation within the endometrium. D: Vagina of stressed mice with what appear to be large amounts of *T. foetus* organisms shown by white arrows.

Lymphocyte Effector Responses are Diminished During Infection in Estradiol-treated and Stressed mice

Lymphocyte numbers within the reproductive tract remained the same during both normal and stressed conditions, whereas estradiol-treatment of mice during *T. foetus*

infection resulted in a significant increase of lymphocyte accumulation after 21 days of infection ($P = .0185$, Figure 2.7). Overall, a low intensity T cell effector response occurred during early *T. foetus* infection and colonization (< 21 days) in normal mice. This was not observed in estradiol-treated and stressed mice infected with *T. foetus*. T cells isolated from reproductive tracts were gated according to CD4 and CD8 expression and grouped according to the expression of activation markers such as CD25, CD62 ligand (CD62L), CD44 and CD43 expression. CD43 expression was similar to CD44 expression as previously reported⁷⁴ thus we only show CD44 expression.

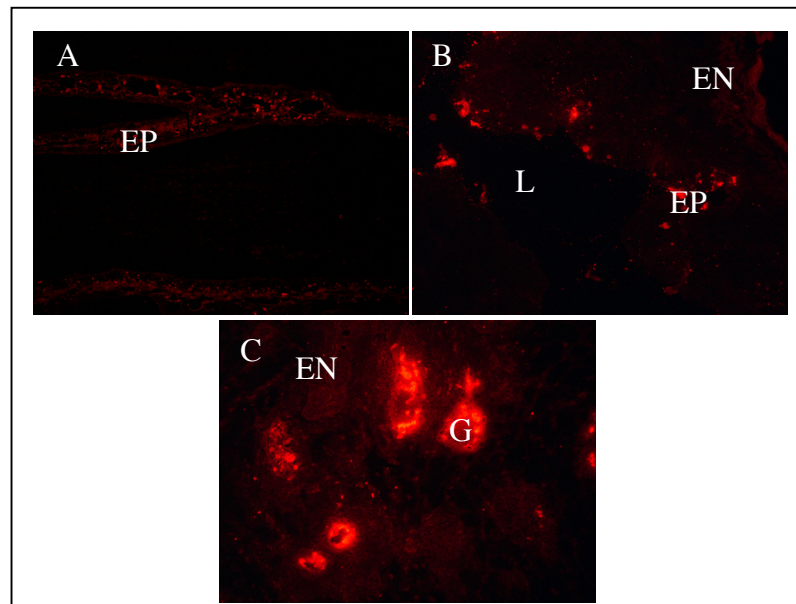


Figure 2.5. Immunohistochemical detection of *T. foetus* parasites within reproductive tracts. A: Parasites were detected in the vagina. B: Parasites detected in the epithelium of the uterus. A and B 200X magnification. C: Parasites were also detected within the uterine glands, especially in estradiol-treated mice. C 400X magnification. EP: epithelium. L: lumen, EN: endometrium. G: uterine glands.

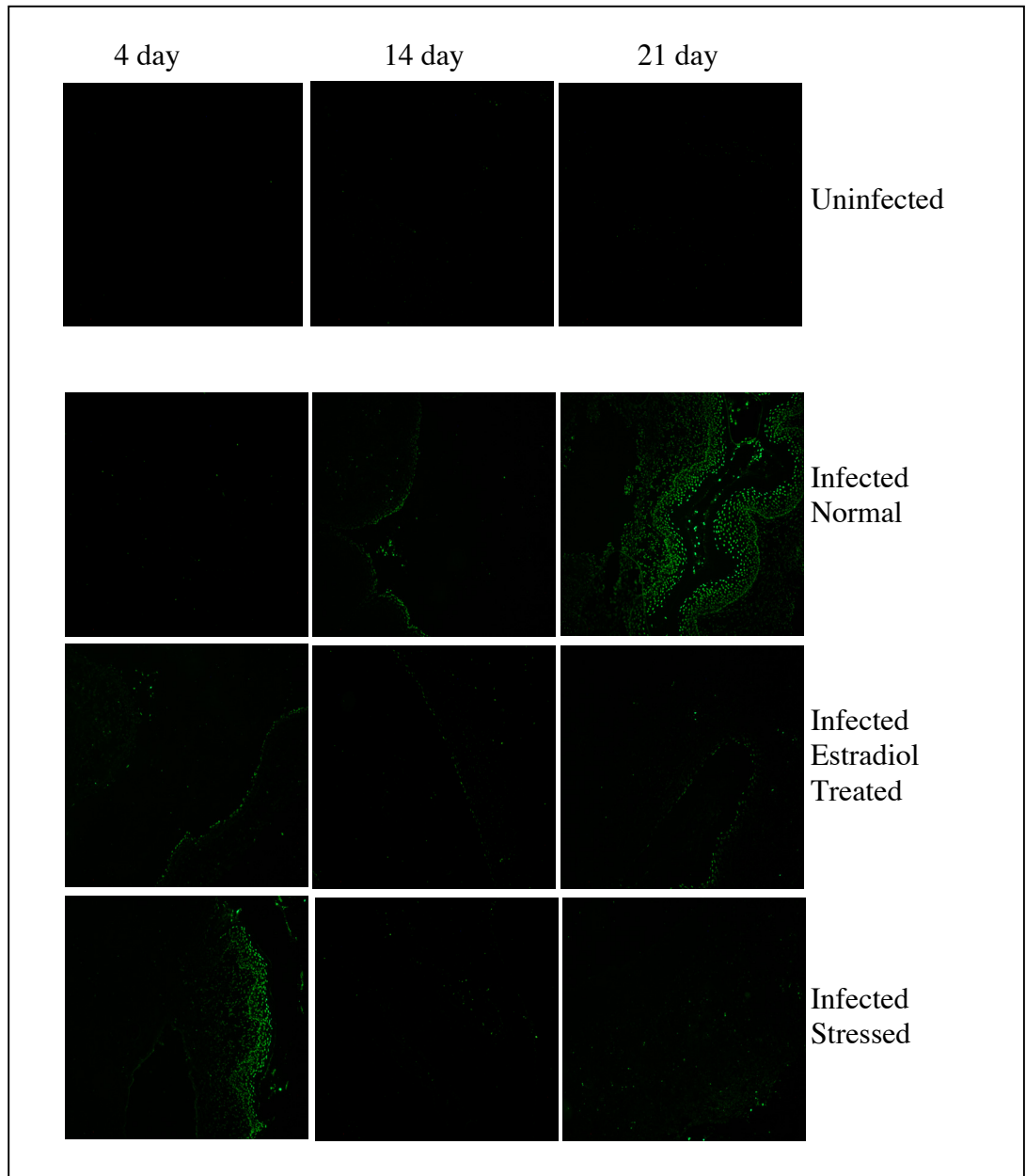


Figure 2.6A: TUNEL stain and detection of apoptosis in the vaginas of mice infected with *T. foetus*. TUNEL staining was compared between infections in normal mice compared to estradiol-treated and stressed mice. Apoptosis was detected in the vagina or lower reproductive tract. All images 200X magnification. Negative controls all had no positive staining and weren't shown.

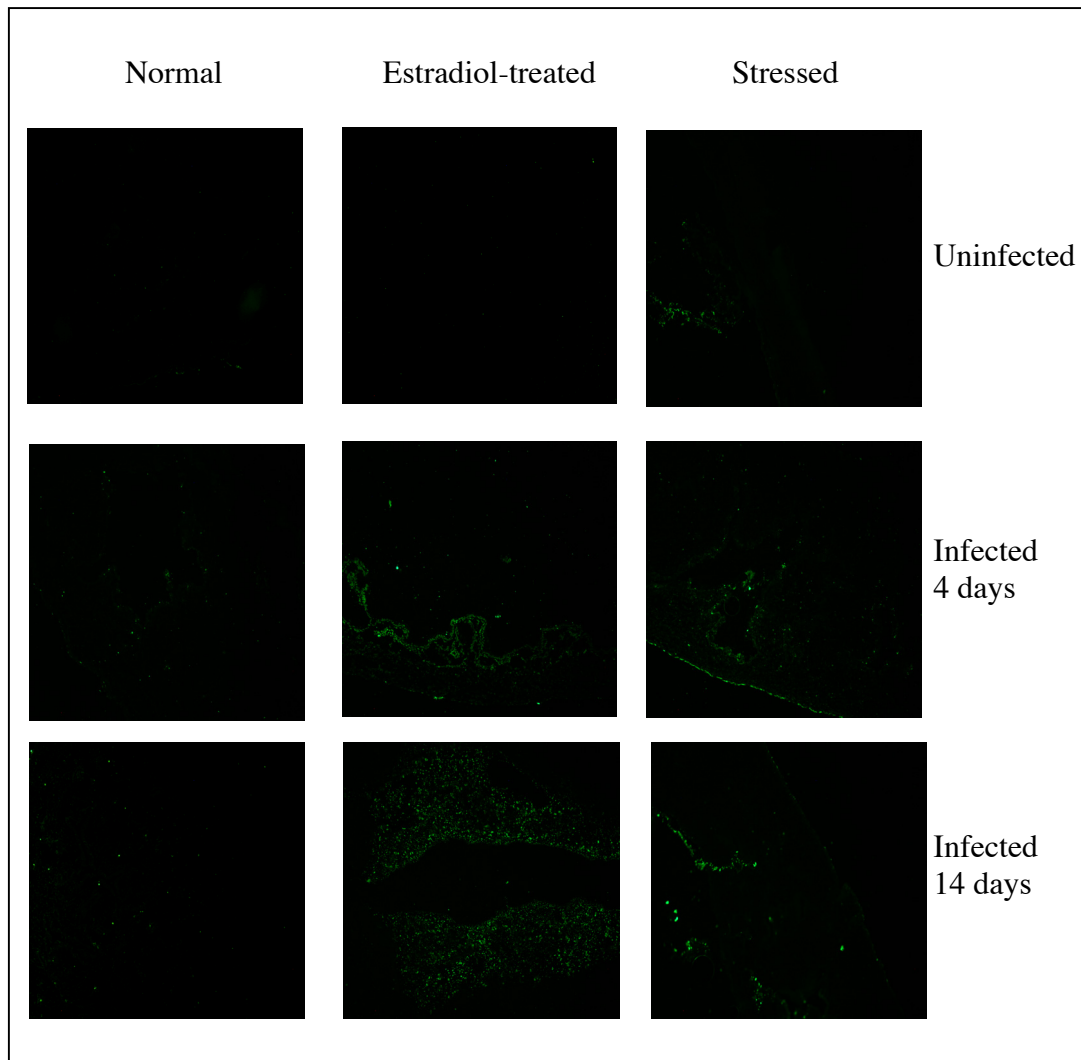


Figure 2.6B: TUNEL stain and detection of apoptosis in the uteri of mice infected with *T. foetus*. TUNEL stain was compared during infection in normal mice compared to estradiol-treated and stressed mice. Apoptosis was detected in the uterus or upper reproductive tract. All images 200X magnification. Negative controls all had no positive staining and weren't shown.

There were no significant changes in the CD4 or CD8 phenotypes of infected mice at day 4 (data not shown). Normal mice had a significant increase in CD4⁺ CD62L

low T cells in the reproductive tract after 14 days of infection ($P = .0220$, Figure 2.8A). By day 21, there was a significant increase of CD4⁺ CD62L low CD44⁺ cells from the reproductive tracts of normal infected mice ($p = .0115$ and $.0011$ respectively, Figure 2.8B). No CD4⁺ CD25⁺ T cells were detected in the reproductive tracts of normal mice. Only after 21 days of *T. foetus* infection in estradiol-treated and stressed mice was there an increase in CD4⁺ T cells with effector phenotypes. Estradiol-treated mice had a significant increase of CD4⁺ CD25⁺ CD62L low CD44⁺ cells after 21 days of infection ($P = .0307$, $.0251$, and $.0097$ respectively, Figure 2.8B). Stressed mice had a significant increase of CD4⁺ CD25⁺ cells on day 14 ($p = .0064$, Figure 2.8A) and on day 21 had a significant increase of CD4⁺ CD25⁺ CD62L low cells into the reproductive tract ($P = .0382$ and $.0381$ respectively, Figure 2.8B). Putative regulatory T cells (CD4⁺ CD25⁺) were present in the reproductive tracts of stressed mice after 14 days of infection, and in both stressed and estradiol-treated mice after 21 days of infection.

Accumulation of CD8⁺ effector T cells within the reproductive tracts of all groups of infected mice was less than that observed for the CD4⁺ T cell subsets. Concurrent with CD4⁺ T cell phenotypes on day 4 of infection, there were no significant differences in CD8⁺ T cell subsets in any of the groups of mice (data not shown). After 14 days of infection, there were no significant differences in CD8⁺ phenotypes in both normal and estradiol-treated infected mice whereas in stressed mice there was a significant increase in CD8⁺ CD62L low cell accumulation into the reproductive tract ($p = .0216$, Figure 2.8C), suggesting CD8⁺ T cells may be homing to the reproductive tract in stressed mice. After 21 days infection with *T. foetus* a significant increase in CD8⁺ CD44⁺ cells in the

reproductive tract of normal infected mice ($p = .0015$) was detected, whereas there were no significant differences CD8⁺ T cell subsets of estradiol-treated or stressed and infected mice (Figure 2.8B). Thus, *T. foetus* infection in normal mice results in a mild T cell effector response which is not observed during infection in both estradiol-treated or stressed mice.

Inflammatory Cytokines Within the Reproductive Tract Increased During Infection in Estradiol-treated and Stressed mice

Since both estradiol treatment and housing animals in LL resulted in increased tissue damage and neutrophil and regulatory T cell accumulation into the reproductive tract, we then asked which cytokine signals may coincide with the observed cellular responses. *T. foetus* infection in estradiol-treated and stressed mice resulted in elevated levels of inflammatory cytokines, although the increases in inflammatory cytokine levels were most dramatic during infection in estradiol-treated mice.

Normal mice had no detectable increases in IFN- γ throughout the course of infection with *T. foetus*. Estradiol treatment and housing mice in LL resulted in decreased levels of IFN- γ in uninfected controls compared to uninfected normal mice. Interestingly, infection with *T. foetus* resulted in significant increases of IFN- γ in both estradiol-treated and stressed mice, compared to uninfected controls, by day 21 ($p = .0496$ and $.0038$ respectively, Figure 2.9A). TNF- α levels did not change during infection in normal and stressed mice. After 14 days of *T. foetus* infection in estradiol-treated mice, there was a significant increase ($p = .0040$) in TNF- α secretion. This increase was not observed after 21 days of infection in estradiol-treated mice (Figure 2.9B). Interestingly, estradiol

treatment of mice led to TNF- α levels that were more than 36 times higher in uninfected mice compared to uninfected controls in the normal and stressed groups. These levels rose to 50 times higher in infected estradiol-treated mice compared to normal and stressed infected mice after 14 days.

MCP-1 chemokine expression in normal and stressed mice were significantly higher in mice infected for 21 days compared to uninfected controls for each group ($p = .0006$ and $.0332$ respectively). MCP-1 chemokine secretion during infection in estradiol-treated mice was only significantly elevated after 14 days of infection ($p = .0040$). Similar to TNF- α patterns of secretion in the reproductive tract, MCP-1 chemokine expression was nearly 17 times higher due to estradiol treatment, compared to MCP-1 levels in uninfected normal and stressed mice (Figure 2.9C). Infection for 14 and 21 days in estradiol-treated mice also resulted in significant increases in IL-6 production ($p = .0290$ and $.0360$ respectively, Figure 2.9D). Estradiol treatment of mice resulted in levels of IL-6 that were nearly 10 times higher in uninfected controls compared to stressed and normal mice. After 14 days of infection in estradiol-treated mice, IL-6 levels were 17 times higher than normal infected mice, and over 60 times higher than stressed infected mice

There were no detectable differences in IL-12p70 and IL-10 secretion between infected and uninfected controls of the three treatment groups (data not shown). These results suggest somewhat similar cytokine patterns for heavily infected mice, as the normal and stressed mice each had similar changes during *T. foetus* infection, with the exception of IFN- γ levels. They also demonstrate increased estradiol levels seem to

increase or change baseline inflammatory cytokine secretion. Increases in cytokine secretion in estradiol-treated mice during infection with *T. foetus* they are greatly enhanced. This is especially evident after 14 days of infection, when *T. foetus* levels are declining within estradiol-treated mice. These results indicate that the observed tissue damage in estradiol-treated and stressed mice may be due to elevated levels of inflammatory cytokines, especially in estradiol-treated mice infected with *T. foetus*.

Estradiol-treated mice had lower parasite burden within the reproductive tract, compared to normal and stressed mice. However, damage was greatest in the estradiol-treated animals and stressed animals. Inflammatory cytokines were greatly elevated in uninfected estradiol-treated mice, although significant increases were observed in IFN γ , MCP-1, TNF α , and IL-6 after infection with *T. foetus* in estradiol-treated mice. These rises in inflammatory cytokines most likely were the cause of the tissue damage observed. Stressed mice only had significant increases in inflammatory cytokines IFN γ and MCP-1. Despite the lowered cytokine levels in stressed animals, similar tissue damage was observed, most likely due to the increased parasite burden during conditions of stress.

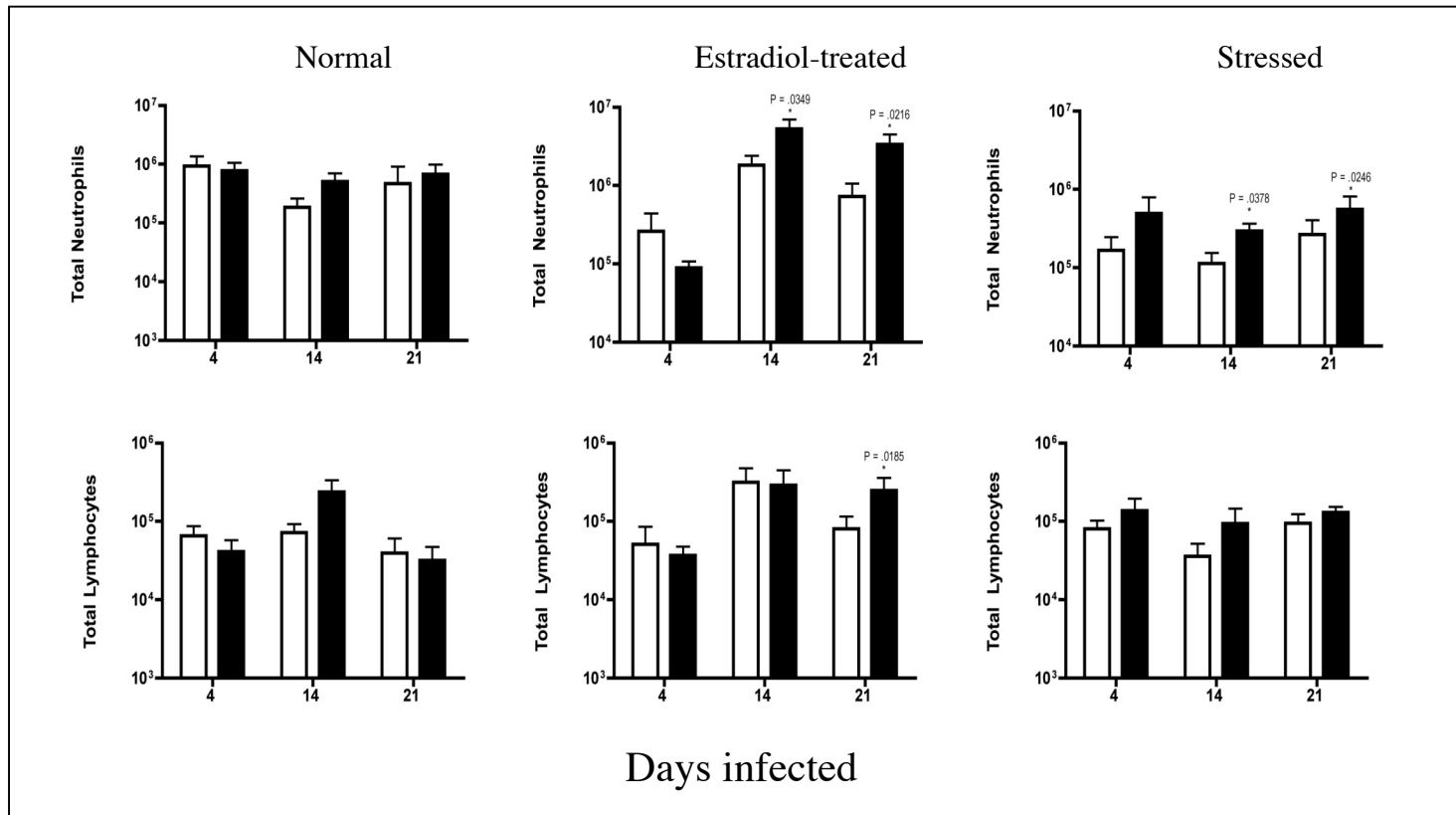


Figure 2.7: Neutrophil and lymphocyte counts in homogenized reproductive tracts of normal, estradiol-treated, and stressed mice after 4, 14, and 21 days of *T. foetus* infection. Neutrophil numbers increased in estradiol-treated and stressed mice infected with *T. foetus* for 14 and 21 days but were unchanged during normal infection. Lymphocyte numbers increased in estradiol-treated mice infected with *T. foetus* for 21 days, whereas there were no differences in normal and stressed mice. Clear bars represent uninfected mice. Filled bars represent infected mice. Data collected from differential cell counts performed on mice with positive cultures of *T. foetus* for each time-point. Comparisons were made between uninfected and infected mice for each group and time-point.

