UNIVERSITY OF CALIFORNIA
RIVERSIDE

Toxoplasma gondii Induced Changes in Human Foreskin Fibroblast Cells

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Biomedical Sciences

by

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September 2021

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ACKNOWLEDGEMENTS

I would like to acknowledge my mentor Dr. Emma Wilson for her guidance in writing this thesis I would like to acknowledge my collaborator Stefanie Sveiven who contributed her knowledge and expertise of extracellular vesicles in order to guide this publication. Zoe Figueroa, Edwina Mambou, Emily Tabaie, Kristina Bergerson, and Edward Vizcarra also deserve acknowledgment for guidance throughout the year.

DEDICATION

I would like to thank Dr. Emma Wilson for being a great mentor. Thank you for always being understanding and supporting throughout my graduate career. I feel blessed to have worked under an advisor who values family and work-life balance. I am deeply grateful to the other members of my thesis committee Dr. Seema Tiwari-Woodruff and Dr. Monica Carson. You have all shaped my experience in graduate school and created an environment where I could be successful.

I could not have successfully executed this experiment without the direct mentorship of Stefanie Sveiven. Thank you for being patient and spending various long hours in lab with me, I am eternally grateful to you.

A special thank you to members of Wilson Lab, specifically Edwina Mambou and Emily Tabaie for assisting me with experiments, Zoe Figueroa consistently advising me and being a direct source of answers to all my questions, and Kristina Bergerson and Edward Vizcarra for their expertise. I will never forget each and every one of you.

I am indebted to the Division of Biomedical Sciences for accepting me into your program and believing in me. I cannot stress how challenging completing a master's degree in one year has been but working alongside the faculty and students made every struggle worthwhile.

Finally, I would like to thank my wonderful family. My parents, Abel and Mercedes who have made more scarifies than I can count to support me. I would not be the person I am today without their guidance and love for me. My sister, Jacqueline, for supporting me and providing me with a safe space that I can escape to after a stressful day. To my niece and nephew, this is for you. I hope that you read this one day and know that there is no limit to what you can accomplish. I love you.

ABSTRACT OF THE THESIS

Toxoplasma gondii Induced Changes in Human Foreskin Fibroblast Cells

by

Stacey Gomez

Master of Science, Graduate Program in Biomedical Sciences
University of California, Riverside, September 2021
Dr. Emma H. Wilson, Chairperson

This study investigates the relationship between extracellular vesicles derived from *Toxoplasma gondii* infected human foreskin fibroblast cells and naïve fibroblasts. It is unknown the role extracellular vesicles play in regulating immune response, parasite replication, and alterations in neurochemistry. For this reason, aimed to analyze the role extracellular vesicles by treating naïve cells with extracellular vesicles isolated from infected cells, then measuring for cell viability and production of the cytokine IL-6. Final results show that extracellular vesicles from infected cells induced cell death and production of IL-6. Therefore, I conclude extracellular vesicles are directly involved in cell-cell communication and participate in initiation of innate immune response during *Toxoplasma* infection.

