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Trichomonas vaginalis Induced Toll-like Receptor Gene Expression in Cervical Epithelial Cells

Research Hypothesis

During the 2021 Lamar University Summer Undergraduate Research program we were able to determine gene expression of Toll-Like receptors in cervical epithelial cells, understand cytokine expression, compare differences in cytokine responses, analyze TLR activation, study innate immune responses induced by *Trichomonas vaginalis*, and understand the pathways that could provide potential targets against *Trichomonas* infections for chemotherapy.

Data Analysis - Development of the Project

Parasites

Trichomonas vaginalis is a pear or circular shaped unicellular protozoan contracted through venereal transmission during sexual contact in intercourse and causes the Trichomoniasis infection in their definitive host – humans (Schumann and Plasner, 2020; Shiadeh, *et al*., 2016). Extracellular trichomonas adheres to the vaginal

epithelium which disintegrates the tissue creating an immune response while also exponentially reproducing by longitudinal binary fission, affecting both the vaginal microbiota and immunity of the host (CDC, 2017; Mercer and Johnson, 2018). The nutrients *Trichomonas vaginalis* needs for survival, vaginal glycogen, are taken by feeding on the epithelial tissue by the trogocytosis mechanism where the protozoan nibbles or will completely phagocytosize the lymphoid cells (Coleman, *et al*., 2013; Mercer and Johnson, 2018). These pathogens are categorized by four flagella which appear to be amphitrichous, however are lophotrichously flagellated with an axostyle, ranging in size of 5 micrometers to 32 micrometers. Unlike organisms with mitochondria, trichomonas has hydrogenosomes creating their ATP and hydrogen, and *Trichomonas vaginalis* is unable to survive outside of a human host as there are no existing intermediate host (Shiadeh, *et al*., 2016). In the United States trichomonas is the highest transmitted sexually transmitted parasite that is not a virus (Tsevat, *et al*., 2017). 7.4 million cases are diagnosed every single year and given its' name, *Trichomonas vaginalis*, infects mostly women, but also infects men, and in both cases most times, one in every two women, the host is asymptomatic (Coleman, *et al*., 2013; Gunn and Pitt, 2012; Mercer and Johnson, 2018). As a self-diagnosis, change in discharge color as in green or unusual textures may indicate the need for further inspection by a gynecologist or urologist to diagnose the infection. For clinical diagnostic testing there are three main tests: one – during a pelvic exam, a swab will collect specimen culture from the infected person to be viewed on a wet-mount with light microscopy, 2 - rapid antigen testing, and 3 – nucleic acid molecular tests (Coleman, *et al*., 2013). Unfortunately, because the host does not know they are infected the parasite infection becomes chronic, which leads to further long-term issues. *Trichomonas vaginalis* has a relation with other sexually transmitted diseases such as a proneness to Human Immunodeficiency Virus and other additional risks include having other sexually transmitted diseases, multiple sex partners, or misusing intravenous drugs

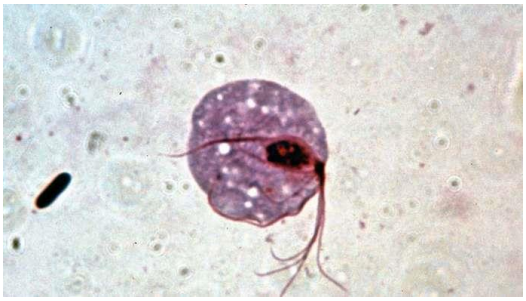


Figure 1. *Trichomonas vaginalis* captured from Britannica (Leu, A. L., n.d.).

(Shiadeh, *et al*., 2016). In the genital tract trichomonas can cause deformities, cervical neoplasia which are new masses on the cervix, and inflammation of the uterus and vagina in women causing pelvic inflammatory disease (Coleman, *et al*., 2013). The protozoan can also infect the upper genital tract leading with 30% giving salpingitis, or inflammation in the fallopian tubes, in women (Tsevat, *et al*., 2017). During pregnancy, the trichomonas parasite can cause low birth weight as well as rupture of the membranes resulting in premature delivery (Shiadeh, *et al*., 2016). Infertility in women with a history of *Trichomonas vaginalis* increase the likelihood of infertility twice as much (Gunn and Pitt, 2012) and treatment to manage the protozoan is with either of the oral metronidazole, which cures nine of ten patients, or tinidazole curing closer to ten of ten patients. Prevention includes protected sex and monogamous sexual relations (Shiadeh, *et al*., 2016). During pregnancy metronidazole medicated treatment is given, and the prescription is not found to correlate with congenital deformities yet is more closely monitored (Coleman, *et al*., 2013). *Trichomonas vaginalis* elicits host inflammatory responses that promotes cell proliferation and dysregulation of cell cycle.