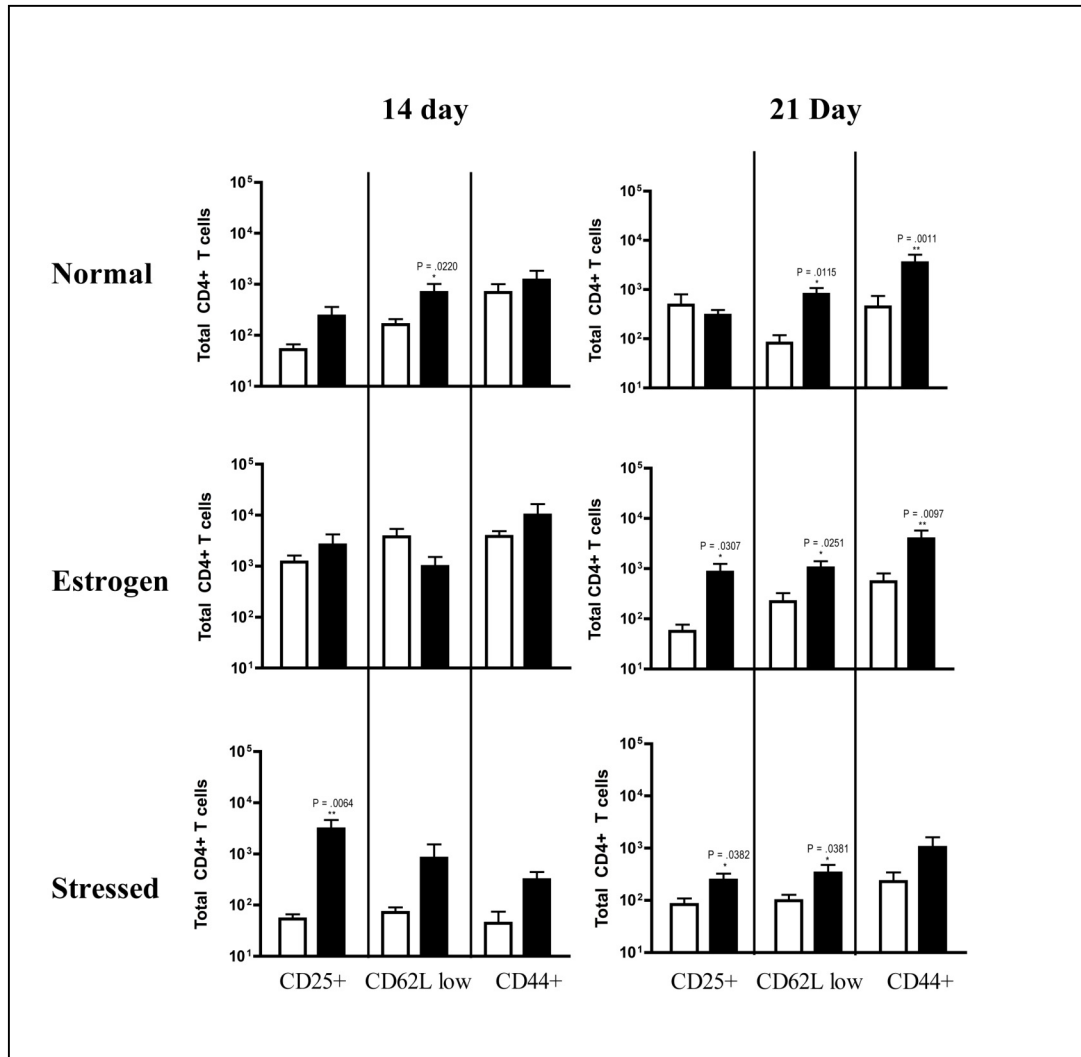


Figure 2.8 A. CD8⁺ T cell counts during infection in normal, estradiol treated, and stressed mice. FACS analysis of the CD4⁺ effector T cell response within the reproductive tract. CD4⁺ T cells after 14 and 21 days of infection were analyzed by staining with CD25, CD62L, and CD44 expression as described in Materials and Methods. Clear bars represent uninfected mice. Filled bars represent infected mice. Data collected from differential cell counts performed on mice with positive cultures of *T. foetus* for each time-point. Comparisons were made between uninfected and infected mice in each group.

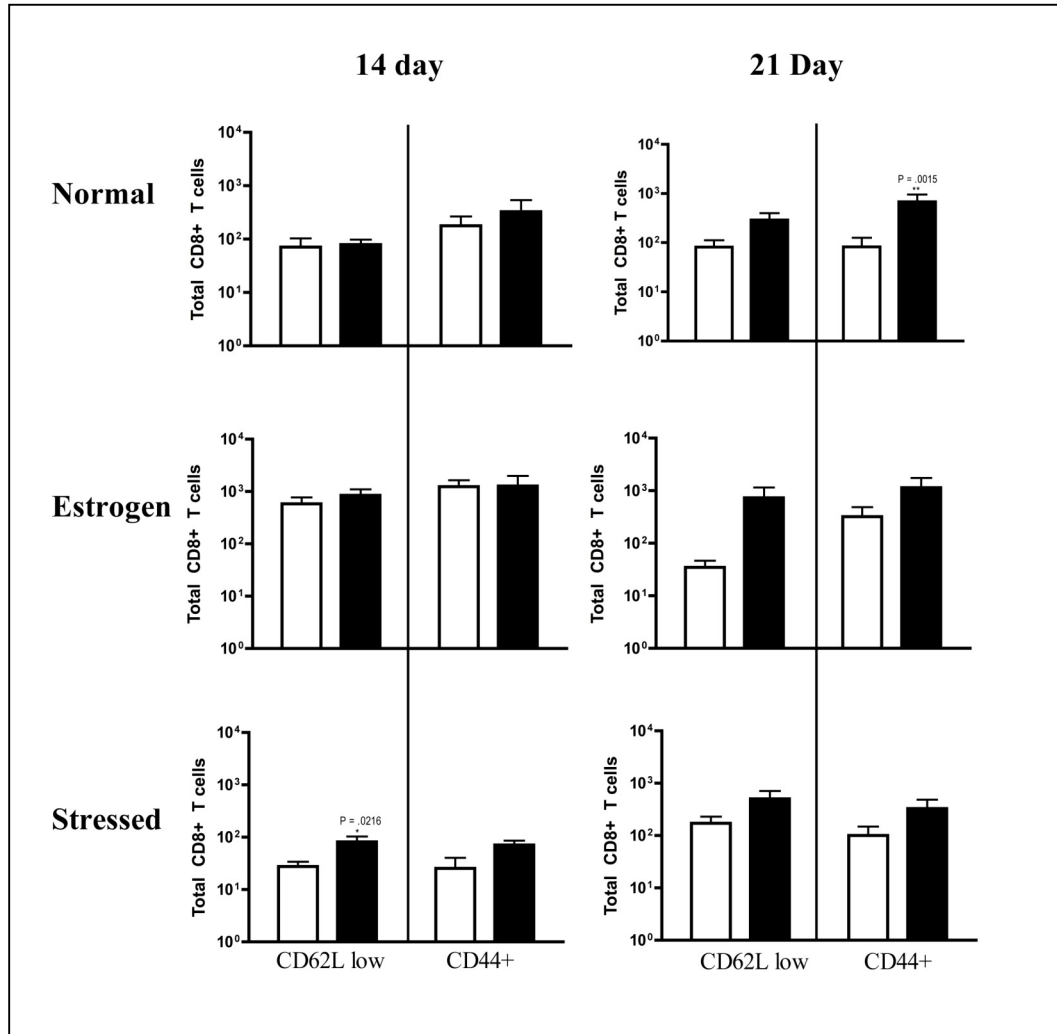


Figure 2.8 B: Infection in normal, estradiol treated, and stressed mice. FACS analysis of the CD8⁺ T cell response after 14 and 21 days of infection was analyzed by staining with CD25, CD62L, and CD44 expression as described in Materials and Methods. Clear bars represent uninfected mice. Filled bars represent infected mice. Data collected from differential cell counts performed on mice detected with positive cultures of *T. foetus* for each time-point. Comparisons were made between uninfected and infected mice in each group.

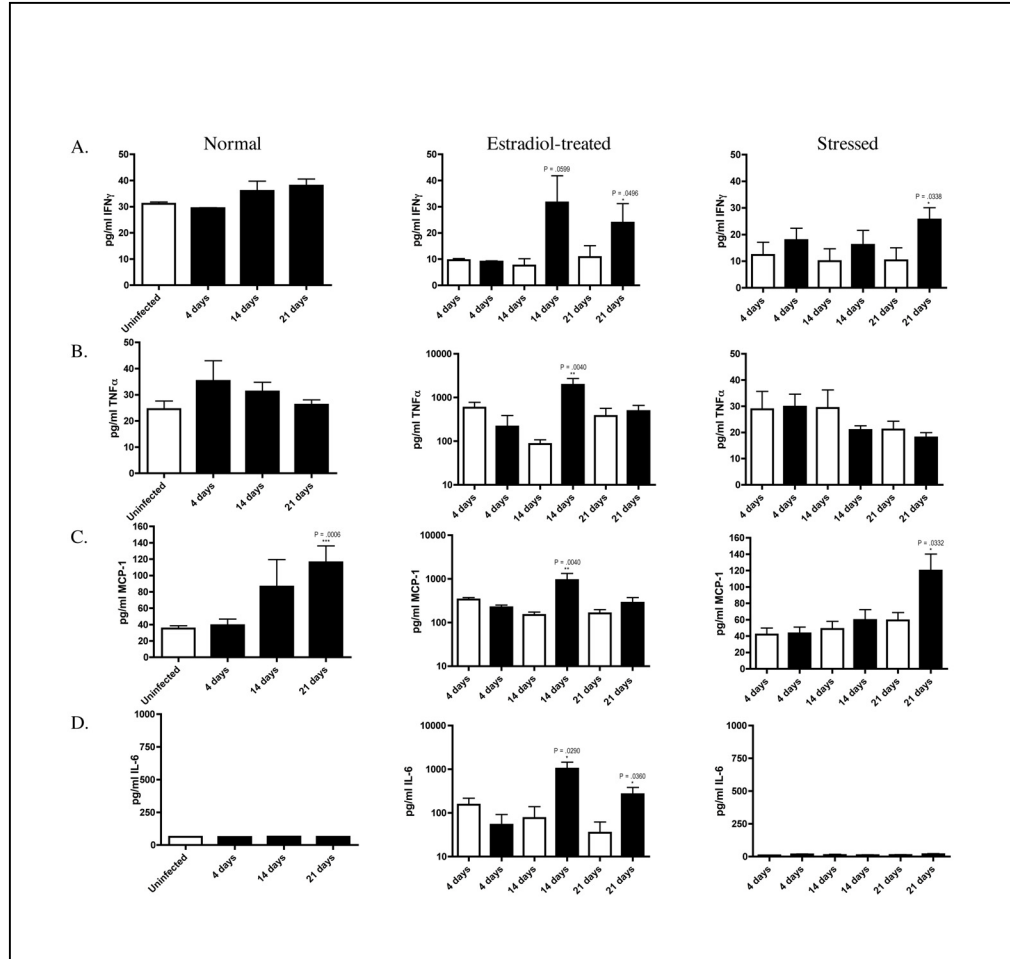


Figure 2.9: Inflammatory cytokine levels within the reproductive tract are increased after *T. foetus* infection in mice with elevated estradiol levels and in stressed mice. A: Levels of IFN γ , B: TNF α , C: MCP-1, and D: IL-6 were quantified in reproductive tract homogenates as described in Materials and Methods. Clear bars represent uninfected mice. Filled bars represent infected mice. Data collected from differential cell counts performed on mice detected with positive cultures of *T. foetus* for each time-point. Comparisons were made between uninfected and infected mice in each group/time-point.

Discussion

These studies were conducted to examine if estradiol-treatment or stress affect *T. foetus* colonization within the reproductive tract and the subsequent disease. *T. foetus* burden and incidence was relatively high in normal mice, however cellular inflammation and histopathology were generally lacking. Consistent with these observations was the lack of apoptosis and epithelial cell damage. This form of cell death results in minimal tissue damage and inflammation, resulting in prolonged survival of *T. foetus*. It has been suggested trichomonads induce apoptosis as a means of immune evasion, establishing infection without initiating severe inflammation and necrosis¹³⁷. Similar to the bovine host, since pathology is not usually observed early in infection, perhaps lack of inflammation and delayed apoptosis indicate a strategy *T. foetus* utilizes to persist within the reproductive tract without clearance.

Normal animals had significant effector CD4⁺ and CD8⁺ responses due to infection by 21 days. Although effector CD4 T cells were observed accumulating in the reproductive tract of mice after *T. foetus* infection, neutrophil and associated inflammatory cytokine production was not observed, with the exception of a significant increase in MCP-1 secretion after 21 days of infection. Large neutrophil and macrophage accumulation within tissues of aborted fetuses¹²² and within the reproductive tracts of cattle with severe endometritis and pyometria¹¹³ have been observed. Thus, heavy macrophage and neutrophil accumulation seem to be associated with severe pathology

such as abortion and endometritis. Additionally, animals with little pathology or impending abortion also have delayed inflammation that is not generally observed until after 60 days of infection in the bovine host¹¹³. Our data regarding early infection support a study by Corbeil et al.³⁹ demonstrating BALB/c mice infected with the same strain of *T. foetus* remained chronically infected for at least 26 weeks³⁹ without clearance of the parasite which is similar to the carrier state described in cattle¹³⁸.

Administration of estradiol resulted in a lower percentage of mice infected after 14 and 21 days. In addition, relatively lower numbers of live trichomonads were recovered from the reproductive tract after 21 days of infection, indicating estradiol-treatment diminishes the ability of *T. foetus* to colonize its host. Lower infection frequency and parasite burden in mice administered estrogen could have been a result of direct hormonal influence on parasite establishment of infection. It has previously been shown that estrogens act as chemorepellents and directly inhibit growth and adherence of the human pathogen *T. vaginalis* to mammalian cells¹⁴⁴. In addition, *T. foetus* adhesion to bovine vaginal epithelial cells was facilitated in the presence of progesterone¹³⁶. Since estradiol and progesterone have opposing effects¹¹, progesterone levels would be low under the influence of high serum estradiol levels. Low *T. foetus* burden in estradiol-treated mice could also be a result of increased mucous secretion within the reproductive tract due to elevated estradiol levels¹¹. Increased mucous secretion and epithelial cornification could result in diminished parasite adhesion to the epithelium, which is critical during establishment of infection^{23,90}.

Infection of estradiol-treated mice resulted in epithelial damage and inflammation within the vagina in addition to large inflammatory foci within the endometrium after 21 days of infection. This damage was not observed in uninfected mice treated with estradiol for equal periods of time, suggesting it is the cumulative effects of both *T. foetus* antigen and estradiol-treatment. Large amounts of *T. foetus* antigen were present within the endometrial glands after 21 days of infection in estradiol-treated mice. Reasons for increased tissue damage observed in estradiol-treated mice, even though there is a lower incidence of trichomonads within the reproductive tract, could be related to the effects estradiol has on epithelial integrity. Transepithelial electrical resistance, a measure of epithelial integrity, is significantly decreased in the presence of estradiol (reviewed by reference ¹⁶⁶). In addition, increased tissue damage could arise due to the greatly increased levels of TNF- α secretion. Estradiol treatment resulted in a 50-fold increase in TNF- α . TNF- α plays a critical role in tissue homeostasis by regulating cellular responses, and when TNF- α levels increase for prolonged periods of time tissue damage occurs ⁸⁰.

Previous studies of estradiol-treated mice after 2 weeks of infection found no inflammation or epithelial damage, which is in contrast to our study. Only after 10 weeks of infection was uterine damage observed, although no vaginal damage was described¹⁵². Van Andel et al. ¹⁵² used *T. foetus* strain ATCC 30003. In the present study, *T. foetus* strain D1 was used to establish infection in mice. This was the same strain Singh et al. ^{135,137} used to demonstrate *T. foetus* induced apoptosis in bovine vaginal and uterine epithelial cells and the strain Mutwiri et al. ¹⁰⁷ demonstrated could infect mice regardless

of the stage of estrus. Since increased numbers of apoptotic cells were observed in estradiol-treated mice much earlier than normal mice, estradiol treatment may also influence parasite-mediated factors that help to establish infection.

Infection in estradiol-treated mice also resulted in significant neutrophil accumulation and an increase in inflammatory cytokines within the reproductive tract, which was not observed in normal mice. In normally cycling mice, moderate estrogen levels (during proestrus and part of estrus) result in decreased neutrophil accumulation within the reproductive tract²⁰. Prolonged administration of unnaturally high levels of estradiol results in purulent inflammation of the reproductive tract¹⁴². Therefore, neutrophil responses during *T. foetus* infection in estradiol-treated mice are most likely the combined result of elevated serum estradiol levels in addition to parasite colonization, albeit minimal, within the murine reproductive tract. Lymphocyte responses in estradiol-treated mice were delayed when compared to the effector response detected in normal mice after 14 days of infection.

Increased levels of inflammatory cytokines in estradiol-treated mice could have been due to the production of cytokines, such as TNF- α , by epithelial cells in response to increased serum levels of estradiol⁸⁸. In addition, increased estradiol concentrations lead to increased epithelial division in the vagina and uterus¹³, resulting in increased TNF- α production. Interestingly, progesterone and estrogens were both found to inhibit MCP-1 production⁷⁶, suggesting both *T. foetus*-induced effector responses and perhaps TNF- α secretion⁷⁶ are influencing MCP-1 production. Elevated inflammatory cytokine responses

in estradiol-treated mice indicate there is a strong hormonal influence upon the magnitude of cellular-induced inflammation in response to early *T. foetus* infection.

Housing mice in LL resulted in nearly 100% animals remaining infected after 21 days in addition to a similar parasite burden to normal mice. Unlike infection in normal mice, stressed animals had exacerbated tissue damage and inflammation similar to that observed during infection in estradiol-treated mice. Neutrophil accumulation in stressed mice was also increased. In addition, there was an increase in CD4⁺ CD25⁺ T cells by day 14 in stressed mice and after 21 days of infection in both estradiol-treated and stressed mice. Inflammatory cytokine levels in stressed mice were moderate compared to those observed in normal and estradiol-treated mice, with significant increases of IFN- γ and MCP-1 chemokine expression after 21 days of infection.

The increase of IFN- γ production in both estradiol-treated and stressed mice may be attributed to the increase in neutrophil accumulation within the reproductive tract after 14 and 21 days of *T. foetus* infection. Intraepithelial neutrophils located within the endometrium have been found capable of producing IFN- γ ¹⁶⁸. Similar to estradiol-treated mice, stressed mice infected with *T. foetus* also had severe epithelial damage within the vagina, most likely due to the inflammation and parasite-mediated destruction and apoptosis of epithelial tissues.

The accumulation of CD4⁺ CD25⁺ T cells correlated with increased inflammation and IFN- γ levels observed in estradiol-treated and stressed mice. Severe inflammation within the reproductive tract has been reported to result in poor pregnancy outcomes¹¹.

This indicates a role for regulatory T cells in maintaining the proper inflammatory homeostasis within the uterus and oviducts. Estradiol-treatment has been shown to augment FoxP3 expression *in vitro* and *in vivo*, helping to induce tolerance of the allogenic fetus during pregnancy by expanding the regulatory T cell compartment¹¹⁵. Regulatory T cells have also been shown to be important in the presence of exogenous antigen from bacterial^{186,104} and parasite-derived antigens^{57,149}. In addition, regulatory T cells have been shown to diminish pathogenesis caused by *Leishmania amazonensis*⁷³ and *Pneumocystis carinii*⁶⁸. These studies demonstrate regulatory T cells may have a role in reducing inflammation, thus preventing damage within the reproductive tract during infection with *T. foetus*.

Although there were no increases in IL-10 cytokine responses in estradiol-treated and stressed mice, IL-4 modulation has also been demonstrated with regulatory T cells. During infection with *Leishmania major*, regulatory T cells were shown to regulate the production of early IL-4 production resulting in progression of a Th2 type response and dissemination of the parasite³. Infection with *T. foetus* in the natural bovine host has recently been reported to result in a Th2-driven inflammation which includes eosinophil infiltration and mast cell degranulation in addition to the production of the Th2 immunoglobulins IgG1 and IgE³. Since estradiol-treatment and stress resulted in early inflammation and secretion of Th1 cytokines such as IFN- γ and TNF- α , which seemed to be associated with increased tissue damage in these two groups of mice, regulatory T cells may be involved in suppression of Th1-driven inflammation by secretion of

cytokines such as IL-4 and TGF- β , which were not measured in this study. This suggests that regulatory T cells may accumulate in the reproductive tract to limit epithelial damage caused during the host response to *T. foetus* during elevated estradiol or stress levels.