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LIST OF ABBREVATIONS

T. gondii……*Toxoplasma gondii*

EV……extracellular vesicles

HFFs……human foreskin fibroblasts

TE ……..toxoplasma encephalitis

PV …….parasitophorous vacuole

CNS …….central nervous system

DC ……dendritic cell

IL ……interleukin

CD…….. cluster of differentiation

APC…… antigen presentation cell

PAMPS ……..pathogen associated molecular patterns

TLR ……..toll-like receptors

IFN- γ……interferon gamma

TFN ……tumor necrosis factor

TCR ……..T cell receptor

CXCR3 ……C-X-C motif chemokine receptor

NO …….nitric oxide

PMNs …….polymorphonuclear neutrophils

BBB ……blood brain barrier

CCR …….chemokine receptor

CCL …….chemokine ligand

Trm ……..tissue resident memory

GABA ……gamma aminobutyric acid

GAD …….glutamate decarboxylase

Glu …….glutamate

Gln …….glutamine

GLT …….glutamate transporter

GS ……glutamine synthetase

PBS …….phosphate buffer saline

BCA ……bicinchoninic acid

ELISA ……enzyme linked immunosorbent assay

LPS ……lipopolysaccharide

2. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite. What makes *T. gondii* unique and successful is its ability to remain dormant in the host for years. Even though one-third of the world's population is infected with *Toxoplasma gondii*, most people are unaware of the parasite's residency (1). This is due to the fact that most people don't experience symptoms associated with infection. Symptoms of toxoplasmosis are generally seen in people who have a compromised immune system (2). However, it is important to note that there are some strains of *T. gondii*, particularly in South America, which are able to cause disease in immunocompetent individuals (3). Nonetheless, immunosuppressed individuals suffer from toxoplasmic encephalitis (TE), which is caused by reactivation of cysts and uncontrolled replication of tachyzoites in the brain (2). More specifically, cell lysis that occurs as the tachyzoites exit the cell causes disease. Symptoms include fever, headaches, muscle aches, and swollen or enlarged lymph nodes (2). *T. gondii* can also cause retinal disease, retinal chorioretinitis, which is caused by active and uncontrolled replication of the parasite in the retina (2). Congenital disease contributes to fetal abnormalities and developmental problems including mental retardation (2).

2.1 Toxoplasma life cycle

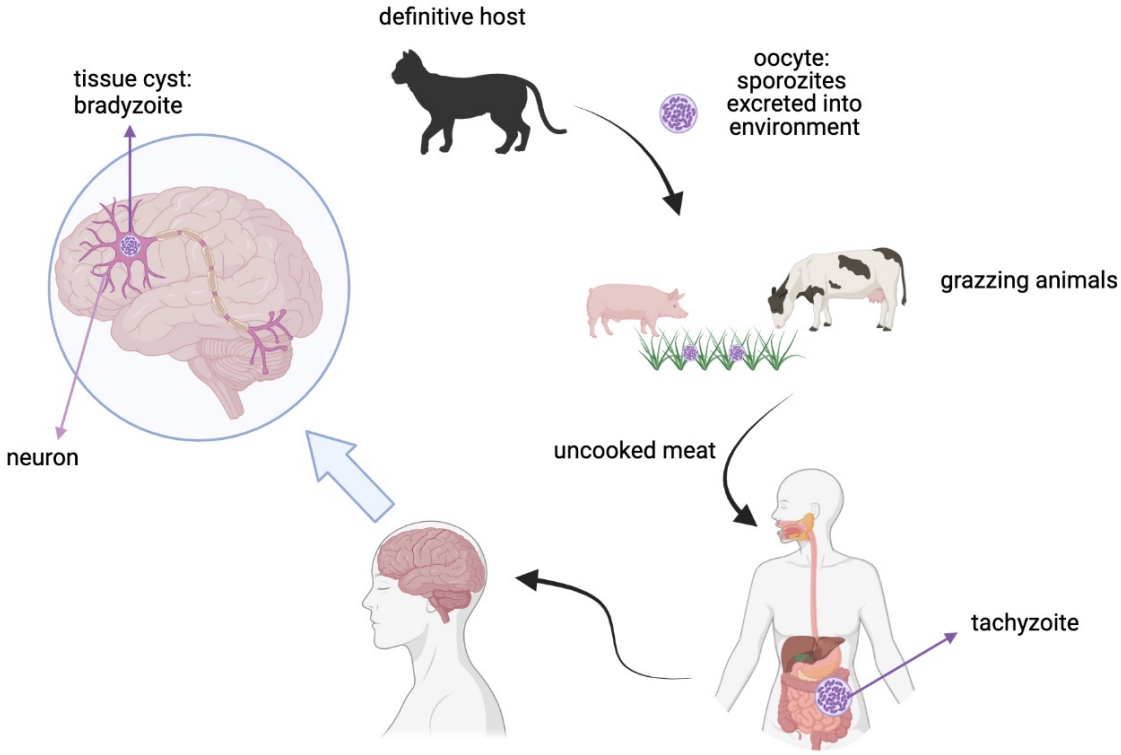

Figure 1. Life cycle of *Toxoplasma gondii*.

T. gondii can exist as a sporozoite, bradyzoite, or tachyzoite. Parasite lives as a sporozoite in a dormant stable oocyte, or bradyzoite in suppressed tissue cyst, or fast-replicating tachyzoite inside parasitophorous vacuole (PV) (2). Replication occurs in two fashions, sexually in its natural host to form oocyst in feline or asexually to form tissue cyst in any other mammal (2).