Toll-like Receptors

Toll-like receptors or Innate Immune System receptors identify pathogens, to determine the molecular mechanism for host defense. There are intracellular and extracellular, which are located on the plasma membrane, receptors to focus on a response and defend the host. TLR 1, 2, 4, 5, 6 and 10 are within the cell, while TLR's 3, 7, 8, and 9 are located externally. Because Trichomonas vaginalis is located outside the vaginal epithelium and most pathogenic protozoans correlate with TLR 4, we chose to focus on Toll-like receptor 4.

Experiment

Several cell cultures, molecular and immunological techniques such as establishing parasite and HeLa cell cultures, co-culturing Parasites and Human Cell Lines (Interaction), Polymerase Chain Reactions, Reverse Transcription-Polymerase Chain Reaction, gel electrophoresis, proinflammatory cytokine response, measurement of

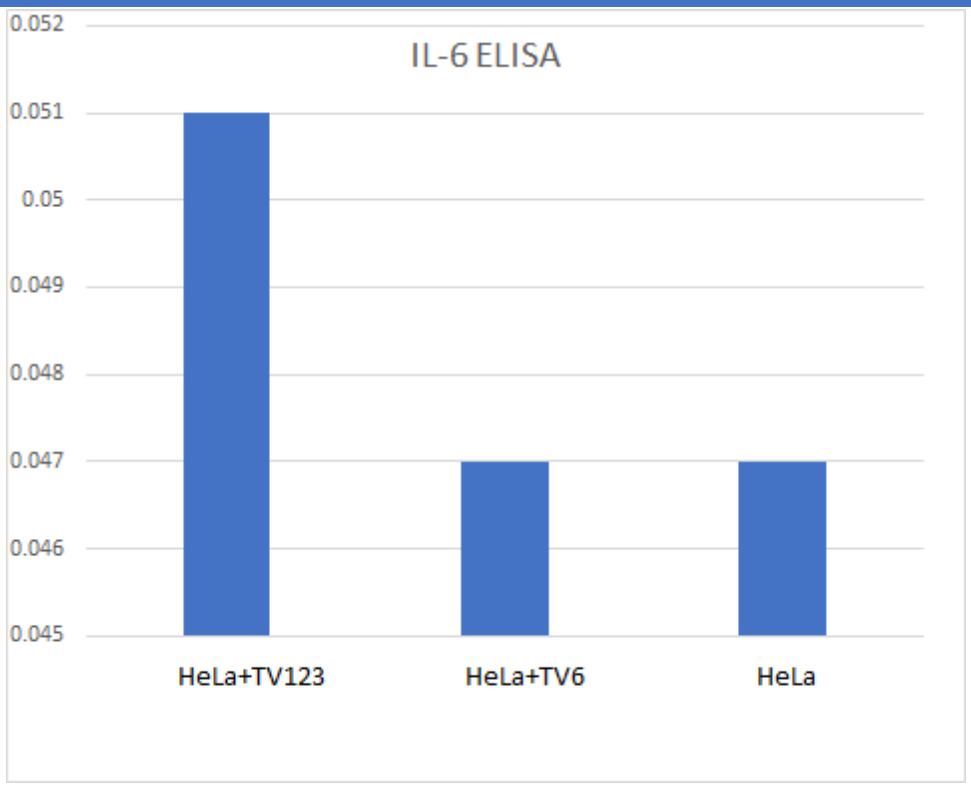
adherence, immunohistochemistry staining, TUNEL staining, and Enzyme Linked Immunosorbent Assay were performed. Initially, the culturing of both *Trichomonas vaginalis* and the epithelial gastrointestinal and cervical cells were established. For the parasites, Tryptose-Yeast Extract Medium 1,000mL Diamond's Medium is composed of 500mL dH20, 20 grams of Tryptose, 10 grams of yeast extract, 5 grams of maltose, 1 gram of cysteine, 0.2 grams of ascorbic acid, 0.8 gm kh2po4, 0.8 grams of k2h2po4. For the complete media when growing the Trichomonas protozoan, per 50 milliliter flask, 36 mL of the Tryptose-Yeast Extract Medium, 4 mL of Horse Donor Serum, 40 microliters of Penicillin-Streptomycin or Antimycotic-Antifungal Antibiotic (and 20 microliters of additional Gentamicin are needed if the cells are contaminated with bacteria), and 500 microliters of *Trichomonas* cells are required. The Cells are then incubated in human-like conditions at 37 Celsius and 95% CO2. The epithelial cells are cultured using a different media, known as DMEM. The complete media for both HeLa and CaCo2 cells both require 5 milliliters of Fetal Bovine Serum, 45 milliliters of plain media, and 0.5 milliliters of AA-Antibiotic. For each 50mL flask, 9 milliliters of complete media, 100 microliters of AA-Antibiotic, and 500 microliters of epithelial cells were used. The epithelial cells were also cultured in the incubation system mimicking a human's carbon dioxide level and temperature. To begin the interaction of Trichomonas and the epithelial cells, counting each portion was done with a light microscope on a 5 by 5 glass slide to measure the exact number of cells. The number of cells were then calculated, with scientific notation, with a 1:3 ratio – *Trichomonas* to epithelial respectively, into microliters. The different variants of Trichomonas were then manually pipetted into the single epithelial tissue layer for the interaction. 2 mL of the Tryptose-Yeast extract, 4 mL of DMEM media along with the epithelial cells and injected Trichomonas vaginalis cells were co-cultured for either 30 minutes or two hours. Next, the flask would be trypsinized with trypsin, which is an enzyme which removes the epithelial cells from the treated side of the flask, enabling the process of centrifugation. After the centrifugation, the remaining cells began the ribonucleic acid isolation process with Trizol, in preparation for both the polymerase chain reaction and reverse transcription polymerase chain reaction. After the amplification process was finalized, the samples sat in 4 degrees Celsius to later be analyzed with gel electrophoresis.