Histological findings described here illustrate an important point regarding *T. foetus* pathogenesis. Apoptosis appears to precede tissue damage during estradiol-treatment and stress. In estradiol-treated and stressed infected animals, whenever large amounts of apoptosis was detected in the vagina or uterus, similar damage would then be detected after the subsequent time points during infection. Apoptosis has never been examined *in vivo* during *T. foetus* infection in either the bovine or mouse hosts. It has been shown that *T. foetus* can induce apoptosis upon host vaginal and uterine epithelial target cells only after 4-20 hours *in vitro*^{135,137}. Therefore, parasite-mediated apoptosis could occur within the first 24 hours of infection. This illustrates the importance of examining the early pathology during *T. foetus* infection.

Early pathogenesis of *T. foetus* infection is poorly understood, and the results presented herein demonstrate a direct relationship between estradiol-treatment or stress upon the magnitude of infection in addition to the inflammation and tissue damage during infection. Clearly, infection is established quickly under normal, estradiol-treatment, and stressed conditions, as heavy parasite burden within the reproductive tract was seen by day 4 of infection. These results are similar to the study by Parsonson¹¹³, which describes *T. foetus* infection within the oviducts of experimentally infected cattle only after two weeks of infection. Although the entire reproductive tract was colonized by two weeks,

gross inflammation and abortion were reported to occur after 60 days of infection¹¹³. This study, together with our data, illustrates early events during *T. foetus* infection, such as elevated estradiol levels and stress in addition to host-mediated inflammation and apoptosis of vaginal and epithelial tissue, clearly determine the course of infection and are perhaps attributable to the varied pathological findings in bovine trichomoniasis.

Since normal and stressed mice had similar incidence and magnitude of infection, it is logical to conclude that stressing the host resulted in increased tissue pathology and inflammatory response. Lack of ovulation due to an absent or erratic LH surge and intermediate serum levels of estradiol in mice housed in LL²⁴ are hormonally similar to suppressive effects of estradiol on LH secretion due to nutritional deprivation⁷¹ alone, or in post-partum anestrous cattle. Clinically stressed mice have similar hormone levels to stressed and nutrient deprived cattle, implying *T. foetus* infection during periods of stress could result in increased pathology such as inflammation and tissue damage.

In summary, this study demonstrates that *T. foetus* cytotoxicity and pathogenesis (defined by elicited inflammatory response and tissue damage) are enhanced due to both estradiol treatment and stress. In addition, this study shows how administration of high doses of estradiol results in the inability of *T. foetus* to establish infection, although inflammation and tissue damage within the reproductive tract of these animals is increased. Thus a picture emerges that illustrates how host response, and more importantly how the environment within the female reproductive tract, can directly affect the course and outcome of *T. foetus* infection.

NEUTROPHILS AND iNOS ARE CRITICAL FOR EARLY RESISTANCE TO THE
ESTABLISHMENT OF *TRITRICHOMONAS FOETUS* INFECTION

Introduction

Trichomoniasis is a sexually transmitted disease in cattle caused by the parasitic protozoan *Tritrichomonas foetus* (*T. foetus*). *T. foetus* is transmitted from asymptomatic carrier bulls harboring the parasite during coitus, thus making trichomoniasis a relevant concern for cattle operations utilizing natural service insemination. Infection in females causes vaginitis, placentitis, and endometritis¹¹³ resulting in preterm delivery and abortion within the first 2-3 months of gestation^{13,113}, although fetuses aborted as late as 7 months of gestation have been reported¹²². On rare occasions, postcoital pyometria will develop¹³. Pyometria and endometritis often result in significant inflammatory changes within the endometrium and uterine tubes², resulting in a reduction of fertility¹¹. However, infection without embryonic loss or overt clinical symptoms has been reported¹³⁸, resulting in the exposure of *T. foetus* infected cattle to bulls assumed to be pathogen free. Undetected infection results in severe economic loss, as doubling the prevalence of *T. foetus* in the bull population results in an exponential reduction in calf crop revenue¹¹⁸. Therefore, *T. foetus* is a disease of economical and veterinary relevance.

Previous studies in the bovine model^{2,15,35,39,62,70,139-141}, the estradiol-treated mouse model^{142,152}, and the non-treated mouse model¹⁰⁷ have focused mainly on acquired immunity and late stages of *T. foetus* infection for two reasons. First, *T. foetus* is an

extracellular parasite and infection in cattle is self-limiting. Following clearance of infection, transient protection against pathogen challenge is observed¹⁷⁰, indicating both acquired immunity and antibody responses are important during infection. Second, cattle bred to an experimentally infected bull have gross inflammation and associated fetal loss after 60 days of infection¹¹³ indicating clearance of the parasite before 60 days may result in increased reproductive efficiency. Vaccination and challenge with *T. foetus* results in local antigenic stimulation within the endometrium and uterine tubes as evidenced by the formation of secondary lymphoid nodules and a strong secretory IgA response within the uterus^{2,107}. These studies indicate that anamnestic IgA and systemic and local IgG1 antibody responses might be critical in developing a vaccine to adequately clear infection. However, these studies do not elucidate how innate reproductive immune responses contribute to the acquired immunity responsible for protection against *T. foetus*.

Both host and parasite-mediated factors most likely contribute to the ability of *T. foetus* to successfully colonize the female reproductive tract, thus determining the eventual outcome of infection. In order to successfully colonize the female reproductive tract, *T. foetus* requires secreted proteases^{22,90,150} and adhesion⁹⁰. Glycoconjugates on the surface of *T. foetus*, such as lipophosphoglycan (TF-LPG), facilitate parasite adhesion to bovine vaginal epithelial cells¹³⁶ with subsequent contact-dependent parasite-mediated cytotoxicity²³. In addition, several reports have demonstrated host responses against both secreted proteases and TF-LPG^{2,134}. Thus, factors such as proteases and TF-LPG, which

facilitate parasite colonization, are directly affected by the host immune response.

Therefore, an effective early immune response could curtail *T. foetus* infection and the resultant pathogenesis.

Because previous studies have focused on acquired responses during an already established infection, little information exists regarding how the innate response affects early colonization and establishment of infection *in vivo*. Neutrophil infiltration into the superficial endometrium and uterine lumen is the initial response to surface infection within the uterus ¹¹. Since *T. foetus* is generally a lumen dweller, neutrophil responses such as the release of antimicrobial peptides, reactive oxygen species (ROS), and reactive nitrogen species (RNS) during early infection may play a role in *T. foetus* colonization and pathogenesis. Since it has been demonstrated that nitrite is toxic to *T. foetus* and inhibits both motility and growth ⁹⁴, oxidative metabolites from macrophages and neutrophils may be important factors that prevent early *T. foetus* infection. Infection in cattle results in a large accumulation of neutrophils and macrophages throughout the endometrium, endometrial glands, and uterine lumen, in addition to an accumulation of macrophages within the vaginal epithelium ¹¹³. Accumulation of macrophages and neutrophils due to *T. foetus* invasion of fetal lungs and intestines has also been reported ¹²², and phagocytized *T. foetus* was detected in the bronchi of an aborted fetus ¹²³, indicating both neutrophil and macrophage responses are important in clearance of *T. foetus*. However, these previous reports do not elucidate the mechanisms whereby neutrophils and macrophages are able to kill *T. foetus*.

The goal of this study was to understand how the innate immune response affects colonization of *T. foetus* within the murine reproductive tract. To determine which innate immune responses may be important during initial infection, we compared parasite burden within the vagina and uterus of IFN- γ ^{-/-}, CXCR2^{-/-}, gp91^{phox-/-}, gp91^{phox-/-}/iNOS^{-/-}, and iNOS^{-/-} mice as well as in SCID and RAG^{-/-} mice to the parasite burden in BALB/c and C57C57BL/6 immune competent mice. We also used the monoclonal antibody RB6-8C5⁶² to deplete BALB/c mice of circulating neutrophils in order to determine if neutrophils affect the ability of *T. foetus* to colonize the reproductive tract. In addition, we examined *T. foetus* killing by bone marrow-derived neutrophils and thioglycolate-elicited peritoneal macrophages from mice with defects in ROS and RNS production to determine whether these oxidative metabolites are important in the elimination of *T. foetus* during the early stages of infection.

We found that neutrophils within the reproductive tract are important in controlling the early dissemination of *T. foetus* throughout the reproductive tract. Mice depleted of neutrophils before infection with *T. foetus* had a significantly higher parasite burden within reproductive tract homogenates, illustrating neutrophils are involved in inhibiting early colonization of the reproductive tract. However, the ability of neutrophils or macrophages to kill *T. foetus* is not dependent upon ROS production. Wild-type and gp91^{phox-/-} macrophages and neutrophils stimulated with PMA to generate ROS were unable to kill *T. foetus*. Additionally, we found that mice unable to produce RNS are more susceptible to infection with *T. foetus*, compared to wild-type control mice.

Peritoneal macrophages from $iNOS^{-/-}$ and $gp91^{phox}/iNOS^{-/-}$ mice were unable to kill *T. foetus* whereas wild-type and $gp91^{phox/-}$ macrophages induced with $IFN\gamma$ and LPS to generate RNS were able to kill *T. foetus*. These data indicate RNS production by macrophages is one of the major innate mechanisms that inhibit *T. foetus* colonization within the reproductive tract.

Materials and Methods

Mice

All Breeder mice were bred in our colony at Montana State University. Breeder Fox Chase SCID C.B-17 mice were obtained from Charles River. Breeder chemokine receptor CXCR2 deficient ($CXCR2^{-/-}$) mice (C.129S2(B6)-*Cmkar2*^{tm1/Mwm}) and breeder interferon gamma knockout ($IFN\gamma^{-/-}$) mice (C.129S7(B6)-*Ifng*^{tm1ts}) were both obtained from Jackson Laboratory. BALB/c mice, purchased from NCI, were used as wild-type controls for SCID, $CXCR2^{-/-}$ and $IFN\gamma^{-/-}$ models. Breeder Rag1 deficient ($RAG^{-/-}$) mice (B6.129S7-*Rag1*^{tm1Mom}), mice with a null allele of the NADPH oxidase gp91^{phox} subunit ($gp91^{phox/-}$) (B6.129S6-*Cybb*^{tm1Din/J}) and mice with a mutation in the nitric oxide synthase 2 gene ($iNOS^{-/-}$) (B6.129P2-*Nos2*^{tm1Lau/J}) were both obtained from Jackson Laboratory. Double knockout breeder $gp91^{phox}/iNOS^{-/-}$ mice were a generous gift from Dr. Robert North at Trudeau Institute. Stock $gp91^{phox}/iNOS^{-/-}$ mice were given itraconazole and sulfamethoxazole-trimethoprim (Bactrim) prior to the beginning of an experiment as previously described^{131,145}. C57C57BL/6 mice, purchased from National Cancer Institute,

were used as wild-type controls for RAG^{-/-}, gp91^{phox^{-/-}}, iNOS^{-/-}, and gp91^{phox}/iNOS^{-/-} mouse models. Mice were housed in microisolator cages and provided with sterilized food and water *ad libitum* in accordance with the *Guide for the Care and Use of Laboratory Animals*. All experimental procedures and protocols were approved by and followed the guidelines set forth by the Institutional Animal Care and Use Committee.

Parasites, Inoculums, and Antigen Preparation

T. foetus strain D1, a low passage isolate originally isolated from a cow with pyometria and that has been previously utilized to successfully infect cattle^{2,39,140}, was a kind gift from Dr. Lynnette B. Corbeil (University of California). Parasites were maintained in axenic culture at 37°C in modified Diamonds TYM media⁴⁶ supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco).

Parasite inoculums were prepared by incubating a thawed aliquot of D1 until logarithmic growth was achieved. Parasites were counted on a Neubauer hemocytometer, resuspended to 6.67×10^7 /ml, washed three times in sterile Hanks balanced salt solution (HBSS) by centrifugation at 250 x *g* for 5 minutes, and resuspended in HBSS. Mice were infected intravaginally with 1×10^6 trichomonads each day, for two consecutive days. Infection was allowed to persist for the indicated times.

Heat-killed *T. foetus* antigen (HKTF) was prepared by washing *T. foetus* 3 times in complete DMEM (cDMEM) with 2 mM L-glutamine, 100 U penicillin, 100 µg streptomycin, 1mM sodium pyruvate, (all from Gibco) and 10% fetal bovine serum

(Atlanta Biologicals). Parasites were resuspended into 1 ml aliquots of 1×10^7 *T. foetus*/ml and incubated overnight at 56°C. After the incubation, preparations were microscopically examined and counted to ensure parasites were killed and remained intact. Antigen preparations were stored at 4°C until further use.

Neutrophil Depletion

Mice were depleted of circulating neutrophils with the monoclonal antibody RB6-8C5⁶³, modified as previously reported by our lab¹⁴⁵. Briefly, mice were administered an intraperitoneal injection of 300 µg/500 µl RB6-8C5 antibody or an equal concentration of isotype control rat IgG1 two consecutive days prior to infection with *T. foetus*. Injections were administered daily throughout the course of infection. On the third day of neutrophil depletion, mice were infected with *T. foetus* as previously mentioned and the infection was allowed to persist for 5 days. Mice were sacrificed with a lethal injection of sodium pentobarbital (100 µg/ml) and exsanguinated. Whole reproductive tracts were removed and processed as described below.

To verify blood and reproductive tract depletion of neutrophils, leukocyte differential counts were performed on blood and reproductive tract homogenates. Total numbers of reproductive tract leukocyte subsets were calculated by multiplying the percentage of subsets obtained in the differential analysis by the total number of cells in the reproductive tract homogenate.

Recovery and Enumeration of *T. foetus*

For analysis of *T. foetus* burden in the lower reproductive tract of mice, parasites were collected from the vagina prior to removal of the whole reproductive tract by lavaging with 50 μ l sterile HBSS. Trichomonads were cultured from the lavage fluid as described above by incubation in 1 ml Diamonds medium for 48 hours. After whole reproductive tracts were removed, each uterine horn was lavaged with 500 μ l of sterile HBSS and cultured in 2 ml Diamonds medium for 48 hours to examine parasite burden in the upper reproductive tract. To quantify parasites remaining in the reproductive tract (considered to be invasive or tightly adhered to the vaginal and uterine epithelium), whole reproductive tracts were gently homogenized through a mesh screen with 1 ml of sterile HBSS. Homogenates were incubated in 3 ml Diamonds medium for 48 hours as described above. Parasites were enumerated from each sample by counting the number of viable (motile) trichomonads with a Neubauer hemocytometer.

Macrophage killing assay

Immature inflammatory macrophages were recruited intraperitoneally by injecting 1 ml of sterile 3% thioglycollate medium into the peritoneum of C57C57BL/6, gp91^{phox}^{-/-}, iNOS^{-/-}, and gp91^{phox}/iNOS^{-/-} mice. After 7 days, mice were sacrificed and the peritoneal cavity was carefully washed with 10 ml sterile harvest medium (cDMEM). Total macrophages were calculated by enumerating total leukocytes and performing a differential cell count, as previously described. Macrophages were then resuspended in harvest medium and plated in a flat bottom 24 well plate at a concentration of 8×10^5

cells/well. Cells were then incubated at 37°C with 5% CO₂ for 1 hour to allow macrophages to adhere firmly to the plastic. After the incubation, wells were gently washed 3 times with warmed cDMEM and 500 µl media was added to the wells.

Macrophages were primed by adding either 500 µl cDMEM containing 10 µg/ml IFN γ or 200 µl/well of heat-killed *T. foetus* antigen into the wells and incubated at 37°C and 5% CO₂ for 4 hours for RNS-induced killing or 24 hours for ROS-induced killing of *T. foetus*. After priming, wells were washed 3 times with HBSS and cells were triggered with 500 µl of cDMEM containing either 100 ng/ml LPS for RNS-induced killing or 10 µg/ml PMA for ROS-induced killing as described previously³⁷. *T. foetus* resuspended in 500 µl of cDMEM was added to each well at a ratio of 5:1 macrophages:parasites⁴⁴ immediately following macrophage triggering, and incubated at 37°C and 5% CO₂ for 48 hours for RNS-induced killing or 16 hours for ROS-induced killing. After incubation, parasites and media were removed from each well and placed into parasite culture tubes. Aliquots of 100 µl were removed for parasite counts. The culture tubes were resuspended in 3 ml of cDMEM, and the samples were incubated for 10 hours at 37°C followed by parasite counts to examine parasite recovery. Percent killing was calculated by $((X-Z)/X) \times 100$, where X is the number of parasites incubated in the 24 well plates without effector cells and with triggering agents and Z is the total number of parasites incubated with macrophages and triggering agents.

Neutrophil killing assay

Whole blood was collected from each group of mice in non-heparinized tubes and incubated at 37°C for 30 minutes followed by centrifugation at 10000 rpm for 10 minutes. Serum was collected from each tube and pooled and diluted in Dulbeccos phosphate buffered saline with 1% FBS (DPBS-F) to make a solution of 20% sera. *T. foetus* parasites were treated with the 20% non-immune mouse serum by incubation for 30 minutes at 37°C, as previously described⁴. After the incubation, serum treated parasites were resuspended in cDMEM and added to neutrophils in the ratios described below.

Bone marrow was flushed from the femurs and tibias of C57C57BL/6, gp91^{phox}^{-/-}, iNOS^{-/-}, and gp91^{phox}/iNOS^{-/-} mice with 5 ml HBSS with 3 mM EDTA. Bone marrow samples were removed from each animal and stored at room temperature until noted. Red blood cells were lysed using standard hypotonic lysis buffer, bone marrow leukocytes were resuspended in DMEM with 10 mM HEPES (DMEM-H). Aliquots were retained for total leukocyte enumeration and differential analysis and total neutrophils were calculated as described for macrophage assays. Neutrophils were plated into flat bottom 24 well plates at a concentration of 8×10^5 cells/well in cDMEM. Neutrophils were activated with 100 ng/ml PMA and stored on ice until parasites were immediately added to the wells. Untreated or serum-treated parasites were added to the wells at a neutrophil:parasite ratio of 20:1 and centrifuged at 250 x g for 7 minutes at 4°C. Plates

were incubated at 37°C at 5% CO₂ for 8 hours and parasites were removed and counted as described for macrophage killing assay.

Statistics

Prism (GraphPad) was used for all statistical tests of significance (P values of ≤ 0.05). For data that had a normal (Gaussian) distribution, unpaired two-tailed t tests were used to compare two groups of data. A nonparametric test (Mann-Whitney test) was used to analyze pairs of data that were assumed to have abnormal distribution. In most cases non-parametric analysis of normally distributed populations gave P values similar to those from the two-tailed t test.

Results

Susceptibility of Mice Deficient in RNS Production is Greater than Immune Competent Mice

In order to determine whether components of the host immune response affected initial colonization of *T. foetus* within the reproductive tract, BALB/c, SCID, CXCR2^{-/-}, IFN γ ^{-/-}, or C57BL/6 and RAG^{-/-} mice were infected with *T. foetus* for 10 days. After 10 days, mice were sacrificed, and parasite numbers were quantified from the vagina, uterus, and vaginal homogenates of entire reproductive tracts. Interestingly, none of the mutant mice had significantly increased parasite burden compared to either wild-type BALB/c or C57BL/6 mice (Figures 3.1A-1C and 3.3A-C). The median number of parasites collected from the vagina and homogenates of IFN γ ^{-/-} mice was elevated compared to wild-type

controls, although not significantly (Figures 3.1A and 3.1C). In addition, there were no significant differences in the total numbers of parasites recovered from the reproductive tracts of each group of mice (Figures 3.1D and 3.3D). Approximately 75% of all groups of mice with a BALB/c background remained infected after 10 days (Figure 3.2), whereas only 15% of the C57BL/6 and RAG^{-/-} mice remained infected (Figure 3.4). These results indicate that lymphocyte deficiencies or absence of either the CXCR2 chemokine receptor or IFN γ did not enhance susceptibility to *T. foetus* infection.

To determine whether reactive oxygen species and nitrogen radicals have an effect upon initial infection, gp91^{phox^{-/-}} or iNOS^{-/-} mice were infected with *T. foetus* for 10 days. In addition, double gp91^{phox^{-/-}}/iNOS^{-/-} knockout mice were used. Compared to the C57BL/6 or RAG^{-/-} mice, gp91^{phox^{-/-}} mice were not more susceptible to *T. foetus* infection, as there were similar numbers of parasites recovered from all regions of the reproductive tract (Figures 3.3A-C). Conversely, iNOS^{-/-} mice had significantly more parasites recovered from the vaginal washes, the uterine washes, and the reproductive tract homogenates compared to the C57BL/6 wild-type mice (Figures 3.3A-C, $p = .0039$, $.0008$, and $.0050$ respectively). gp91^{phox^{-/-}}/iNOS^{-/-} mice had significantly more parasites recovered from the homogenate than C57BL/6 mice (Figures 3.3A-C, $p = .0152$). iNOS^{-/-} and gp91^{phox^{-/-}}/iNOS^{-/-} mice had significantly more total parasites recovered from the entire reproductive tract than C57BL/6 mice (Figure 3.3D, $p = .0006$ and $.0252$ respectively) in addition to both having significantly more mice infected than wild-type after 10 days (Figure 3.4, $p = .0351$ and $.0429$ respectively). These results indicate that mice unable to

produce RNS are more susceptible to initial infection with *T. foetus*, resulting in significant parasite colonization throughout the entire reproductive tract.