In felines, *T. gondii* enters the host orally, infiltrates the epithelial tissue cells in the intestine and begins oocyte formation and sexual reproduction (2) (Figure 1). Once sporozoite containing oocytes are formed cats begin to excrete oocytes via fecal matter. Cats can shed millions of oocytes into the environment and can survive in the soil for months (4). *T. gondii* is an extremely successful parasite because an oocyte can survive outside of the host in feces, resist concentrations of bleach, and spread throughout the surrounding environment (2). In addition, earthworms, flies, and cockroaches can spread oocytes mechanically (4). Thus, infection can occur by direct ingestion of oocytes through unwashed produce (2). But more commonly grazing animals such as cows, pigs, and goats ingest the cells. After ingestion of oocytes by any mammal other than cat, the parasite will penetrate epithelial tissue in the intestine; however, since the host is not a cat, *T. gondii* will reproduce asexually to form tissue cysts in the brain (2). Tachyzoites, the fast-replicating form, are released from the lumen and travel to the brain (1,2). Once in the brain tachyzoites switch to bradyzoites and form tissue cysts in neurons, where they either cause fatal TE or remain dormant (2,5) (Figure 1). *T. gondii* residency is for life, once an animal is infected the parasite can never be removed. Therefore, creating a route of entry for humans, ingestion of tissue cysts via uncooked contaminated meat (Figure 1).

T. gondii can essentially infect any cell with a nucleus, after replication tachyzoites burst out of the cell causing lysis (2). Most of the symptoms occur as a result of damage to cells. *T. gondii* can virtually penetrate and persist all central nervous system (CNS) cells,

including neurons, astrocytes, and microglia (6). Thus, all cells play a role in both parasite killing and survival along with inflammation. However, it is specifically in neurons and skeletal muscle where cysts are spontaneously formed (2,5,6). It is truly interesting how the parasite only forms cysts in the neuron and not in other cells. The reason for this exclusivity is still unknown.

2.2 Immune response to Toxoplasma gondii

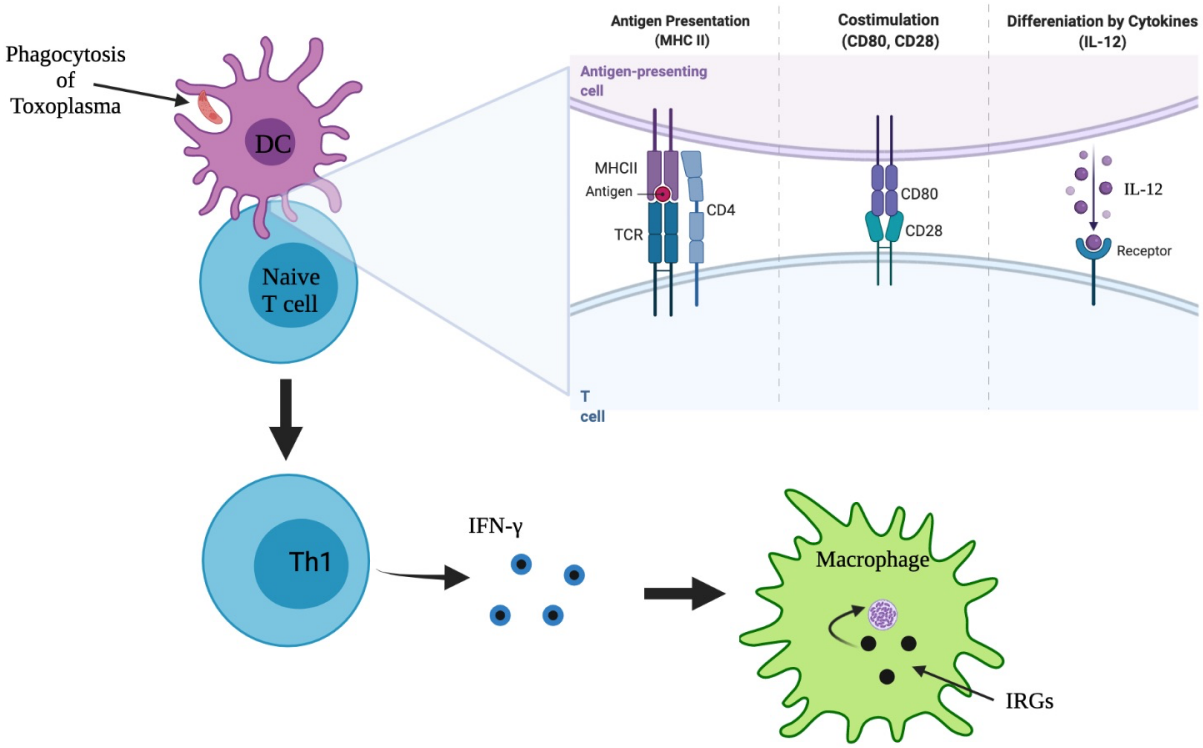

Figure 2. T cell activation and differentiation into T helper cell (Th1) by dendritic cell (DC). IFN- γ promotes macrophages to produce interferon-inducible p47 GTPases (IRGs).