Results

Experiment 1 - Polymerase Chain Reaction to determine the primer annealing temperatures and TLR gene amplification. Genomic DNA from Hela Cells were isolated and verified on the gel to determine the integrity of the DNA. Next, primers corresponding to TLR genes and an internal control GAPDH genes were used to amplify the respective genes. PCR samples were verified on the agarose gel to determine the expected amplicon sizes. All primers successfully amplified the expected amplicons.

Experiment 2 – Reverse Transcription – Polymerase Chain Reaction- Total RNA was isolated from Hela cells alone and Hela cells interacted with Trichomonas cells. Total RNA was separated on gel to determine the quality. 1ug of total RNA was used in reverse transcription PCR to determine the expression of TLRs in response to Hela activation with Trichomonas cells. Results showed that TLR 4 was increased in expression when HeLa cells encountered Trichomonas, TV-123 isolate when compared to HeLa cells alone or HeLa interacted with another TV isolate TV-6.

Experiment 3 – Enzyme Linked Immunosorbent Assay- ELISA assay was performed using the supernatant collected from cell culture flasks after the interaction with Trichomonas cells. The supernatant was diluted in various ratios and used in the ELISA assay to determine the cytokine gene expression using the Iinterleukin-6, antibody. As shown in the graph below, there was an induction of IL-6 over the basal levels (~200 pg/ml) in presence of Trichomonas cells. TV123 isolate showed enhanced induction of IL-6 when compared to TV6, indicating that different strains of Trichomonas will have different degree of host immune response. This result is consistent with the previous experiment results, in that TV123 interaction resulted in induction of TLR-6 as well as the pro-inflammatory cytokine IL-6.



Experiment 4 – Measurement of Adherence Assay, TUNEL Staining, Immunohistochemistry staining – To correlate the TLR and cytokine gene expression with Trichomonas binding an adherence assay using fluorescent dyes is underway.

Conclusion and future directions:

TLR gene expression analysis showed that TLR-4 was induced in Epithelial cells that came in contact with *Trichomonas vaginalis*. We also verified the cytokine gene expression (IL-6) using ELISA, and the cytokine gene expression correlated with that of the TLR-4 gene expression, as expected.

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