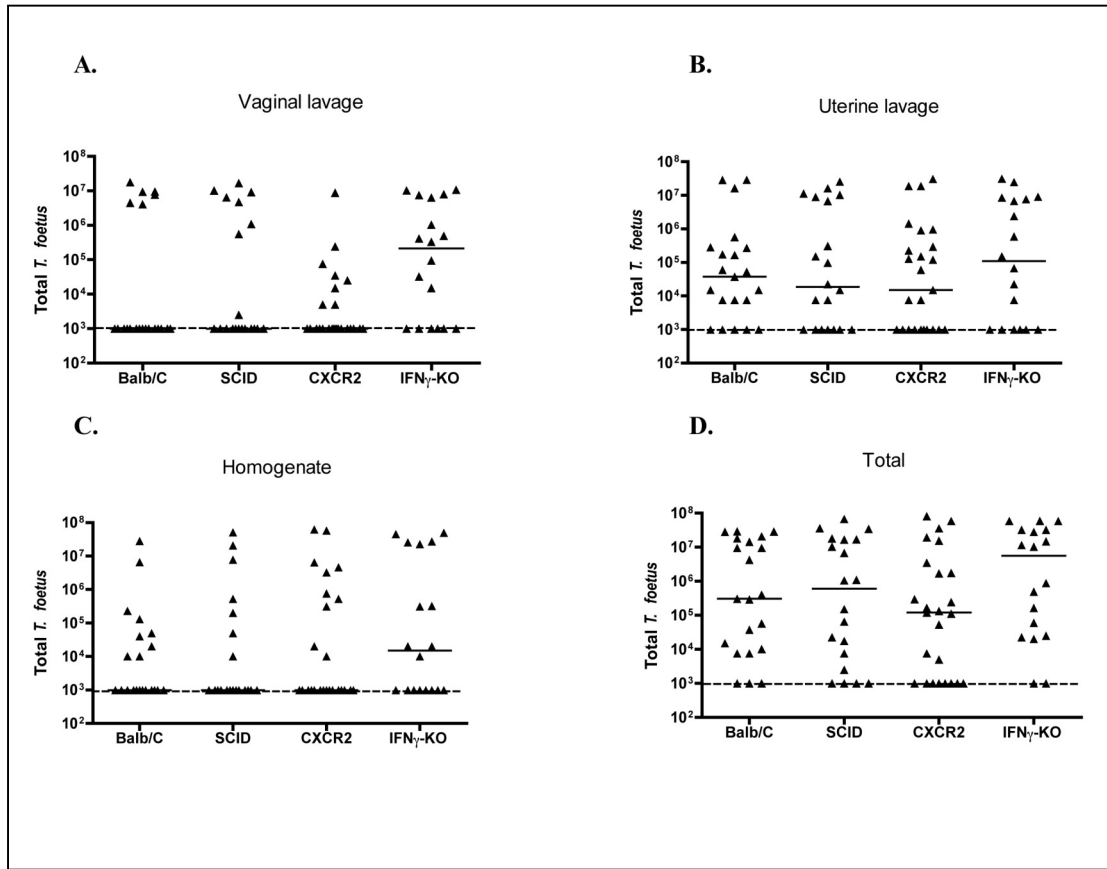


Figure 3.1: Mice with leukocyte deficiencies (SCID), or the inability to generate IFN γ , and without the chemokine receptor CXCR2 are not more susceptible to *T. foetus* infection compared to wild-type control mice. Mice were infected for 10 days with *T. foetus*. The dotted line indicates threshold of detection (<1000). A: Number of parasites recovered from the vagina. B: Number of parasites recovered from uterus. C: Number of parasites recovered from reproductive homogenates. D: Total number of parasites recovered from entire reproductive tract. The median number of parasites from the immune compromised mice were compared to the median number of *T. foetus* in BALB/c mice.

Depletion of Circulating Neutrophils Renders Mice
More Susceptible to Colonization with *T. foetus*

In order to determine if neutrophils play a significant role in the ability of *T. foetus* to establish infection, BALB/c mice were depleted of circulating neutrophils with the antibody RB6-8C5, as previously reported^{63,145}. Control mice were mock depleted with rat IgG1 before being infected with *T. foetus*. The percentage of circulating blood neutrophils was significantly decreased in depleted mice compared to mock-depleted mice (Figure 3.5A, $p = .0001$). Neutrophils play a critical role in ovulation and homeostasis of the reproductive tract²⁰, so we determined whether depletion also diminished the neutrophil population within the reproductive tract. Total neutrophils were enumerated from reproductive tract homogenates significantly depleted compared to mice administered rat IgG (Figure 3.5B, $p = .0295$).

Previous studies have shown that neutrophils begin to reappear after 5-7 days of depletion with RB6-8C5 and become refractory to the effects of the antibody¹⁴⁵. Therefore, mice were only depleted for 7 days and infected for a total of 5 days. Parasite colonization of the vagina was not significantly different between each group of mice, although neutrophil-depleted mice had higher numbers of parasites within the vagina. Additionally, colonization of the uterus was not evident after 5 days of infection in neutrophil-depleted or mock-depleted mice (Figures 3.6A-B). However, neutrophil-depleted mice did have significantly higher numbers of parasites recovered from the vaginal homogenates compared to mock-depleted mice (Figure 3.6C, $p = .0289$). Total

parasites recovered from the entire reproductive tracts of neutrophil-depleted mice indicated a significant increase in susceptibility due to the lack of neutrophils within the reproductive tract (Figure 3.6D, $p = .0401$). One hundred percent of the neutrophil-depleted mice were infected by 5 days, whereas only half of the mock-depleted mice were infected by 5 days (Figure 3.7, $p = .0370$). These results indicate that neutrophils inhibit *T. foetus* colonization of the reproductive tract.

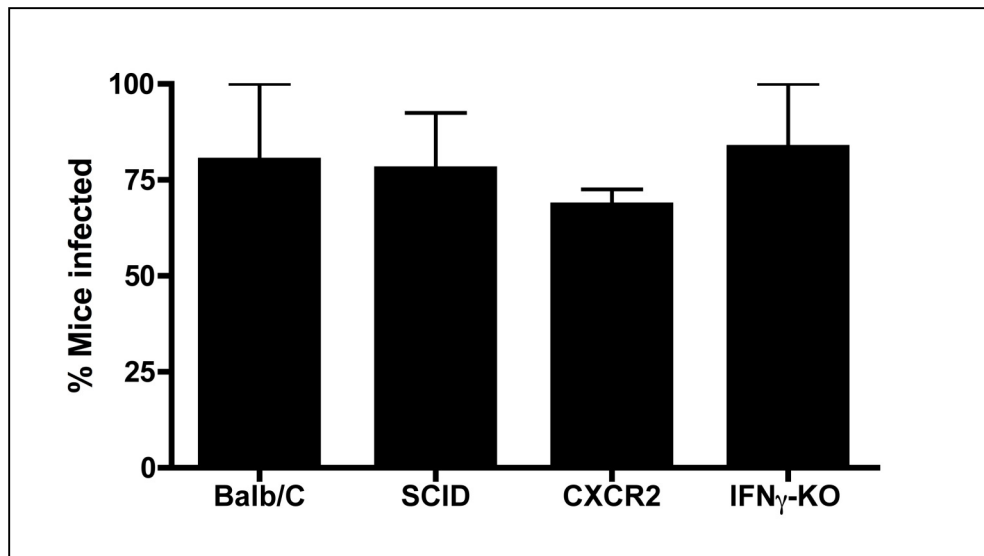


Figure 3.2: Incidence of *T. foetus* infection in BALB/c, SCID, CXCR2^{-/-} and IFN γ ^{-/-} mice was similar. Bars represent at least 8 mice/group with at least 2 repeats.

Macrophages With the Ability to Produce RNS Can Kill *T. foetus* Better than Macrophages Unable to Produce RNS

Since both neutrophils and RNS appear to play an important role during initial *T. foetus* colonization and infection, we then determined whether there was a direct relationship between the ability of neutrophils and/or macrophages to produce ROS or RNS and to kill *T. foetus in vitro*. Bone marrow neutrophils and thyoglycollate elicited

peritoneal macrophages were isolated from $gp91^{phox^{-/-}}$, $iNOS^{-/-}$, and $gp91^{phox}/iNOS^{-/-}$ mice and incubated with *T. foetus*. Killing capability of these cells was compared to killing of neutrophils and macrophages isolated from C57BL/6 mice.

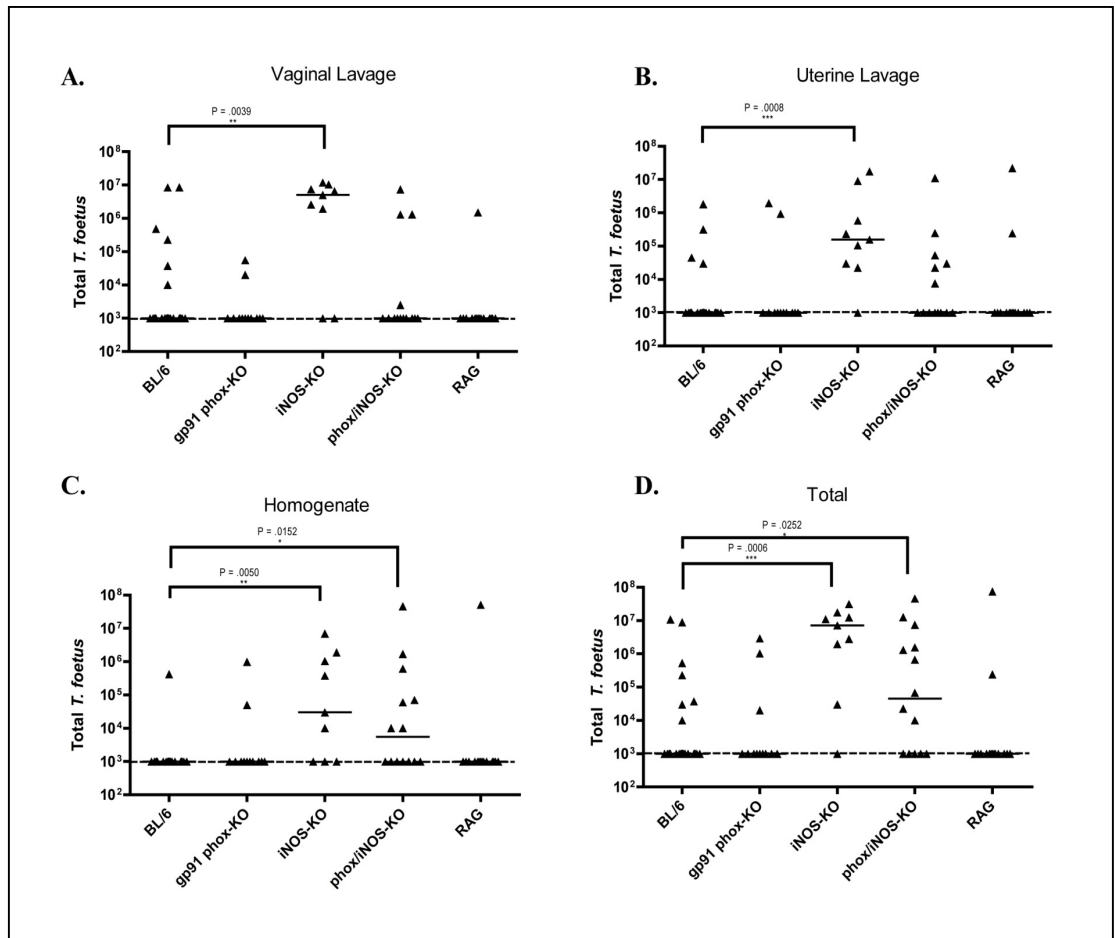


Figure 3.3: Mice deficient in the ability to generate RNS ($iNOS^{-/-}$ and $gp91^{phox}/iNOS^{-/-}$) are more susceptible to *T. foetus* infection than mice deficient in the ability to generate ROS ($gp91^{phox^{-/-}}$). The dotted line indicates threshold of detection (<1000). A: Number of parasites recovered from the vagina. B: Number of parasites recovered from uterus. C: Number of parasites recovered from reproductive homogenates. D: Total number of parasites recovered from entire reproductive tract. The median number of parasites from immune compromised mice were compared to the median number of *T. foetus* in C57BL/6 mice.

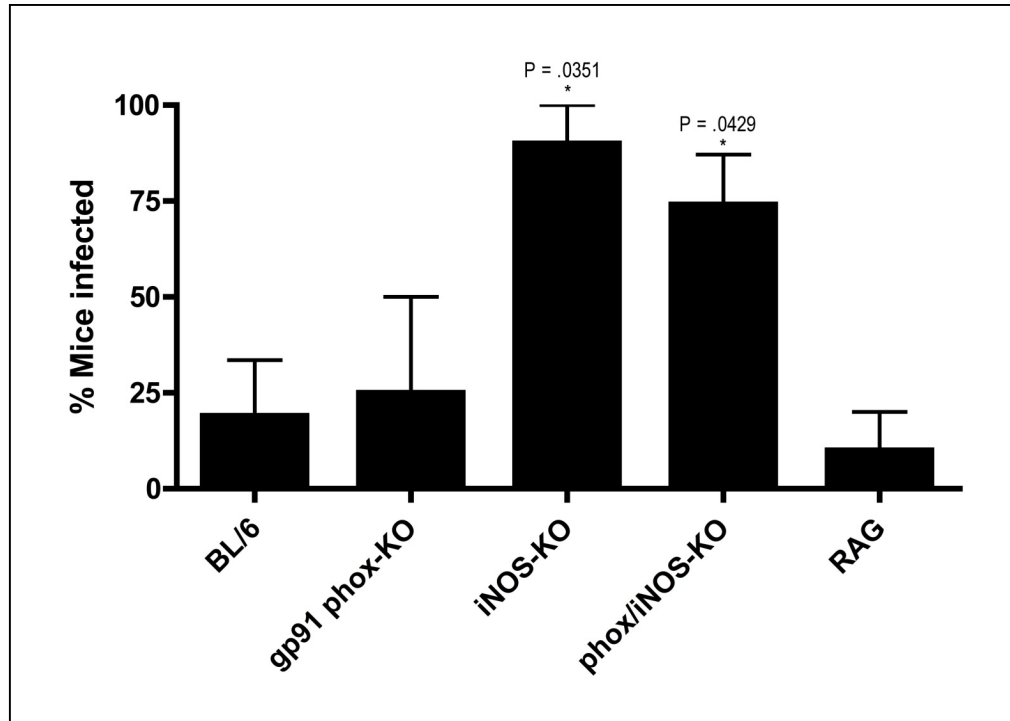


Figure 3.4: $iNOS^{-/-}$ and $gp91^{phox}/iNOS^{-/-}$ knockout mice have a greater incidence of *T. foetus* infection compared to wild-type C57BL/6 mice and $gp91^{phox^{-/-}}$ mice. Bars represent at least 5 mice/group with at least 2 repeats.

Bone marrow neutrophils isolated from all groups of mice were unable to kill *T. foetus*, even when wild-type neutrophils were activated to produce ROS with PMA. *T. foetus* was incubated with serum to determine if neutrophil killing was enhanced, possibly by allowing the neutrophils to phagocytose the parasites, and again there was no killing of *T. foetus* observed (Figure 3.8A). Macrophages stimulated with either $IFN\gamma$ or HKTF and activated to generate ROS using PMA³⁷ were also unable to kill *T. foetus* after 16 hours of incubation. These data indicate that although neutrophils may be

important during establishment of *T. foetus* infection, generation of ROS alone is not able to induce efficient killing of the trichomonads.

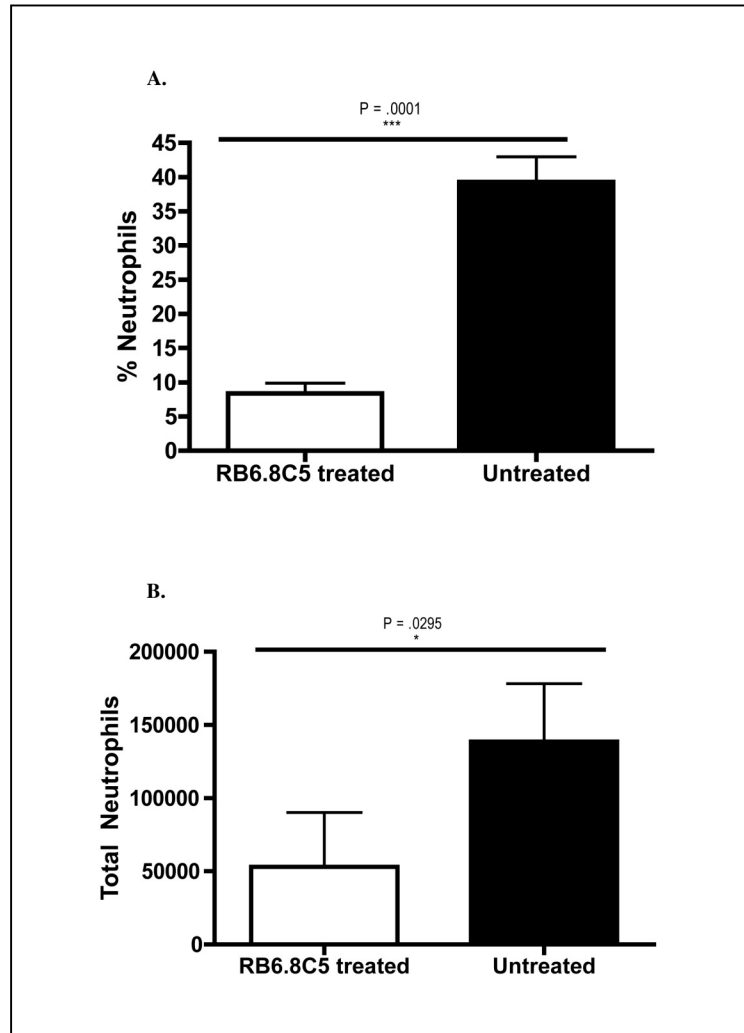


Figure 3.5: The number of neutrophils from the blood and reproductive tract were significantly depleted by RB6-8C5 treatment for 7 days. A: Percent blood neutrophils. B: Total neutrophils within the reproductive tract. Bars represent 4 mice per group with 2 repeats.

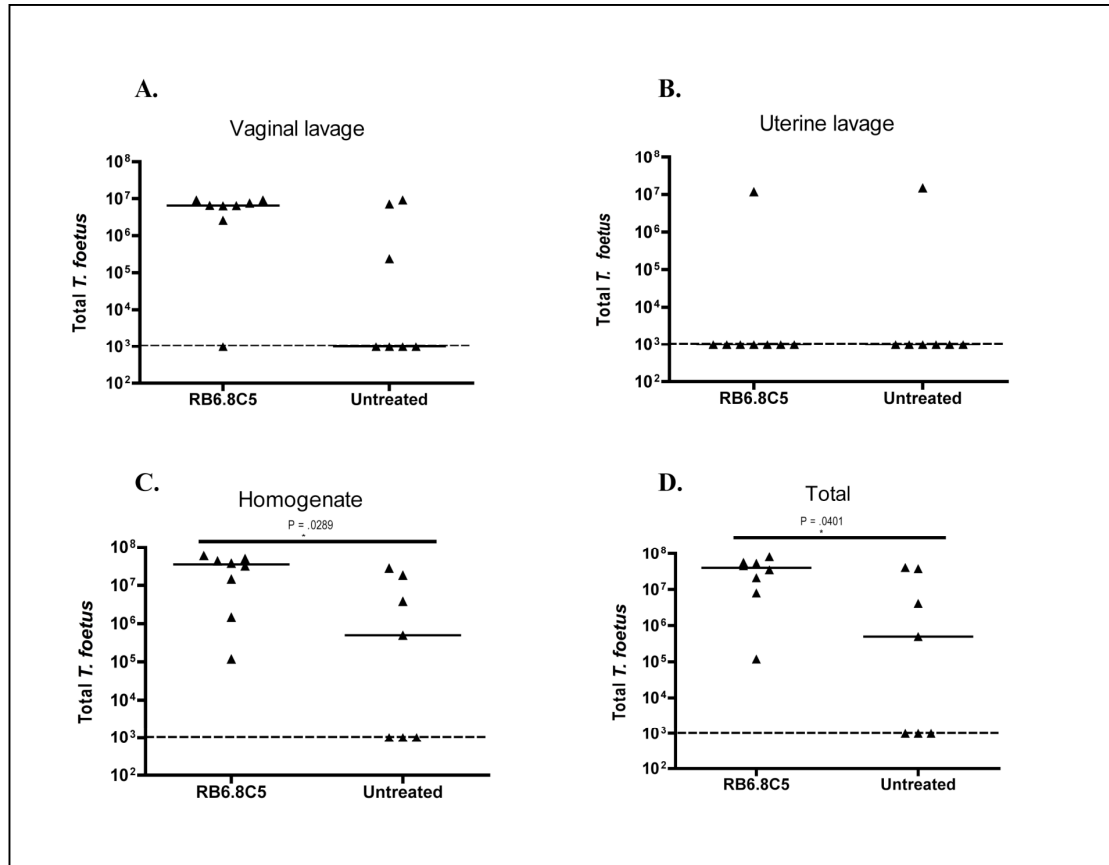


Figure 3.6: Neutrophils are important in initial infection with *T. foetus*. BALB/c mice depleted of neutrophils before initial *T. foetus* infection had significantly higher parasite numbers within reproductive tract homogenates and total parasite numbers than mock-depleted animals after 5 days infection. The dotted line indicates threshold of detection (<1000). A: Number of parasites recovered from the vagina. B: Number of parasites recovered from the uterus. C: Number of parasites recovered from reproductive tract homogenates. D: Total number of parasites recovered from the entire reproductive tract. The median numbers of parasites recovered from each group of depleted mice were compared to the median numbers of parasites recovered from the mock-depleted animals.