T cells are one of the main defense mechanisms in keeping *T. gondii* dormant. T cells play a critical role in controlling parasite reactivation during chronic infection. The two main types of T cells are CD4+ T helper cells and cytotoxic CD8+ cells. We know T cells provide critical protection because depletion of CD8+ and CD4+ T cells leads to rapid reactivation of tachyzoites and death (7). In order for T cells to become activated there must be antigen presentation (MHC), costimulation (CD80, CD28), and differentiation by cytokines (2,6,7). Dendritic cells (DC) and professional antigen presenting (APC) cells, have toll-like receptors (TLR) that recognize pathogen associated molecular patterns (PAMPS) that allow DCs to pick up antigens associated with parasite and present them to naïve T cells (2). *T. gondii* profilin is a type of PAMP recognized by TLR-11, which activates dendritic cells to release interleukin-12 (2) (Figure 2). TLR-2 and TLR-4 recognize GPI-anchored proteins (2). Phagocytosis of pathogen leads to production of MHC II to use as presentation to CD4+ T cells. Together IL-12 and MHC II induce an immunological synapse that drives T cells to become T helper cells (Th1) to elicit a Th1 response (Figure 2). A Th1 response is produced specifically since *T. gondii* is an intracellular pathogen. Th1 cells produce IFN-γ, which destroys infected cells. IFN-γ is important in recruiting macrophages, which also phagocytose parasites and upregulate interferon-inducible p47 GTPases (IRGs) that target the PV (2,8) (Figure 2). The PV is a vacuole that the parasite makes inside the cell, where it rapidly replicates. By having IRGs that target the vacuole it will lead to parasite destruction within the cell (2,8).