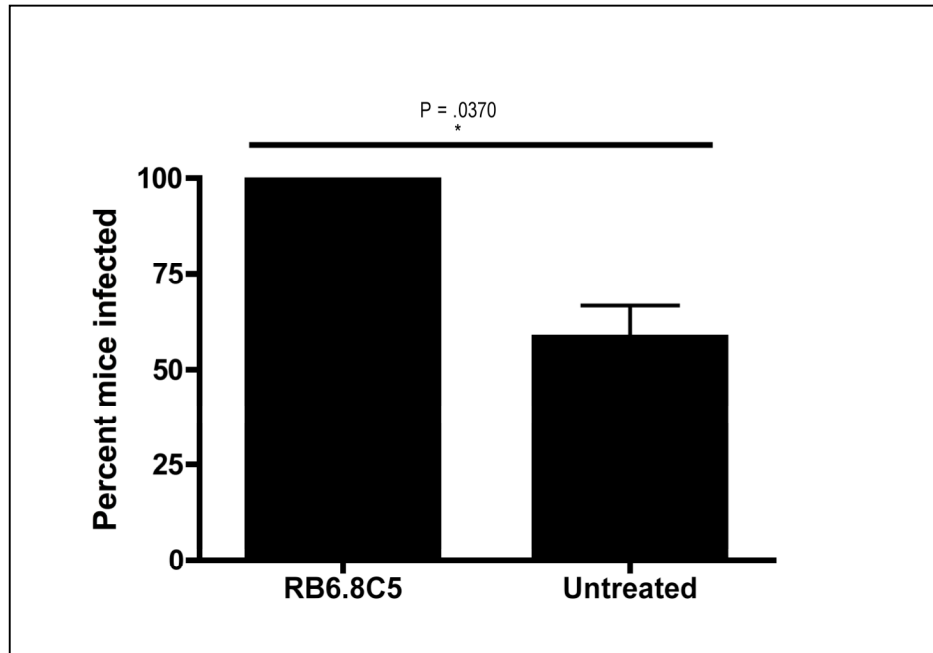


Figure 3.7: Neutrophils affect the incidence of *T. foetus* infection. RB6-8C5 treated mice had a significantly higher incidence of infection compared to mock-treated control mice. Bars indicate at least 4 animals/group with at least 2 repeats.

Macrophages that are unable to produce RNS are unable to kill *T. foetus*, pinpointing a role for RNS in the resistance of establishment of initial infection in mice. In addition, HKTF was a sufficient stimulant for both wild-type macrophages and gp91^{phox-/-} macrophages activated with LPS to kill *T. foetus* more effectively than iNOS^{-/-} mice. Wild-type macrophages stimulated with HKTF and activated with live trichomonads were also able to kill *T. foetus* significantly more effectively than iNOS^{-/-} mice (Figure 3.9A, $p = .0267$). In addition, wild-type macrophages stimulated with IFN γ and activated with LPS were also able to kill parasites more effectively compared to

iNOS^{-/-} and gp91^{phox}/iNOS^{-/-} mice (Figure 3.8C, $p = .0066$ and $.0349$ respectively).

Macrophages isolated from gp91^{phox} mice were also able to kill *T. foetus* when activated with LPS compared to iNOS^{-/-} or gp91^{phox}/iNOS^{-/-} mice (Figure 3.8C, $p = .0184$ and $.0071$ respectively). Similar to the wild-type macrophages, macrophages from gp91^{phox} knockout mice primed with IFN γ or HKTF and activated with LPS were able to kill *T. foetus* more effectively compared to both iNOS^{-/-} and phox/iNOS^{-/-} mice (Figure 3.9A, $p = .0053$ and $.0349$ and $p = .0065$ and $.0002$ respectively). Interestingly, gp91^{phox} mice primed with HKTF and stimulated with LPS were able to kill *T. foetus* better than than wild-type macrophages.

To determine if RNS inhibited growth of *T. foetus* or resulted in sufficient trichomonad killing, parasites were allowed a 10-hour recovery period in media without RNS or other macrophage derived products. Before and after the recovery period, motile parasites from each group were enumerated. After the recovery period, total numbers of parasites removed from unstimulated macrophages were significantly higher than the numbers of parasites removed from macrophages activated to produce RNS (Figure 3.9B). These results indicate parasites were killed by macrophages stimulated to produce RNS, and not immotile due to presence of RNS in the culture media of the 24 well assay plates.

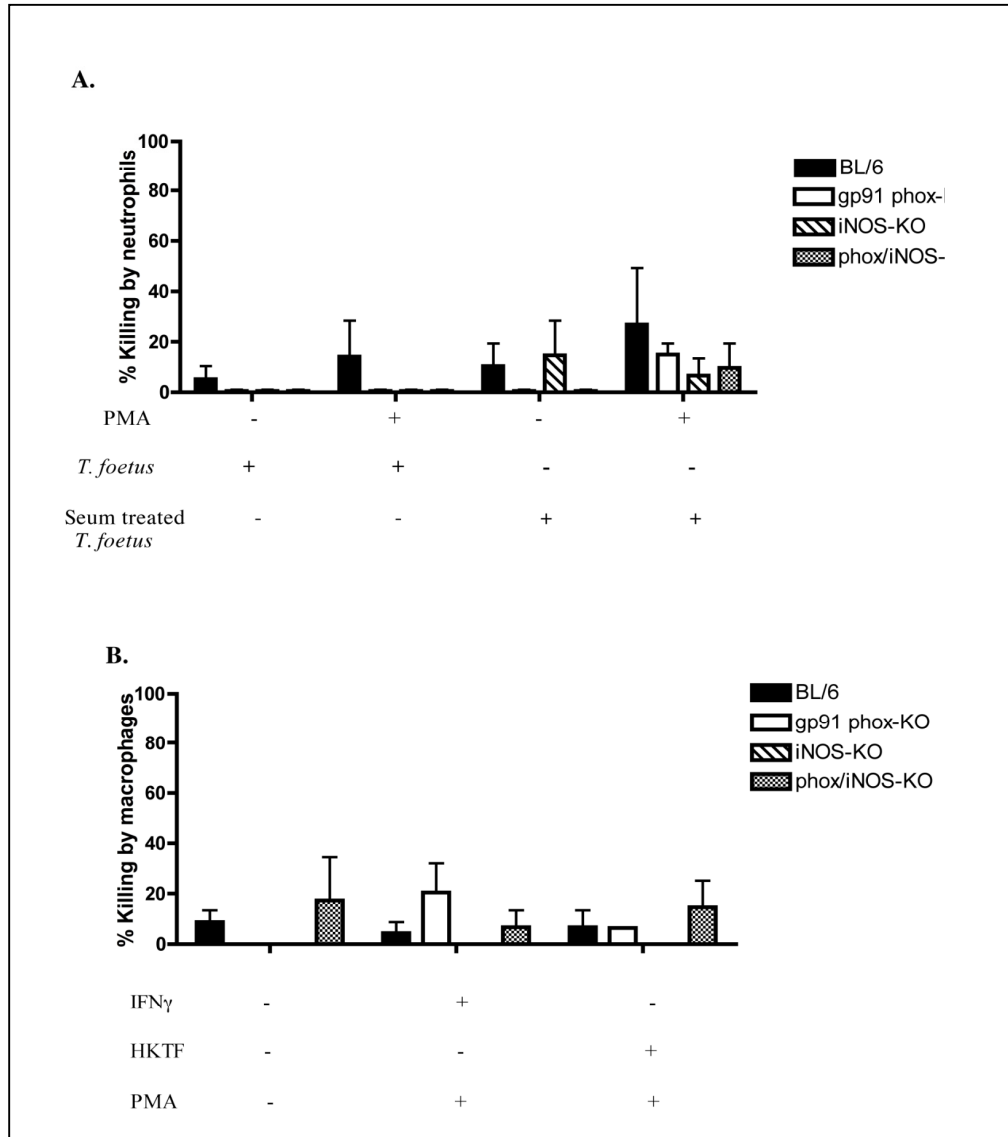


Figure 3.8: ROS generated from neutrophils and macrophages is not important in killing of *T. foetus in vitro* A: Bone marrow derived neutrophils from C57BL/6, gp91^{phox-/-}, iNOS^{-/-}, and gp91^{phox-/-}/iNOS^{-/-} mice given various treatments, including activation with PMA for oxidative burst, and incubated with live *T. foetus* for 8 hours were unable to kill *T. foetus*. B: Thioglycolate elicited peritoneal macrophages induced to produce ROS were unable to kill *T. foetus*. HKTF indicates heat killed *T. foetus* antigen. Percent killing was calculated as described in materials and methods.

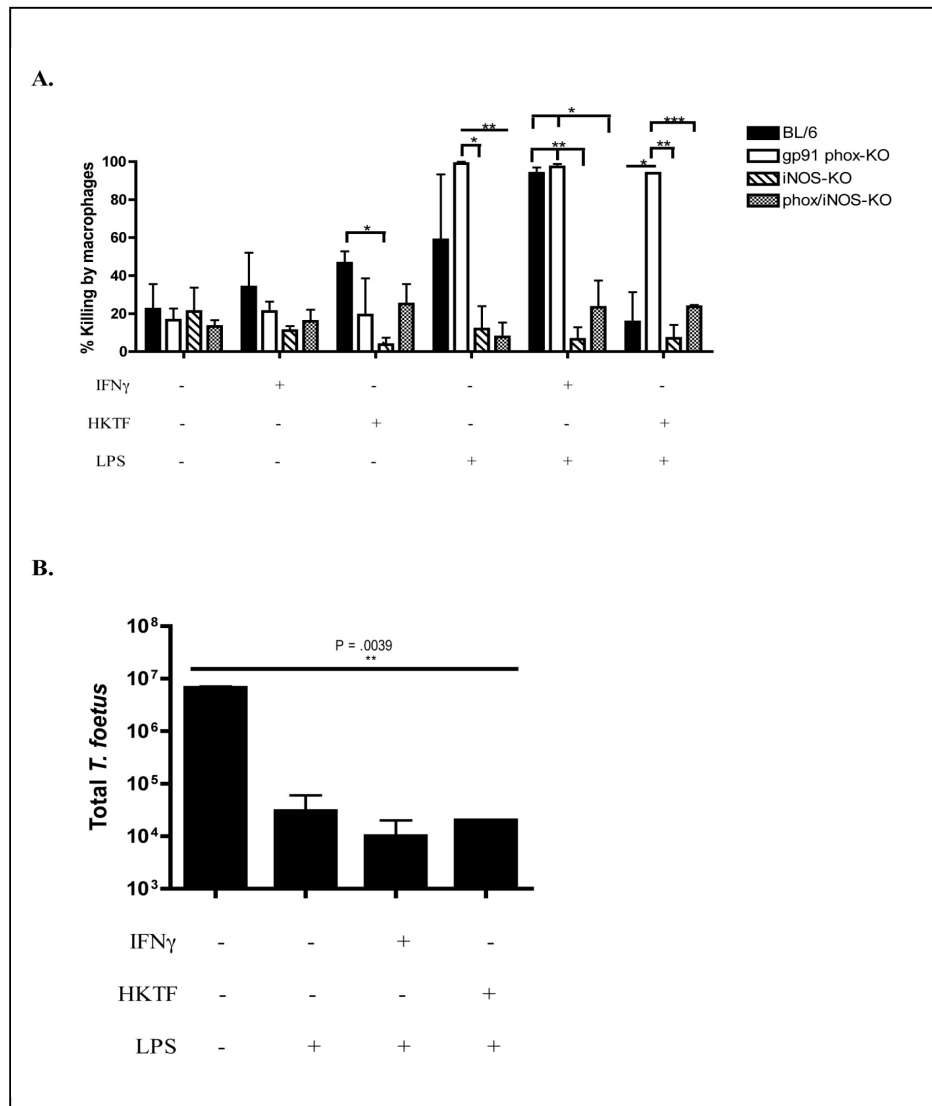


Figure 3.9: *T. foetus* killing is dependent upon the ability to generate RNS A: Macrophages from C57BL/6, gp91^{phox-/-}, iNOS^{-/-}, and gp91^{phox}/iNOS^{-/-} mice stimulated to produce RNS. Percent killing is calculated as described in materials and methods. HKTF denotes heat killed *T. foetus* antigen. B: *T. foetus* recovery after incubation with macrophages during *in vitro* killing assay. Parasites were removed from macrophages isolated from gp91^{phox-/-} mice with the indicated treatments. Parasites were recultured in the absence of macrophage products in fresh cDMEM and allowed to recover for 10 hours. Before and after the recovery period, parasites were counted.

Discussion

We found that neutrophil-mediated responses are important during initial infection with *T. foetus*, as mice depleted of neutrophils had a significantly higher parasite burden and incidence of infection when compared to mock-depleted animals. Depletion of neutrophils from the blood and the reproductive tract resulted in 100% of the animals being infected, in addition to significantly higher numbers of *T. foetus* recovered from the reproductive tract of mice, as compared to mock-depleted mice. However, the generation of ROS by neutrophils is not required for control of *T. foetus* dissemination throughout the reproductive tract. Infection of gp91^{phox-/-} mice with *T. foetus* resulted in a low parasite burden throughout all regions of the reproductive tract, similar to the infection in immune-competent C57BL/6 mice. When neutrophils and macrophages were isolated from gp91^{phox-/-} and C57BL/6 mice, there were no significant differences in the ability of these cells, when stimulated to produce ROS, to kill *T. foetus*. In addition to ROS production, serum treatment of parasites did not enhance neutrophil-mediated parasite killing.

Since *in vivo* and *in vitro* neutrophil-mediated killing of *T. foetus* differed so dramatically, it is possible neutrophils require additional signals or factors from cell types that were not present with the bone marrow-derived neutrophils. For example, neutrophil-generated ROS can interact with NO⁻ released during infection, resulting in the formation of peroxynitrite⁷, an unstable highly toxic microbiocidal agent⁸⁹. Although it is not well established, neutrophils can also produce RNS¹¹¹, which may account for the discrepancy

between neutrophil-mediated control of *T. foetus* infection in the mice and the parasite killing with bone marrow-derived neutrophils. Endothelial cells can be stimulated for iNOS⁹² signaling and RNS production, which could be an additional *in vivo* source of NO⁻, contributing to peroxynitrite formation and subsequent killing of *T. foetus*.

Neutrophils within the endometrium of the uterus also eliminate pathogens by releasing antimicrobial peptides such as α -defensins or the WAP protein elafin⁷⁸. These proteins may not have been released by bone marrow-derived neutrophils stimulated with PMA. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another potent neutrophil activator produced by endothelial cells within the uterus and allows neutrophils to persist at the site of infection by inhibiting neutrophil apoptosis¹⁹. This persistence at the site of infection may be critical in neutrophil elimination or control of *T. foetus* infection. Therefore, without GM-CSF *in vitro* neutrophils may have quickly underwent apoptosis resulting in inefficient killing of *T. foetus*.

iNOS^{-/-} mice were significantly more susceptible to infection with *T. foetus* than wild-type C57BL/6 mice. Peritoneal macrophages isolated from iNOS^{-/-} mice were unable to kill *T. foetus* as well as macrophages isolated from wild-type or gp91^{phox-/-} mice. Since NaNO₂ affects *T. foetus* growth and motility⁹⁴ and RNS products have antiproliferative effects on microorganisms⁹¹, we determined whether the diminished numbers of *T. foetus* during the *in vitro* macrophage experiments were due to increased numbers of immotile live parasites. We reasoned that when immotile parasites were cultured without the inhibitory effects of RNS they would recover and attain similar

levels of growth compared to the levels of parasites after incubation with unactivated macrophages. Our data support RNS killing of *T. foetus*, as the differences in parasite numbers between unactivated and activated C57BL/6 and gp91^{phox}^{-/-} macrophages before the recovery period were similar to those after the recovery period.

We found that iNOS^{-/-} mice were more susceptible to *T. foetus* infection than gp91^{phox}/iNOS^{-/-} mice. Although gp91^{phox}/iNOS^{-/-} mice are severely immune-deficient, macrophages isolated from gp91^{phox}/iNOS^{-/-} mice were able to kill *S. Typhimurium*, *E. coli*, and *Listeria*¹³¹, suggesting these animals have evolved another mechanism that compensates for these extreme immune deficiencies. This compensation may not be readily apparent until both NOS2 and gp91^{phox} are absent. Perhaps the inability of macrophages and neutrophils to produce ROS and RNS results in enhanced release of additional microbicidal compounds such as defensins or lysozyme. Recently, it was shown that uterine mast cell-degranulation results in clearance of *T. foetus* in experimentally infected cattle⁴⁰. This is a potential mechanism that results in clearance of *T. foetus* without generation of ROS or RNS.

Previous studies have indicated NO⁻ is capable of killing *Trichomonas vaginalis*, the human venereal pathogen closely related to *T. foetus*. It has been demonstrated that NO₂⁻ is an effector molecule involved in killing *T. vaginalis*¹¹². Murine peritoneal macrophages primed with IFN γ produced low levels of NO₂⁻ but were able to kill *T. vaginalis*. When stimulated with IFN γ and LPS, NO₂⁻ production was increased, although *T. vaginalis* killing was diminished to levels observed with unstimulated macrophages.

Conversely, when the RAW264.7 macrophage cell-line was stimulated with IFN γ and LPS, *T. vaginalis* killing correlated to production of NO $_2^-$ ¹¹². These data are contrary to the data in this study, as macrophages stimulated with both LPS and IFN γ were able to kill *T. foetus*. There are reports indicating a major immunogenic surface ligand of *T. foetus*, TF-LPG, is vastly different from LPG purified from the surface of *T. vaginalis*¹³⁶. Since macrophage killing depends on specific activation from microbial stimulants, recognition of surface ligands between the two parasites could result in different requirements for killing each of these distinct parasites.

Both bovine neutrophils⁴ and macrophages⁴⁴ are able to kill *T. foetus*. The study that examined neutrophil killing of *T. foetus* illustrated unstimulated neutrophils were able to kill *T. foetus*, although killing was most effective when parasite-specific antibodies and complement were present⁴. The study by De Azevedo et al.⁴⁴ illustrated macrophage phagocytosis of mitogen Concanavalin A coated-*T. foetus* parasites through microscopic examination and macrophage-parasite association indices. This study confirmed the ability of macrophages to ingest *T. foetus*, although they did not demonstrate how the activated macrophages destroyed the parasites. These studies supported previous *in vitro* and histological findings that both macrophages and neutrophils have important roles in resistance to infection, although results from our study indicate the mechanism of killing or control of *T. foetus* infection is dependent upon the generation of RNS.

In an early histological study of natural *T. foetus* infection, cows with pyometria and impending abortion had large numbers of trichomonads present throughout the entire reproductive tract that were often associated with neutrophils and macrophages¹¹³. Animals with less severe histopathology and inflammation also had fewer trichomonads present. In another study that examined *T. foetus* within the tissues of aborted fetuses, large numbers of trichomonads were found fragmented inside of macrophages¹²⁴. These previous studies suggest that macrophages and neutrophils are important in initial control of *T. foetus* dissemination throughout the reproductive tract. In support of early histological findings, results presented herein suggest that both neutrophils and the generation of RNS are critical in the control and dissemination of *T. foetus* throughout the reproductive tract. If *T. foetus* is not controlled, abortion and invasion of fetal tissues may occur.

In the current study, *T. foetus* was found in significantly greater numbers within the vagina, uterus, and reproductive tract homogenate in iNOS^{-/-} mice, compared to wild-type immune competent mice. In gp91^{phox}/iNOS^{-/-} mice, elevated levels of parasites were only observed within the homogenates, compared to wild-type C57BL/6 mice. In neutrophil-depleted mice, significant differences in *T. foetus* colonization were only observed in the reproductive tract homogenates, compared to mock-depleted mice. Differences in the ability of the parasites to colonize different parts of the reproductive tract in these various strains of knockout mice may have been due to epithelial cells and region-specific innate defense mechanisms. Epithelial cells most likely play an important

role in disease progression. They can secrete mediators including defensins, cytokines, and chemokines that attract leukocytes to the area of inflammation ¹¹⁷. Bacterial commensals that colonize the vagina may also be important in vaginal defense mechanisms ¹¹⁷, although this is not the case within the uterus. Cervical mucous has powerful antimicrobial activity in the periovulatory phase ¹¹⁷. Toll-like receptor expression within the uterus and vagina differ, which may also result in differential colonization of *T. foetus* throughout regions of the reproductive tract due to different immune activation throughout the reproductive tract. TLRs 1-6 are observed in the fallopian tubes, uterine endometrium, cervix and extocervix and TLR2 levels are high in fallopian tube and cervix, whereas TLR4 expression is highest in vagina ¹¹⁴. Therefore, stimulation of epithelial cells within the vagina may induce a different TLR-mediated response than in uterine or cervix epithelial cells stimulated with the same antigen. These differences may become more apparent due to the lack of RNS and neutrophils.

Interestingly, wild-type macrophages primed with HKTF were more efficient at killing *T. foetus* as compared to similarly treated macrophages from iNOS^{-/-} mice, suggesting HKTF was sufficient to activate wild-type macrophages resulting in killing of *T. foetus*. These results suggest *T. foetus* surface antigens are able to stimulate macrophages, inducing killing of the parasites. *Leishmania* LPG activates NK cells through TLR2 to induce IFN γ and TNF α ⁶. LPG isolated from the surface of *Leishmania* can also stimulate macrophages to produce RNS through the ligation of TLR4⁴⁸. Additionally, *Leishmania* LPG can also induce the synthesis of RNS ¹¹⁶. Since TF-LPG is

similar to LPG from *Leishmania*, antigens on the surface of *T. foetus* could initiate RNS production and facilitate trichomonad killing through ligation with various TLRs.

In summary, we have demonstrated that RNS plays a critical role in controlling dissemination of *T. foetus* throughout the reproductive tract during early infection. We also demonstrate killing of *T. foetus* is dependent upon the production of RNS *in vitro*. In addition, we demonstrate the importance of neutrophil-mediated killing of *T. foetus* in neutrophil-depleted mice. This ability of neutrophils to control initial *T. foetus* infection is not dependent on ROS production, indicated by similar levels of infection in gp91^{phox-/-} mice and with the *in vitro* killing assays.

Understanding the factors involved with initial *T. foetus* infection is critical, as deficiencies can result in the dissemination of trichomonads throughout the reproductive tract and may result in negative pregnancy outcomes, such as damage and inflammation within the reproductive tract. Early innate factors such as RNS and neutrophils help to determine the course of infection, which when absent result in increased trichomonad burden within the reproductive tract. This increase in trichomonad burden and colonization may eventually result in severe disease outcomes, such as abortion and transient sterility.

TARGET CELL-MEDIATED INDUCTION OF PARASITE CYTOTOXICITY
AND SECRETION OF A NOVEL CYSTEINE PROTEASE FROM THE
PROTOZOAN *TRITRICHOMONAS FOETUS*

Introduction

Tritrichomonas foetus (*T. foetus*) is the cause of trichomoniasis in cattle, a sexually transmitted disease that can lead to abortion and substantial economic losses. Little is known regarding the basis of virulence in this parasite, although certain virulence factors (e.g. adhesion, cytotoxicity, protease secretion) may be responsible for the range of inflammation and pathogenicity observed during infection with *T. foetus*. Disease in cattle ranges from a mild asymptomatic infection in bulls, to severe inflammation in the endometrium and uterine tubes¹¹³ temporary infertility, or preterm delivery and abortion in cows¹³. Invasion of fetal tissues has also been observed with gastrointestinal lesions associated with mucosal epithelial injury and detachment, as well as invasion of fetal lungs¹²².

Most studies regarding pathogenesis of *T. foetus* have focused on the role of adhesion and protease-mediated cytotoxicity. It has been observed that *T. foetus* can bind to bovine vaginal and uterine epithelial cells *in vitro*^{41,136,137}. Besides binding to vaginal epithelial cells, it has been shown that the parasites can bind to and are cytotoxic towards various other cell types such as the human cervical cell line, HeLa cells, and an early bovine lymphosarcoma cell line, BL-3²², bovine erythrocytes^{22,132}, Maden Darby canine

kidney cells (MDCK, ^{22,132}), Chinese hamster ovary cells (CHO, ¹⁶), and murine fibroblast cells and a murine macrophage cell line (WEHI 164 and J774.A1, respectively, Higgins et. al, unpublished data). *T. foetus* showed less cytotoxicity towards African green monkey cells (Vero) and Maden Darby bovine kidney cells (MDBK) compared to HeLa ²². In addition, *T. foetus* was unable to kill human vaginal epithelial cells, suggesting *T. foetus* cytotoxicity is species-specific and is in part mediated by receptors that may differ on the various target cell types.

Adherence of *T. foetus* to host cells may play a fundamental role in the parasite's cytotoxicity. Adhesion and cytotoxicity can be mediated by TF190, a 190 kDa LPG-containing moiety on the surface of *T. foetus* ¹²⁹. Antibodies generated against this surface molecule inhibited adhesion and cytotoxicity ^{23,129}, suggesting parasite cytotoxicity is mediated through a contact-dependent mechanism. The extracellular matrix glycoproteins laminin and fibronectin may also be important in parasite adhesion ^{9,25}. It has been suggested that the parasites possess receptors that recognize laminin and fibronectin, which further increase cytotoxicity⁹. Therefore, adhesion may mediate several aspects of cytotoxicity in *T. foetus*; such as acceleration of host/parasite contact, mediation of signaling to initiate various virulence pathways (e.g. secretion of proteases and upregulation of other adhesion molecules), and facilitation of direct damage of host cells.

It is well established that *T. foetus* produces extracellular and intracellular proteases, mostly belonging to the cysteine protease family^{72,95,110}. Studies of these proteases have demonstrated their ability to decrease the integrity of host tissues by

cleavage of extracellular matrix glycoproteins¹⁴⁸ and assist in immune evasion via cleavage of immunoglobulins⁵⁹. To date, proteases mainly of the cysteine family have been identified and characterized, although a 40 kDa cytotoxin has been identified and purified from *T. foetus* that does not appear to be a cysteine protease⁷⁷. Phospholipase C identified from *Trichomonas vaginalis*⁹⁷, the human pathogen that most closely resembles *T. foetus*, can be induced by co-culturing of *T. vaginalis* with HeLa cell monolayers⁹⁷. Since previous studies in which virulence factors were purified from *T. foetus* used unstimulated parasites, novel virulence factors could be identified using stimulated using *T. foetus*.

The goal of this study was to isolate and characterize novel virulence factors from *T. foetus* upon co-culture with target cells. We hypothesized that *T. foetus* cytotoxicity and adhesion are induced by the recognition of various target/host cells and are phenotypically “primed”, resulting in increased cytotoxicity, adhesion, and protein expression. This would in turn increase the likelihood of identification of novel virulence factors. In this study, we demonstrate protein expression is altered in *T. foetus* stimulated by co-culture with either BOMAC cells or HeLa cells. In addition, secretion of cytotoxic substances was increased in stimulated parasites. Thus, we have identified a previously uncharacterized 30 kDa cysteine protease from stimulated parasites that causes target cells to detach and which is eventually cytotoxic towards those cells. We also demonstrate addition of this cysteine protease to target cells results in apoptosis.

Materials and Methods

Tritrichomonas foetus Culture

TFE4, derived from MT85-330.13 originally isolated in 1985, was sorted and cloned based on TF190 epitope expression. TFE4 was maintained in axenic culture at 37°C in complete Diamond's⁴⁶ medium with 10% fetal bovine serum (Atlanta Biologicals) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

Mammalian Target Cells

WEHI 164 cells (a murine fibroblast cell line) and HeLa cells (human cervical carcinoma cell line) (ATCC; American Type Culture Collection) and BOMAC cells (bovine macrophage cell line, Ballantine et. al) were maintained in complete DMEM (CDMEM) with (2 mM L-glutamine, 100 U penicillin, 100 µg streptomycin, 1mM sodium pyruvate (all from Gibco) and 10% fetal bovine serum (Atlanta Biologicals)) at 37°C in 5% CO₂.

Parasite Stimulation

TFE4 were grown to mid log phase (around 5×10^6 parasites/ml), 3 ml of parasite suspension was added to 75 mm flasks with fixed monolayers of BOMAC or HeLa cells (stimulator cells were fixed with 4% buffered formalin for 30 minutes, washed with 200 ml of DPBS, and equilibrated for 10 minutes in complete Diamonds pH 7.2 (prepared as described above)) with 30 ml of complete Diamonds pH 7.2. Control TFE4 were also added to 75 mm flasks without monolayers at the same volumes. Parasites were

incubated at 37°C for 24 or 48 hours. Prior to cytotoxicity and adhesion assays, flasks were gently washed several times to remove adhered parasites from the flask or monolayers and counted using a hemacytometer.

DiI Labeling of Parasites for the Adhesion Assay

T. foetus were washed two times in DPBS, and resuspended to 5×10^6 parasites in 5 ml of DPBS. The cell suspension was labeled with 30 $\mu\text{M}/10^6$ parasites of Vybrant™ DiI (Molecular Probes) for 10 minutes at 37°C. Cells were then washed two times and resuspended in complete DMEM (final concentration of 5×10^6 parasites/ml). Unlabeled parasite controls, treated identically except for the addition of DiI, were also prepared.

Adhesion Assay

WEHI 164 cells were plated into a 96 well plate at a density of 1×10^5 cells/well. WEHI cells were replenished 1 hour before the assay by adding 100 μl fresh complete DMEM. Parasites were added to the wells containing WEHI cells and to wells without target cells in duplicate, at a starting concentration of 5×10^5 cells/well and serially diluted to a final concentration of 1.63×10^4 cells/well. Labeled and unlabeled parasite controls were also added to wells without target cells to measure spontaneous release of the DiI label and parasite background, respectively. After the addition of parasites, plates were incubated 4 hours, allowing for parasite adherence. Wells with parasites adhered to target cells were gently washed with 100 μl warm DPBS 5 times. After the final wash, 100 μl warm DPBS was added to the wells with the addition of an equal volume of acetic

acid solution (used in the cytotoxicity assays) to solublize parasites remaining adhered. Labeled and unlabeled parasite controls were also solublized with an equal volume of acetic acid solution.

Solubilized parasite suspension in a volume of 100 μ l of was transferred to each well of a 96 well plate. The plate was centrifuged for 10 minutes at 1000 x g, and 100 μ l of supernatant was added to the 96 well fluorescent assay plate. Plates were read (with filters for an excitation wavelength of 544 nm and an emission wavelength of 590 nm) on a Fluoroskan Ascent FL instrument (Labsystems).

Standard curves, generated from the extracts of serially diluted DiI-labeled parasites without monolayers, were used to calculate the mean fluorescence/parasite left adhered to the WEHI cells after background was subtracted from the total fluorescent signal.

96 Well Plate Cytotoxicity Assay

WEHI-164 cells were plated at 1×10^5 cells/well in a 96 well plate and allowed to adhere by cultivation at 37°C with 5% CO₂ overnight. Media was replenished with 100 μ l/well of cDMEM before the assay, or wells were washed three times with HBSS and replenished with 100 μ l/well of serum-free cDMEM. Crude or purified CDF was added to the wells at desired concentrations, and plates were incubated at 37°C and 5% CO₂ for 24 hours, followed by washing 3 times with DPBS, and fixation with 4% buffered formalin for 10 minutes. Cells were stained with 0.2% crystal violet solution for 10 minutes. The plates were washed gently with Mili-Q water, and allowed to dry over

night. Samples were solubilized with 100 µl/well acetic acid solution (0.05M acetic acid, 0.5 % SDS) and read on a spectrophotometer (Thermomax Molecular Devices) at A_{550nm}.

Percent cytotoxicity was calculated by using the following equation:

$$\% \text{ Cytotoxicity} = \frac{(\text{Mean OD 550 nM control}) - (\text{Mean OD 550 nM unknown})}{(\text{Mean OD 550 nM control})} \times 100$$

Transwell Cytotoxicity Assays

To determine whether parasites secreted cytotoxic components, WEHI cells were plated at 1 x 10⁶ cells/well in a 24 well plate and incubated at 37°C at 5 % CO₂ overnight. Wells were washed 2 times in warm DPBS followed by the addition of serum free cDMEM to each well. Costar™ Transwell™ plate inserts (24 well plate inserts, pore size 0.4 µm; Fisher Scientific) were added to wells, and allowed to equilibrate for 1 hour. 1 x 10⁷ *T. foetus* (stimulated on target cell monolayers, or unstimulated) in 200 µl serum free cDMEM was added to the plate inserts, and plates were incubated for 24 hours, after which parasites were removed from the inserts, and counted for viability.

Adherent cells were gently washed 3 times with 2 ml of DPBS and fixed for 10 minutes using 500 µl of 4% buffered formalin solution, stained with 250 µl of .2% crystal violet solution for 10 minutes and washed. Stained cells were solubilized with 250 µl acetic acid solution. 100 µl of the supernatant was transferred to a 96 well plate, plated in triplicate and read. Percent cytotoxicity was calculated as previously described.

Protein Extraction of Parasites

Whole *T. foetus* extract was prepared by washing 1×10^7 parasites in DPBS two times, followed by resuspension in 1 ml of extraction buffer (50 mM Tris pH 8, 100 mM NaCl, 5 mM ethylenediaminetetracetic acid (EDTA), 1% Triton-X 100 (Sigma), 100 μ M leupeptin (Sigma), and 10 μ M trans-epoxysuccinyl-L-leucylamido-(4-guanidine) butane (Sigma)). Extracts were incubated on ice for 30 minutes with gentle mixing every 5 minutes. Cellular debris was pelleted (1000 x g at 10°C, 30 min.), the collected supernatant was stored at -20°C, and protein concentrations were determined with the bicinchronic acid protein assay (Pierce Chemicals).

SDS-PAGE and Western Blot

T. foetus extracts were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% T) according to the method of Laemmli⁸⁷. Gel sample patterns were visualized using silver stain (Owl Silver Stain kit, Fisher Scientific) according to manufacturer's instructions.

Preparation of CDF

TFE4 were grown to mid log phase (5×10^6 parasites/ml), 6 ml of parasite suspension was added to 150 mm flasks with fixed monolayers of HeLa cells (stimulator cells were fixed with 4% buffered formalin for 30 minutes, washed with 500 ml of DPBS, and equilibrated for 10 minutes in cDiamonds pH 7.2 (prepared as described above)) with 60 ml of cDiamonds pH 7.2. Flasks were incubated at 37°C for 24 hours

and gently washed several times to remove adherent parasites from the flask or monolayers. Parasites were counted using a hemacytometer and resuspended in aliquots of 1×10^7 parasites. Parasites were washed three times in HBSS, resuspended in 5 ml of HBSS, and incubated at 37°C for 2 H. Parasites were removed from the conditioned media via centrifugation, after which the conditioned medium was pooled and concentrated by centrifugation through a 10,000 molecular weight cutoff filter (Amicon Ultra, Fisher Scientific). CDF was stored in 1 ml aliquots at -20°C until further use.

Chromatography

CDF was purified using gel filtration chromatography with a Superdex 200 10/300 G1 column (Amersham Biosciences). The column was equilibrated with two column volumes of degassed Dulbecco's phosphate-buffered saline (DPBS). Before purification of CDF, the column was calibrated by loading 1 ml of DPBS containing equal concentrations of each molecular weight standard; cytochrome C, carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase onto the column. Eluant flow of 1 ml/min was maintained using BioLogic Duoflow system. Proteins eluting off the column were monitored with a UV absorbance detector set at optical density of 280 nm and BioLogic software that plotted fraction and UV during the run. A standard curve was calculated by plotting standard size versus fraction collected.

After equilibration, CDF was loaded onto the column in 1 ml of DPBS and run over the column at room temperature with 1 column volume of DPBS. Fractions were collected in 0.5 ml volumes. Protein peaks were eluted at fractions 4-8, 17-28, 39-41.

Fractions were then tested for cytotoxic activity using the assay described below.

Sequential fractions with cytotoxic activity were then concentrated by centrifugation through a 10,000 molecular weight cutoff filter (Amicon Ultra, Fisher Scientific).

Purified CDF was stored at 4°C until further use.

Protease Characterization of CDF

CDF activity was characterized by incubation with the fluorogenic cathepsin substrate III Z-RR-AMC beads and protease inhibitors as previously described (North MJ, 1990). Briefly, 10 µg Z-RR-AMC substrate beads (Calbiochem) were added to 10 µl purified CDF with or without indicated amounts of cysteine protease inhibitors; E-64 (*trans*-Epoxy succinyl-L-leucylamido(4-guanidino)butane), TLCK, and Antipain. Beads, CDF, and inhibitors were mixed into 1 ml final volume of reaction buffer with or without DTT (500 mM Tris-HCl, 150 mM NaCl, and 5 mM DTT (only in buffer containing DTT)) (Sigma). Samples were incubated for 10 minutes at room temperature and 200 µl/well was added in triplicate of a 96 well fluorescent assay plate. A kinetic assay was performed on a Fluoroskan Ascent FL instrument (Labsystems) for 5 minutes with readings every 30 seconds. Filters were set to an excitation of 380 nm and an emission of 450 nm.

CDF was heat-inactivated by incubation at 95°C for 5 minutes and added to WEHI cells plated in 100 µl complete serum free cDMEM. Equal volumes of CDF in serum or serum-free conditions were used as positive controls.

Apoptosis Assays

WEHI cells were seeded into wells of 24 well plates at a concentration of 1×10^6 cells/ml. Cells were incubated as previously described for 24 hours. To induce apoptosis as a positive control, 10 μ M camptothecin (Campto) from Sigma, was added to wells. Purified CDF was added to wells and incubated in serum free DMEM for 24 or 48 hours. Cells were then stained for caspase 3 activation and propidium iodide with carboxyfluorescein FLICA apoptosis detection kit caspase assay (Immunochemistry Technologies), according to manufacturer's instructions. WEHI cells gently lifted from wells with trypsin were used as negative controls. Samples were read on a FACSScan flow cytometer (Becton Dickinson) and analyzed using CellQuest software (BD Pharmingen).

Statistics

One-way ANOVA with a Newman-Keuls Multiple Comparison test was performed using Prism™ version 3 (GraphPad Software).

Results

T. foetus Induction of Cytotoxicity and Adhesion After Incubation Upon Monolayers:

Parasites were stimulated upon fixed monolayers of either HeLa or BOMAC cells for 24 or 48 hours. After incubation upon monolayers, an increase in adhesion of parasites to WEHI target cells was observed, compared to parasites incubated in similar

conditions without a monolayer of adhered cells. Increases in adhesion were initially observed for parasites incubated on HeLa cells for 24 hours, although this was not observed for parasites incubated on BOMAC cells (Figure 4.1A) compared to unstimulated parasites. There were more *T. foetus* parasites adhered to WEHI cells after 48 hours of culture on fixed BOMAC monolayers compared to parasites incubated with HeLa cells (Figure 4.1B). Adhesion did not seem to be cell-specific, as parasites cultured upon HeLa or BOMAC cell lines could readily adhere to WEHI target cells.

T. foetus-induced cytotoxicity towards WEHI target cells was also increased after 48 hours. After 24 hours of incubation on HeLa target cells, parasites were less cytotoxic towards WEHI cells compared to unstimulated parasites and parasites stimulated upon BOMAC target cells (Figure 4.2A). Additionally, cytotoxicity was not enhanced from parasites incubated upon BOMAC monolayers for 24 hours (Figure 4.2A). After 48 hours of incubation upon monolayers of either HeLa or BOMAC target cells, there was increased cytotoxicity compared to unstimulated parasites (Figure 4.2B). Parasites were able to kill nearly 100 % WEHI target cells even at very low starting dilutions of trichomonads. These data indicate that parasites are stimulated to increase adherence and cytotoxicity after incubation upon fixed monolayers of target cells. Enhanced adhesion and cytotoxicity was not cell-type specific, as parasites stimulated upon either HeLa or BOMAC cells were able to adhere and kill WEHI target cells.

Induction of Protein Expression was Observed After Stimulation of Parasites Upon Monolayers

Because stimulation of parasites enhanced parasite-mediated adhesion and cytotoxicity, we reasoned this could be due to an induction of protein expression. Equal concentrations of protein extracts from parasites incubated without monolayers or with fixed monolayers of HeLa or BOMAC cells were resolved by SDS-page gel electrophoresis. Several bands of various molecular weights were present in the lanes of parasites incubated upon the monolayers (designated by red arrows) compared to unstimulated parasites (Figure 4.3). There were also high molecular weight proteins present in the protein extracts from unstimulated parasites not observed in the lanes of stimulated parasites (designated by blue arrows, Figure 4.3). Contribution of protein contaminants from fixed monolayers was minimal; verified microscopically and with multiple washing and gentle centrifugation of live and intact parasites. These results indicate there are changes in protein expression when parasites are incubated upon monolayers. These changes in protein expression are supported by the increased adhesion and cytotoxicity observed after parasites are incubated upon monolayers for 24 and 48 hours.