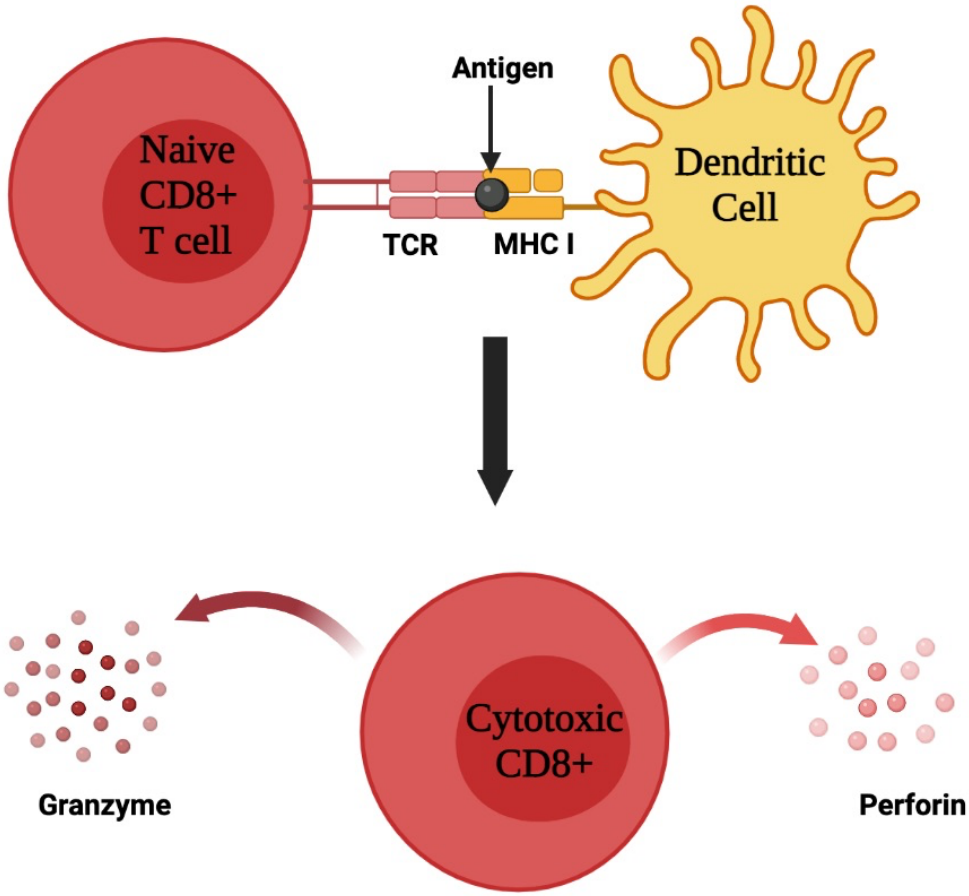

Figure 3. Antigen presentation to naive CD8+ T cells induces mature cytotoxic CD8+ T cells which is characterized by release of granzyme and perforin. Dendritic cell expresses MHC I which presents antigen to TCR on naive CD8+ T cell.

In addition, any cell that has been infected can present peptide in the pocket of MHC I to naive CD8+ T cell to induce cytotoxic CD8+ that will specialize in releasing granzymes and perforin to directly kill infected cells (2) (Figure 3). Compared to wildtype mice, perforin knockout mice succumb to chronic infection vastly faster and display significantly increased parasite burden (9). So, in addition to IFN-γ, perforin is also required for control of parasite reactivation. In IFN-γ knockout mice, perforin has been

found to be sufficient enough to control cyst burden (7). Although IFN-γ is still required to avoid parasite reactivation, it is suggested that CD8+ T cells play a role specifically in controlling cyst burden (9). Therefore, it is essential that T cells are present consistently in the brain in order to avoid TE.

Studies also show in chronic infection, there is a distinct population of macrophages in the brain which express CXCR3, MMR, stabilin-1 and arginase-1 (10). What is special about these macrophages is that they have chitinase activity, which is dependent on the enzyme AMCase to lyse cysts (10). Importantly, this distinct macrophage population is found in clusters surrounding cyst and can only produce chitinase activity when in close proximity to the cyst wall (10). Specifically, the chitin in the cell wall causes the phenotype of the distinct macrophage population (10). This demonstrates yet another mechanism that the immune system uses in battling the persistent parasite.

Neutrophils also contribute to inflammation, phagocytizing, releasing granules and IL-12 production. Depletion of antibody Gr-1, which is found on the surface of neutrophils, causes susceptibility to *T. gondii* and indicates their requirement in clearance of parasites and protective immunity (11). Macrophages are also a source of IL-12 production. Macrophages are activated by IFN-γ and contribute to the immune response by phagocytizing and directly killing the parasite (2). In addition, IFN-γ activated macrophages release nitric oxide (NO) that kills infected cells (2). As previously stated, macrophages are induced to produce IRGs that target PV which also directly kills parasites through degradation within the cell (8).

IL-12 is essential for stimulating Th1 response managed by IFN-γ (12). Interaction of CD40-CD40L can contribute to the production of IL-12 during infection with *T. gondii* (12). DCs express high levels of CD40 during infection and are able to bind to CD40L on T cells to promote IFN-γ production. Even though studies show that CD40L defect mice can produce sufficient levels of INF-γ, patients with CD40-CD40L fail to control parasite replication in the brain and fall victim to TE (12). This suggests that CD40 signaling must be involved in multiple mechanisms fundamental for the control of toxoplasmosis.

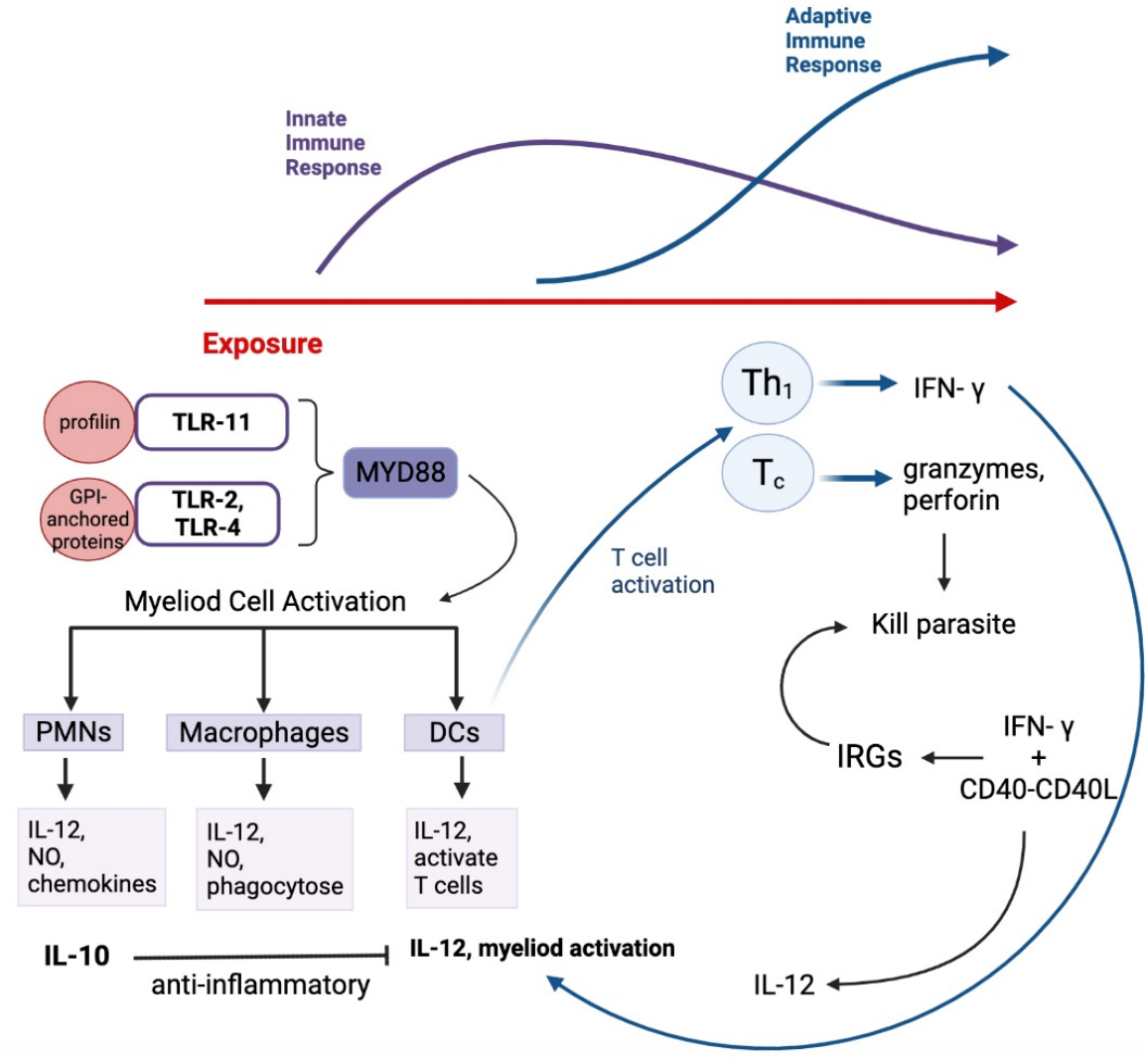

Figure 4. Overview of human immune response. Innate immune response drives adaptive immune response drives innate.

Innate immune cells are responsible for recognizing the PAMPS, profilin and GPI-anchored proteins by binding to TLRs on dendritic cells (Figure 4). This ultimately leads to activation of polymorphonuclear (PMNs) (neutrophils, basophils, eosinophils), macrophages, and dendritic cells. Antigen presentation in the presence of IL-12 leads to

Th1 adaptive immune response and IFN-γ induces production of the innate immune response, IL-12 (Figure 4).

T cells are needed for life, if an infected person acquires an immunogenicity where T cell count is significantly reduced (e.g., AIDS or cancer) this can lead to reactivation of cyst and TE (13). The HIV/AIDS epidemic served as an example of how *T. gondii* can stay in a latent stage until an opportunity strikes and can cause severe damage to CNS (3,13). The ability of *T. gondii* to remain inactivated through a resilient tissue cyst, in part, contributes to its triumph as an opportunistic parasite.

2.3 Neuroimmunology of Toxoplasma

Mice studies show there is a dramatic shift in gene expression over time in genes related to neuropathy, neuroinflammation and inflammation during *T. gondii* infection. Genes associated with neuropathology and neuroinflammation *C4A*, *CTSS*, *IFITM3*, and *PSMB8* are upregulated across chronic infection (8). The upregulation of these genes activates an increase in vascular permeability which aids in infiltration of immune cells into the brain (14). Genes associated with inflammation transition from low expression, compared to uninfected, to high expression by late chronic state. There is an increase in T cell abundance, specifically, CD8+ T cells over time, as well as IFN-γ (8). Additionally, innate immune cells, macrophages and microglia increase significantly over the course of infection (8). Even though gene expression changes begin at the early chronic state

where tachyzoites are replicating rapidly and cyst formation is starting to occur, the most significant change in expression levels is seen during mid chronic state which is when cysts are prevalent in the brain.