Induction of Soluble Cytoactive Factors After Incubation Upon Target Cells

Because a change in protein expression was observed after stimulation upon monolayers, parasites stimulated upon HeLa monolayers were incubated in transwell filters. Cytotoxicity of stimulated parasites was compared to unstimulated parasites. The

goal was to determine whether incubation upon monolayers increases secretion of factors that are cytotoxic to the WEHI target cells on the opposing side of the filters. There was a significant increase in cytotoxicity towards WEHI cells due to secretion of cytoactive factors after parasites were activated on HeLa target cells (Figure 4.4, $p = .0016$).

In order to isolate cytotoxic factors responsible for increased cytotoxicity towards WEHI cells in the transwell assay, parasite-conditioned medium was prepared as described in Materials and Methods. Purified conditioned medium contained cytoactive factor/factors that caused rounding and detachment of WEHI cells within 24 hours of exposure (Figure 4.5B) and was subsequently named cell-detaching factor (CDF). Killing of WEHI cells was observed within 24 hours, and resulted in 100% cytotoxicity after 74 hours of exposure (Figure 4.5C). These results indicate activation of a secreted cytoactive substance after culture of parasites upon monolayers of HeLa cells. In addition, these data indicate parasite mediated cytotoxicity upon target cells is contact dependant. However, after parasites establish contact, they are more cytotoxic than parasites that have not had prior contact to target cells. Parasite cytotoxicity is most likely facilitated by the secretion of cytoactive factors.

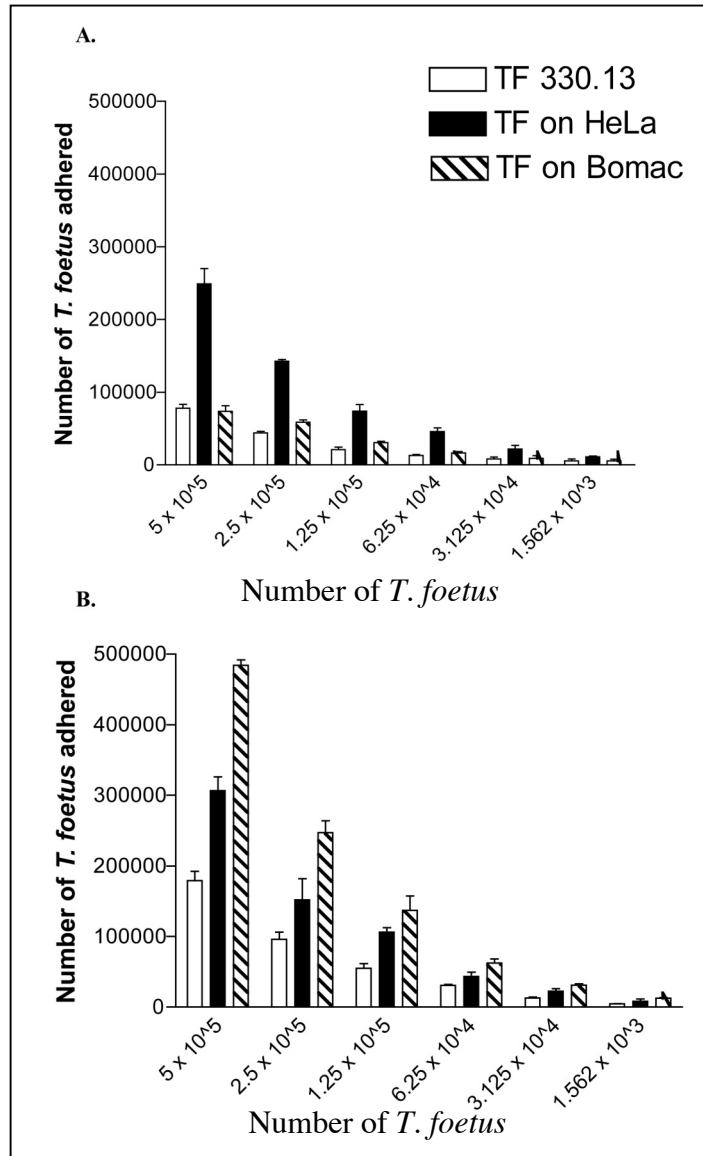


Figure 4.1: Induction of *T. foetus* adhesion to WEHI cells after incubation on fixed monolayers of HeLa or BOMAC monolayers. A: Adhesion after 24 hours of incubation. B: Adhesion after 48 hours incubation.

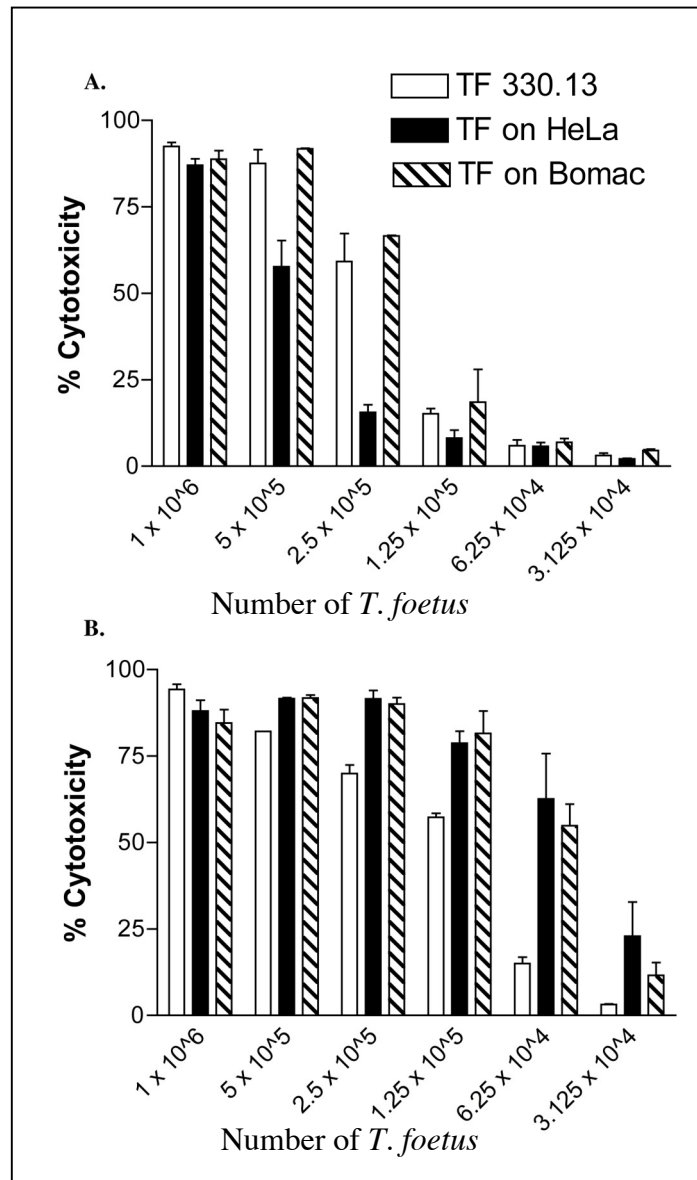


Figure 4.2: Induction of *T. foetus* cytotoxicity to WEHI cells after incubation upon HeLa or BOMAC monolayers. A: 24 hours after incubation on monolayers. B: 48 hours after incubation on monolayers.

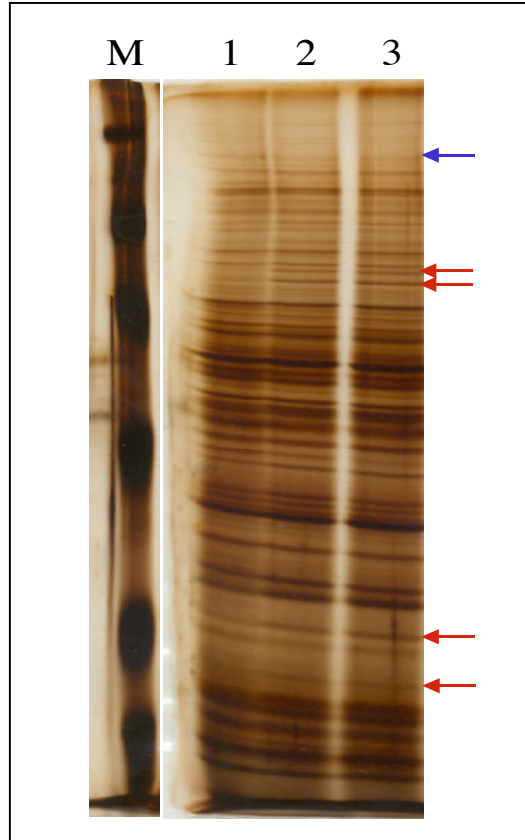


Figure 4.3: Change in *T. foetus* protein expression after incubation upon HeLa (lane 2) or BOMAC (lane 3) monolayers. Lane 1: unstimulated parasites. Equal amounts of protein were loaded onto the gel. Red arrows: proteins that are up-regulated. Blue arrow: protein that is down-regulated.

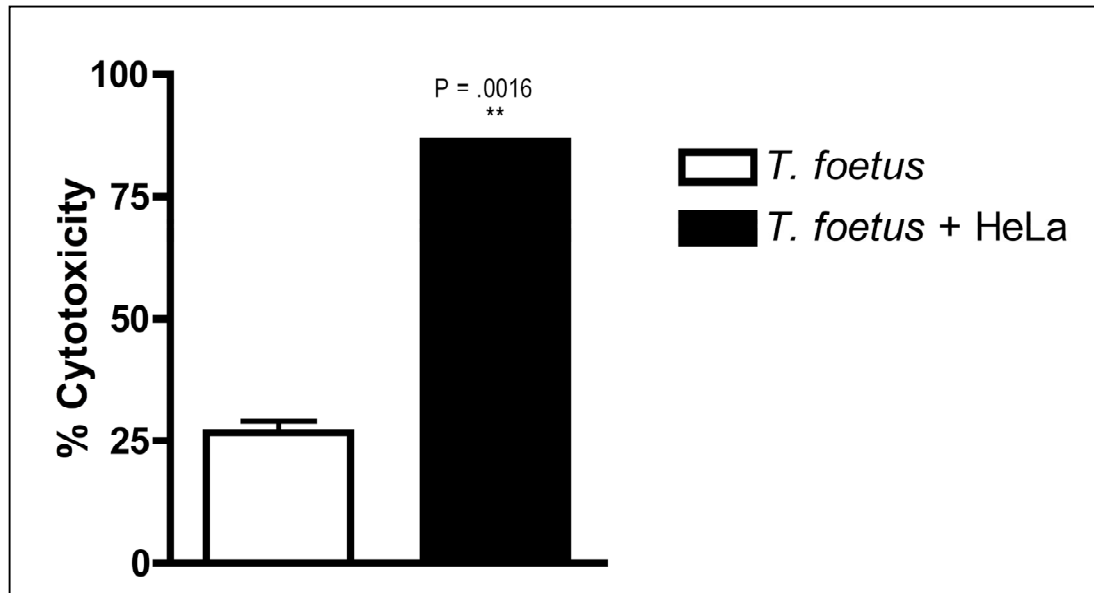


Figure 4.4: Incubation of *T. foetus* upon HeLa monolayers results in secretion of a cytoactive factor that results in cell death for WEHI target cells.

Purification of a 30 kDa Cysteine Protease From the Secreted Cytoactive Factor

Because parasite-conditioned medium contained parasite-secreted factors capable of inducing epithelial cell death, we reasoned fractionation of parasite-conditioned medium over a size exclusion column could result in isolation of novel virulence factors. Parasite-conditioned medium was prepared as described and fractionated by size exclusion chromatography. Protein peaks were recovered from fractions 4-8, 17-28, and 39-41 (Figure 4.6A). The broad middle peak of protein yielded recoverable cytotoxicity within fractions 24-28 (Figure 4.6B). Based upon column calibration curves (data not shown) and SDS-PAGE of fractions 24-28 (Figure 4.7), the molecular weight of the isolated substance was approximately 30 kDa.

The cytotoxic factor was a protein and was inactivated by heating at 95°C for 5 minutes (Figure 4.8). In addition, incubation of the purified protein with fluorescent cathepsin substrate III beads, in the presence or absence of DTT, indicated the protein was activated by DTT, suggesting it was a cysteine protease. Incubation of the purified protein in the presence of varying concentrations of inhibitors E-64, Antipain, or TLCK resulted in diminished cleavage of the cathepsin substrate beads (Figure 4.9A-C), further demonstrating the protein was a cysteine protease. These results indicate activation of *T. foetus* upon HeLa cells results in the increased expression of a novel secreted 30 kDa cysteine protease.

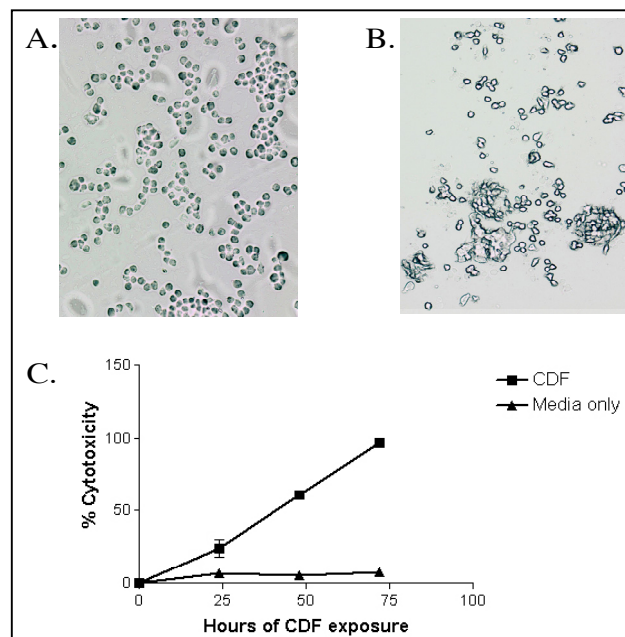


Figure 4.5: Partial purification of parasite conditioned media from *T. foetus* incubated upon HeLa monolayers for 24 hours. The soluble cytoactive factor (CDF) causes rounding, detachment, and eventually death to WEHI target cells. A: WEHI cells only. B: WEHI cells after 24 hours exposure to cytoactive factor. C: Exposure of WEHI cells to CDF results in 100% cell death after 74 hours. Cells were incubated in serum free medium.

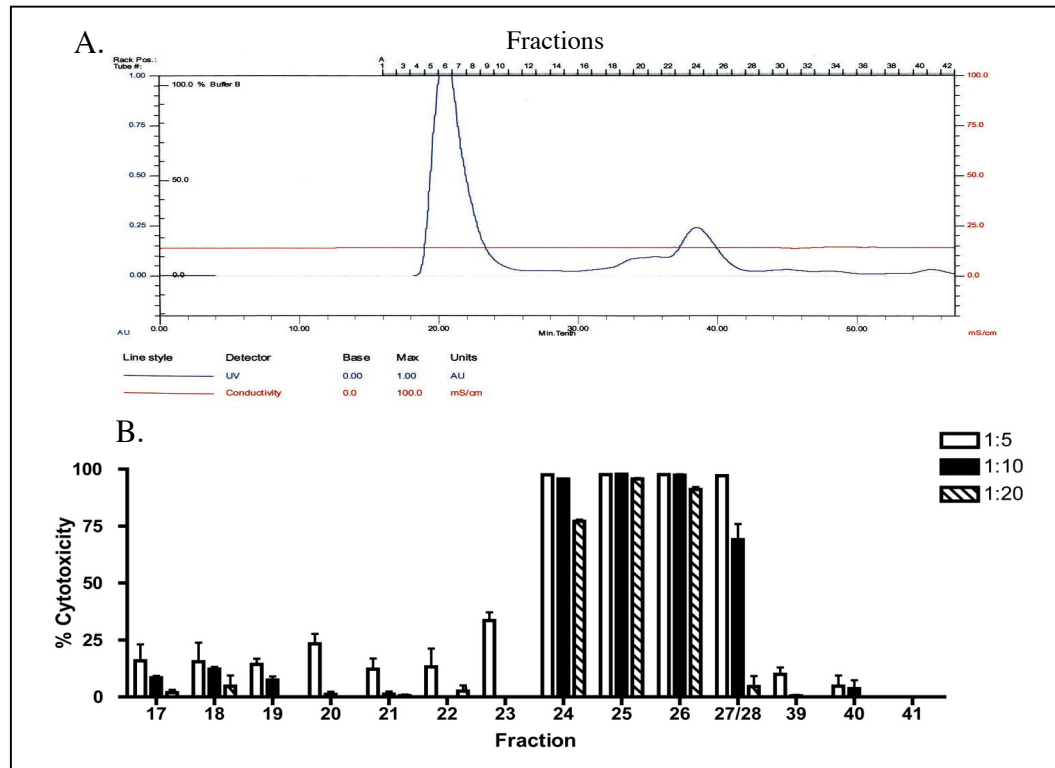


Figure 4.6: Purification of a 30 kDa protein from conditioned medium of *T. foetus* incubated upon HeLa monolayers. A: Protein fraction peaks of CDF eluted off the column. B: Cytotoxicity of each diluted fraction upon WEHI target cells. WEHI cells were incubated for 24 hours in serum free media in the presence of 10 μ l of each fraction diluted as indicated in the legend.

CDF Induces of Apoptosis to WEHI Cells

Purified CDF was applied to WEHI target cells for 24 or 48 hours in serum-free culture conditions. After each time-point, cells were stained with an antibody against activated caspase 3 (FLICA) and propidium iodide. Compared to camptothecin-treated cells, which is a positive control for apoptosis induction, CDF induced similar amounts of

FLICA staining. This indicated caspase 3 activation of WEHI cells treated with CDF, illustrating CDF-induced apoptosis of epithelial cells.

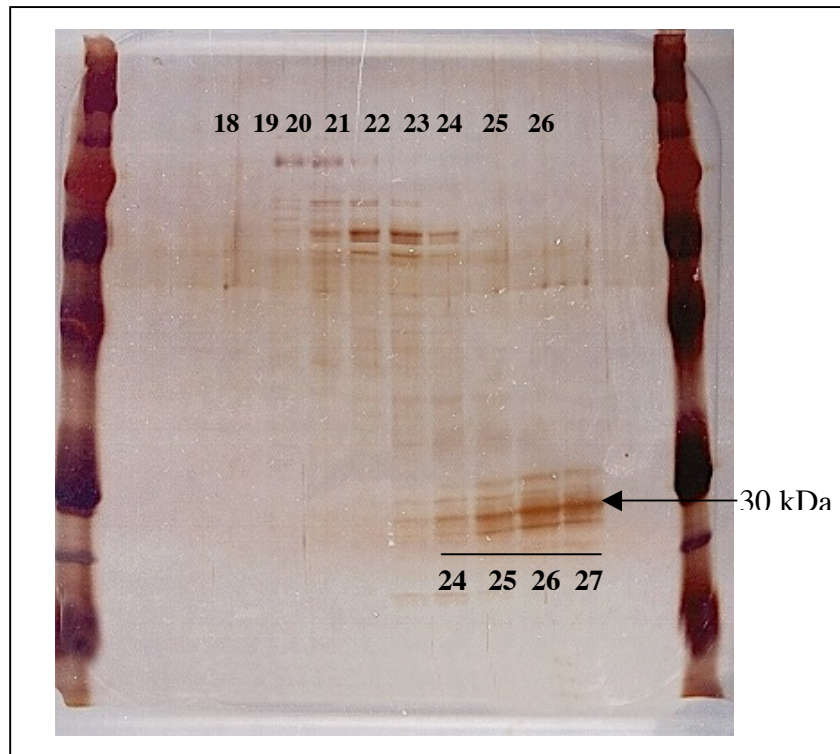


Figure 4.7: SDS-PAGE gel electrophoresis of fractions recovered from biologic column. Fractions 24-27, which had the most cytotoxic activity, had sharp bands at a molecular weight of 30 kDa, depicted by arrow. This indicates CDF activity was partially purified and concentrated to a molecular weight of 30 kDa.

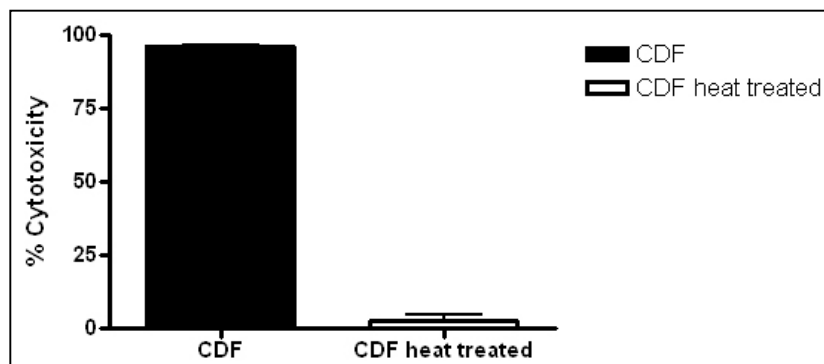


Figure 4.8: Heat inactivation of purified CDF, indicating soluble factor is a heat labile protein.