As tachyzoites enter into the CNS they infect neurons, astrocytes and microglial cells. Parasite invasion is followed by influx of CD4+ and CD8+ T cells that aim to control parasite proliferation by releasing IFN-γ, granzymes, perforin and recruiting innate immune cells. However, there is a balance between parasite proliferation and immune response during chronic infection that is truly interesting. An immune response is necessary to control parasite burden, but a hyperimmune response could cause dangerous inflammation in the brain. Nonetheless, to avoid reactivation and TE, T cells must consistently be present in the brain.

The brain is not an immunologically pristine site. We do not expect to see any immune cells persistently present in the brain. Yet, during infection with *T. gondii* it is essential to have immune cells preventing reactivation of cysts within neurons (7). The brain is unique, there is a blood brain barrier (BBB) which blocks the entry of unwanted ions, molecules, or cells (6). The BBB is composed of vascular endothelial cells, basement membrane, basal membrane, and astrocyte end feet (15). In order for T cells to first enter into the brain, there must be adhesion molecules that aid in slowing down and stimulate the rolling of cells on the endothelial layer of the BBB (9). The cellular adhesion molecules responsible for this motion are PECAM-1, ICAM-1, and VCAM-1 which are

expressed on the surface of the endothelial cells (9). Studies reveal that upregulation of the cellular adhesion molecules occur during acute and chronic infection, suggesting that the upregulation proceeds after invasion of the CNS, or in this case after parasites. Adhesion molecule, VCAM-1 is the integrin VLA-4 which is expressed on activated T cells and is necessary for brain infiltration (9). Mice models with anti-VLA-4 antibodies, which block VLA-4 function, were shown to inhibit VLA-4/VCAM-1 interaction and blocking of this interaction demonstrated an inability of CD8+ T cells to be recruited into the brain, which ultimately lead to a significant increase of parasite burden (9).

Next, immune cells must pass through the endothelial barrier. It is observed during chronic infection that the CCR7 ligands, CCL21 and CCL19 are upregulated (9). CCR7 is a chemokine receptor which is involved in transcriptional regulation and binds to CCL21 and CCL19 exclusively (16). CCR7 acts by transiting cells from the previously described rolling state into an arrest state followed by activation and displacement of integrin molecules that will allow lymphocytes to migrate into peripheral tissues (16). CCR7 deficient mice illustrated a deficiency in T cell recruitment as well as decreased levels of IFN-γ (16). This suggests that CCR7 is a key regulator of lymphocyte migration into the BBB, either directly or indirectly. Additionally, in the absence of CCL21, CD4+ T cells are unable to migrate into the brain and uncontrolled infection occurs (9). Whereas chemokine receptor CXCR3 is imperative in order to maintain influx of CD8+ T cells into the brain (9). Recruitment of CD8+ T cells into the brain and their ability to produce perforin is an absolute requirement for protective immunity against *T. gondii*.

Although protective immunity is mediated by continuous circulation of immune cells, there is evidence of a subset T cell population, tissue resident memory cells, which contribute to protection against chronic infection. Tissue resident memory cells (Trm) are characterized by the expression of activated CD69 along with suppression of tissue egress axis KLF2/S1PR1, together assuring that this population is localized in the tissue and not recirculating (17). Additionally, Trm cells are able to recruit lymphocytes and activate dendritic cells through the production of proinflammatory cytokines and chemokines. Most Trm express CD103, which allows the Trm cells to bind to epithelial tissue (17). CD103 binds to ligand E-cadherin in epithelial cells. This interaction optimally arranges Trm cells to function as an alarm during local infection. For these reasons it is suggested that Trm cells function as a first line of response at the site of infection.

2.4. T. gondii -induced changes in neurochemistry

Infection with *T. gondii* leads to changes in neurochemistry. A key factor affecting CNS physiology is changes in neurotransmitters (18). Neurotransmitters, also referred to as chemical messengers, are molecules released by neurons that are used for interneuronal communication (18). In order to maintain homeostasis, there must be a balance in the neuronal environment. Transmitters must be properly regulated, and neuronal connectivity protected. *T. gondii* infection causes a change in neuron function and morphology (18).