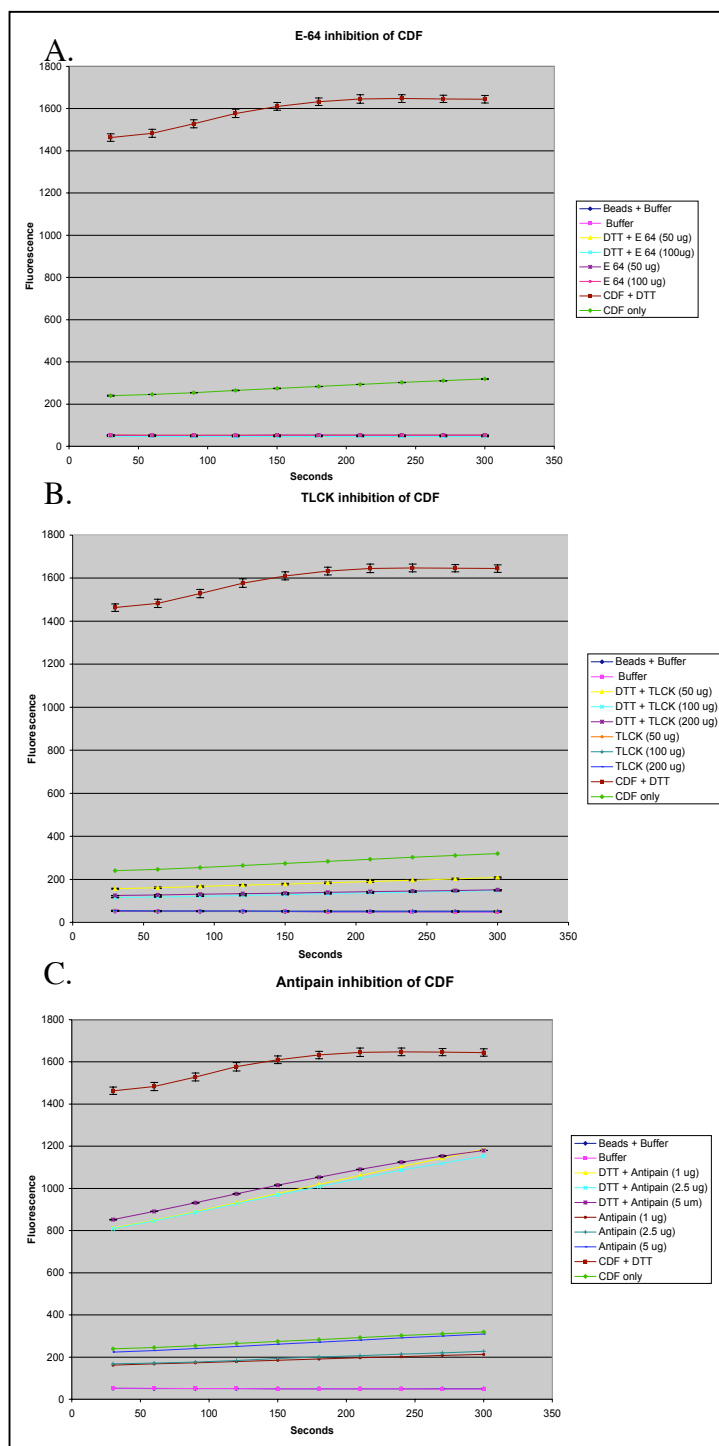


Figure 4.9: Incubation of CDF with Z-RR-AMC cathepsin substrate III fluorescent beads, DTT, and protease inhibitors indicate purified CDF is a cysteine protease. A: Incubation with CDF and E-64. B: Incubation of CDF with TLCK. C: Incubation of CDF with Antipain.

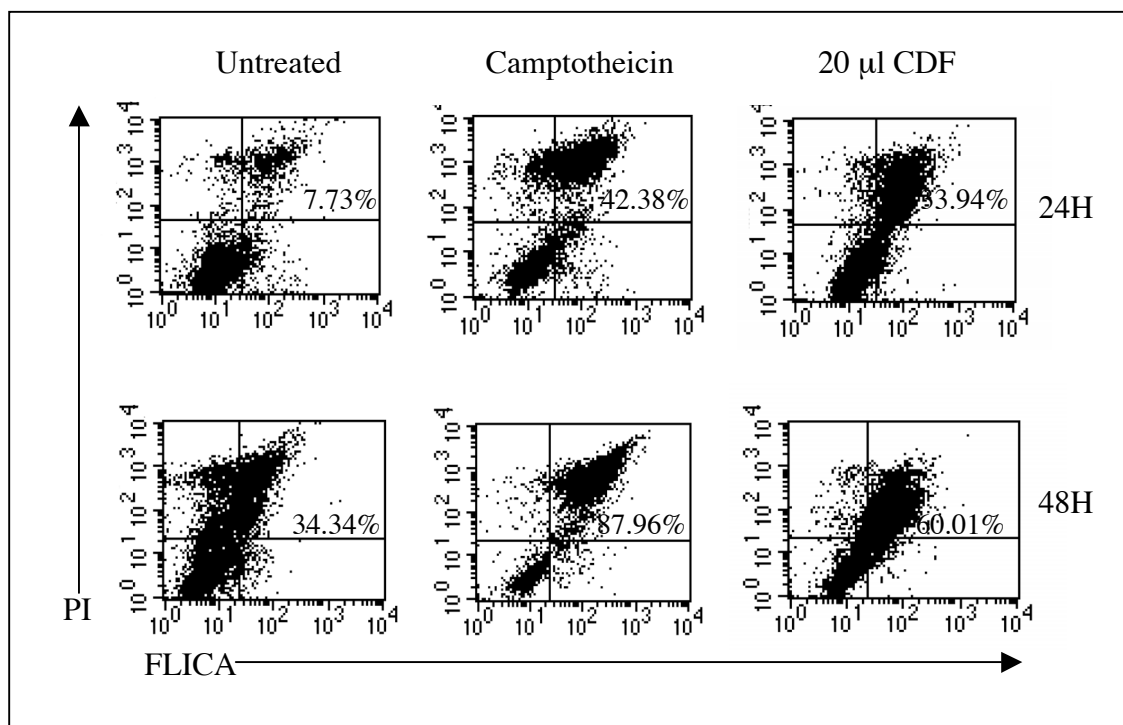


Figure 4.10: CDF induction of apoptosis after 24 and 48 hours exposure to WEHI cells. CDF induced similar intensities of FLICA staining (activated caspase 3) and propidium iodide (PI) staining than camptothecin treated WEHI cells.

Induction of apoptosis was rapid, and occurred within 24 hours of treatment of cells with CDF. This was similar to the effects of camptothecin upon the WEHI target cells. Apoptosis was increased from 7.73% in untreated cells to 42.38 and 33.94% in camptothecin-treated and CDF-treated cells, respectively (Figure 4.10). In addition, after 48 hours of treatment in serum free medium, 34.34% of untreated WEHI cells stained for both PI and FLICA whereas camptothecin-treated and CDF-treated cells had 87.96% and 60.01% double stained cells (Figure 4.10). Thus, CDF-induced apoptosis increased with time of incubation and perhaps reached a peak at 74 hours, as demonstrated in Figure 4.5.

Discussion

It is well established that *T. foetus* produces several intracellular and secreted proteases, mainly belonging to the cysteine proteinase family¹¹⁰. In this study we purified and characterized a novel cysteine proteinase derived from parasites that had been stimulated upon fixed monolayers of cells. Activation of parasites resulted in increased adhesion and epithelial cytotoxicity, presumably due to increased secretion of cytoactive factors. We demonstrate alteration of protein expression when parasites are stimulated upon monolayers upon either HeLa or BOMAC cells, and the resulting increase in cytotoxicity is not cell specific, as parasites activated upon both human cervical carcinoma cells and a bovine macrophage cell line were cytotoxic towards a murine fibroblast cell line.

Initial reports involving adhesion-mediated cytotoxicity of *T. foetus* suggested parasite-mediated cytotoxicity was contact dependent, as parasites incubated in chambers separate from monolayers were unable to kill the target cells underneath²³. Additionally, it has previously been shown antibodies directed against surface adhesions of *T. foetus* inhibited cytotoxicity towards bovine vaginal epithelial cells⁴¹ and towards HeLa target cells²³. Results from this study support previous reports, although we demonstrate that in order to maximize killing and detachment of target cells, parasites need to be stimulated upon target cells initially.

In our studies, stimulation of parasites was critical for secretion of cytoactive factors that we demonstrate are able to kill target cells, contrary to the reports by Corbeil

et al.⁴¹ and Burgess et al.²³. Reasons for this discrepancy are most likely attributed to the fact that parasites weren't exposed to monolayers, or activated, before the transwell or antibody experiments. Therefore, parasite-mediated cytotoxicity was contact-dependent in that parasites needed to be exposed to target cells in order to maximize cytotoxicity towards target cells. After initial contact was made, an up-regulation of virulence factors occurred such as epithelial adhesion and cytotoxicity. No further adhesion towards the surface was needed in order to induce killing of the target cells. An early study that examined *T. foetus* interaction towards epithelial cells using microscopy indicated parasites treated with trypsin were unable to adhere to the host cells, although parasites were still able to damage the cells²⁵. The authors suggested both adhesion and secretion of factors was responsible for damage towards target cells.

T. foetus adhesion to epithelial or immortalized cells most likely results in an alteration in gene expression. A laminin-binding protein was detected on the surface of both *T. foetus* and *T. vaginalis*, and based upon cytotoxicity assays with laminin-specific antibodies, the authors speculated that levels of laminin-binding protein on the surface of trichomonads correlated to cytotoxicity towards host cells²⁵. It was later shown that binding of *T. foetus* to laminin globular domains induces metalloprotease activity¹³³, which in reduced iron conditions, results in diminished ability of parasites to adhere to and kill target cells¹⁰⁶. These results suggest binding of *T. foetus* to laminin induces signals important to initial colonization and subsequent cytotoxicity within the host.

Perhaps laminin binding proteins upon the surface of *T. foetus* induce signals that induce cytotoxicity or protein expression and facilitate colonization of host epithelium.

Early studies examining the extracellular endopeptidases of trichomonads indicated they were mainly cysteine proteases with a broad range of specificities^{95,110} although putative virulence factors have been identified that were not cysteine proteases⁷⁷. The cysteine proteinase was most likely similar to the sequenced TFECP, a 31 kDa cysteine proteinase with sequence similarities towards two *T. foetus* cysteine proteinase genes TFCP1 and TFCP2¹⁵⁰. Although this proteinase has been previously sequenced, its specific activity has not been elucidated. In this report we demonstrate that purified CDF is able to cause detachment of target cells in serum free conditions, which eventually results in cell death. A CDF has been identified from *Trichomonas vaginalis*, the human pathogen closely related to *T. foetus*, although TV-CDF was unable to cause cell death to target cells⁵⁴.

Our results suggest that CDF is a cysteine proteinase that is able to induce detachment of adherent target cells, resulting in rounding and sloughing of the cells and eventually death. This could be a possible virulence mechanism, as invasion of the reproductive tract and subsequent colonization are dependent upon destruction of mucous layer in addition to adhesion to epithelium⁹⁰. Histological studies have indicated trichomonads are able to cause epithelial damage to the epithelium of the uterus, although this has not been observed within the vagina¹¹³. In addition, *T. foetus* has been shown to be tissue invasive causing significant damage within fetal lungs, intestines, the liver, and

the brain ¹²². Little information is available regarding invasion of mucosal tissues.

Perhaps secretion of CDF is enhanced after binding upon the host epithelium, resulting in tissue destruction and trichomonad invasion of the uterine endometrium. Trichomonad invasion of the reproductive tract then induces inflammation, resulting in abortion and transient sterility.

Induction of epithelial cell apoptosis by CDF indicates a strategy that *T. foetus* may employ in order to colonize the reproductive tract without eliciting a strong inflammatory response. Apoptosis may result in immune evasion while tissue damage is occurring. Necrosis results in rapid destruction of host cells including the release of intracellular proteases and other cellular products. Macrophages respond to clean up the debris in order to prevent further tissue destruction due to the release of intracellular proteases. Recently, other investigators purified a cysteine proteinase with an approximate molecular weight of 30 kDa that was able to induce rapid apoptosis upon bovine vaginal epithelial cells ¹³⁷. Although sequence analysis is not available for the CDF characterized in this report, characteristics between the two 30 kDa cysteine proteases suggest they are the same or similar proteases causing apoptosis directly to target cells. Although the study in 2004 did not establish how the cysteine proteinase caused apoptosis, results in this report indicate this activity is enhanced upon stimulation of target cell monolayers.

In summary, we have identified a cysteine proteinase with a relative molecular weight of 30 kDa. This cysteine proteinase, termed CDF, was able to cause rounding and

detachment of target cells, resulting in cell death within 74 hours. Additionally, it was determined that mechanism whereby CDF caused cell death was apoptosis. This study helps illustrate how initial interaction of *T. foetus* with target cells induces a change in the parasite's ability to kill target cells, one of which is secretion of CDF that induces apoptosis in target cells. Induction of apoptosis may be a mechanism whereby *T. foetus* colonizes and invades the host epithelium without causing an early inflammatory response. Advantages to this mechanism of cell death include the ability to sufficiently colonize the host without interference from host innate defenses, such as macrophage and neutrophil infiltration.

SUMMARY AND CONCLUSIONS

Despite the availability of artificial insemination, trichomoniasis still remains a disease of veterinary relevance. A large body of literature has focused upon virulence factors of *T. foetus* in addition to the acquired immune response against this pathogen. Studies in the murine model have indicated early host-parasite interactions may dictate the course and eventual outcome of disease. A robust inflammatory response can control the dissemination of *T. foetus* throughout the reproductive tract, although it can be detrimental to the fecundity of the cattle. Conversely, parasites may evade the host immune response, resulting in chronic infection of the host. Chronic infection may result in infection of virgin bulls during the next breeding season. An understanding of the events during initial colonization may result in increased methods to control the spread of *T. foetus* throughout the herd.

The goal of chapter two was to determine whether conditions that directly affect the environment within the female reproductive tract alter the ability of *T. foetus* to establish infection and change the subsequent disease progression. Acute *T. foetus* infection was compared in female BALB/c mice to infections in mice treated with either high doses of estradiol or housed in constant bright illumination (stressed). In untreated mice, *T. foetus* readily colonized the reproductive tract although colonization resulted in little epithelial damage, inflammation, or cytokine expression. Infection in estradiol-treated or stressed mice resulted in increased tissue damage, inflammation, and

inflammatory cytokine expression. However, estradiol-treatment or stress did not result in enhanced *T. foetus* colonization within the reproductive tract. Additionally, estradiol-treatment of mice resulted in reduced *T. foetus* colonization compared to untreated mice. These results indicate infection during estradiol-treatment and stress cause the pathogenesis often observed during severe cases of trichomoniasis, such as epithelial damage and inflammation. Infection in non-treated mice results in chronic colonization with little inflammation or pathology.

Studies in chapter three set out to address which components of the innate immune response are critical in the control of early establishment of infection with *T. foetus*. Infection with *T. foetus* and parasite burden within the vagina and uterus of IFN γ ^{-/-}, CXCR2, gp91^{phox-/-}, gp91^{phox-/-}/iNOS^{-/-}, and iNOS^{-/-} mice as well as in SCID and RAG^{-/-} mice was compared. In addition, mice depleted of neutrophils were used to examine the role of neutrophils within the reproductive tract in control of *T. foetus* infection. In addition, *T. foetus* killing with bone marrow-derived neutrophils and thioglycolate-elicited peritoneal macrophages from mice with defects in ROS and RNS production was examined, to determine whether these oxidative metabolites were important in elimination of *T. foetus* during early infection. Results indicated neutrophils were important for controlling initial establishment and colonization of the reproductive tract.

Mice without the ability to produce RNS were also more susceptible to infection with *T. foetus*, compared to wild-type control mice. Peritoneal macrophages from iNOS^{-/-}

and gp91^{phox}/iNOS^{-/-} mice were unable to kill *T. foetus*, whereas iNOS-stimulated wild-type or gp91^{phox}^{-/-} macrophages were able to kill *T. foetus*. The ability of neutrophils or macrophages to kill *T. foetus* was not dependent upon ROS production, as unstimulated wild-type and gp91^{phox}^{-/-} macrophages and wild-type macrophages stimulated to generate ROS were also unable to kill *T. foetus*.

Since *T. foetus* depends upon the host for a large amount of metabolic molecules, *T. foetus* most likely closely interacts with host cells. Such interactions would result in increased virulence of *T. foetus*. The goal of chapter four was to examine the interaction of *T. foetus* to target epithelial cells. After the parasites were exposed to target epithelium, protein expression was altered in addition to increased adhesion and cytotoxicity towards host cells. In addition, *T. foetus* stimulated upon epithelial cells had increased secretion of cytoactive factors, which when partially purified and concentrated resulted in rounding, detachment, and eventually death of target cells. A novel 30 kDa cysteine protease was further purified from the secreted cytoactive factors that induced apoptosis upon WEHI cells within 48 hours of exposure to the cells.

The data in this report support the hypothesis that initial parasite/host cell interactions affect trichomonad virulence, as *T. foetus* virulence (adhesion and cytotoxicity) was increased after exposure to target epithelium. In addition, innate host-responses directly affect the ability of *T. foetus* to colonize the reproductive tract. Specifically, neutrophils and the generation of RNS diminish parasite numbers within the reproductive tract during early colonization. Lastly, factors that directly affect the

reproductive environment also directly affect *T. foetus* colonization and disease pathology. Estradiol-treated and stressed mice infected with *T. foetus* had increased tissue damage and inflammatory cytokine expression and neutrophil accumulation.

In summary, this report demonstrates how factors such as estradiol-treatment and stress enhance pathogenesis of *T. foetus* infection. Results also show that neutrophils and the generation of RNS act as innate immune-mediators in the control of *T. foetus* dissemination throughout the reproductive tract. If neutrophils or the generation of RNS does not kill *T. foetus*, interactions with host epithelium will result in an induction of virulence. This induction of virulence will result in the secretion of cysteine proteases that induce apoptosis, detaching, and cell death to target cells. Altogether, this interaction will enhance colonization and most likely shape the course of the disease.

FUTURE STUDIES

Since both exposure to host epithelium and estradiol-treatment and stress result in cell death and apoptosis, it will be of great interest to determine whether these results are due to the secretion of the 30 kDa cysteine proteinase characterized in chapter four. Specifically, it would be interesting to associate estradiol-treatment or stressing the mice during infection to increased activation and secretion of the CDF. Infecting mice *in vivo* and staining parasites directly with an anti-CDF antibody would be an approach to defining the relationship between activation and CDF secretion. Immunohistochemistry of tissue sections from infected mice would be another strategy to define CDF mechanism of virulence in a murine infection. If CDF staining co-localized with stained parasites or was significantly associated with tissue damage or apoptosis, it would be logical to conclude that CDF plays a direct role in the virulence of *T. foetus*. In addition, it could be concluded that stress and/or estradiol-treatment enhance secretion and deposition of CDF within the reproductive tract tissues.

Since chapter three identified two innate mechanisms whereby parasites are controlled by killing, it would be informative to determine how the neutrophils are killing the parasites. Is it due to the generation of peroxynitrite or the neutrophil's need for anti-apoptotic signals such as GM-CSF to induce parasite-mediated killing? A co-culture of isolated neutrophils and murine epithelial cells could be developed in order to systematically define the cells or co-factors involved in neutrophil/trichomonad control. Also it would be interesting to determine whether purified surface moieties from *T. foetus*

directly activate macrophages to produce RNS. Can trichomonads activate Toll-like receptors? *T. foetus* establishment of infection throughout the female reproductive tract may depend upon the TLR expression upon the epithelium throughout the entire tract. It would also be important to define which surface antigens are able to stimulate innate cells through TLR engagement.

Since infection in normally cycling mice results in a chronic infection with high trichomonad burden and little tissue damage and inflammation, it is possible the parasites suppress the local immune response. A uterine inflammation model could be developed by injection of individual murine uteri with inflammatory agents such as LPS to determine whether *T. foetus* can suppress inflammation. Immune suppressive cytokines such as TGF- β or other TH2 type cytokines could also be examined. Infection in estradiol-treated and stressed mice resulted in an increase of regulatory T cells into the reproductive tract. It would be important to define the role regulatory T cells have in the control of trichomoniasis, especially during severe inflammation and tissue damage.

These studies would help to clarify the relationship between host and early trichomonad colonization. Learning how *T. foetus* can bypass innate barriers of the female reproductive tract would be important in designing therapeutic measures that effectively treat trichomoniasis. Additional information regarding early colonization may clarify the factors that influence the course of trichomoniasis and result in severe pathologic outcomes such as abortion and transient sterility.

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