Genes associated with neuronal activity, more specifically those directly related to neurotransmitter production and function, are decreased significantly throughout infection compared to those uninfected. For instance, gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter of the brain, is improperly localized (20). In normal conditions glutamate decarboxylase (GAD) processes glutamate into GABA, then GABA is released into the synaptic cleft where it binds to GABA receptors on postsynaptic neurons that cause a decrease in excitability of the neuron (20). During infection, GAD is re-localized to the cytosol of the presynaptic neuron, which prevents GABA from being released into the synaptic cleft, therefore causing a decrease in inhibition of the postsynaptic neuron (20) (Figure 5). Recent studies have shown that over the course of chronic infection the gene associated with GABA signaling, *GABRA1*, were significantly decreased (11). This supports the idea that *T. gondii* directly interferes with the imbalance of neurotransmitters and provides a mechanism for developing neurological complications.

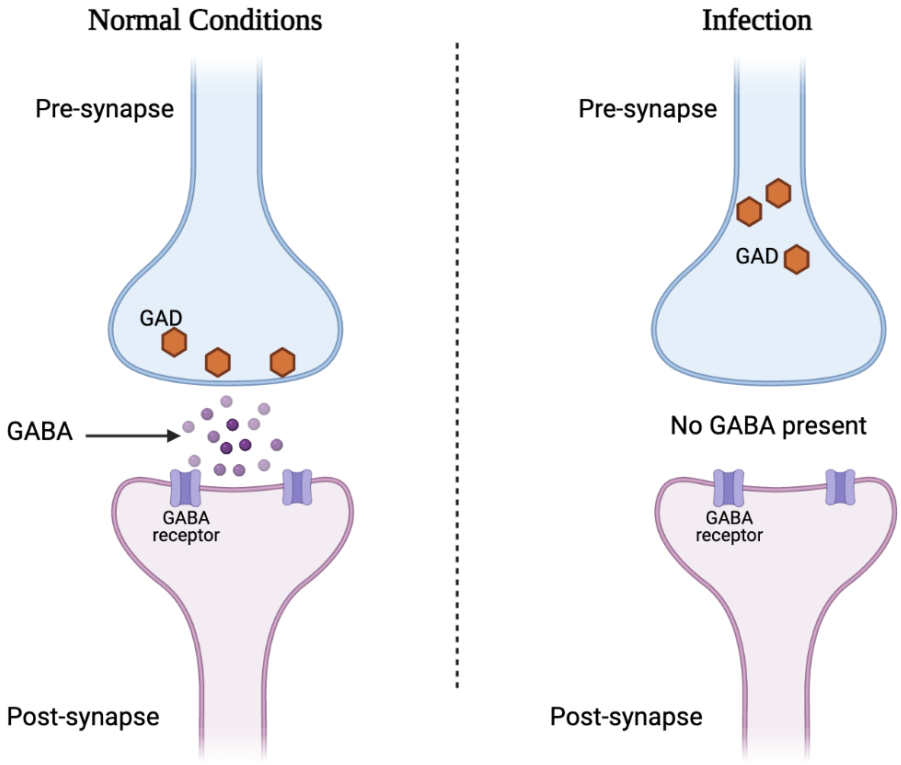

Figure 5. Gamma-aminobutyric acid displacement during *Toxoplasma* infection. Glutamate decarboxylase (GAD) processes glutamate into gamma-aminobutyric acid (GABA) which is released from the presynaptic neuron into synaptic cleft where it binds to the GABA receptor on a postsynaptic neuron. During infection GAD is displaced which causes GABA to not be released into the synaptic cleft therefore leading to decreased inhibition of the postsynaptic neuron.

Additionally, the gene associated with the intake of excitatory neurotransmitter glutamate, Slc17a6 is significantly decreased (21). Also, the gene that is correlated with a receptor that functions as neurotransmitter ion gated channel, Glrb, is downregulated consistently (22). An increase in excitatory neurotransmitters and decrease in inhibitory neurotransmitters leads to neuronal imbalance and toxicity.

Furthermore, pathways associated with myelination are consistently upregulated as infection persists (11). Myelination of neuronal axons is important for neuronal signaling, function and survival (23). Throughout infection, genes correlated with promoting myelination, Hexb, Tgfb1, and CXCR4 are increased (11). Processes of re-myelination is not uncommon; drugs are developed for remyelination in treatment for multiple sclerosis. Upregulation of the myelination pathway correlates with the neuronal damage occurring throughout infection and provides a mechanism to help in repair (11).

2.5 Astrocytes and Toxoplasma

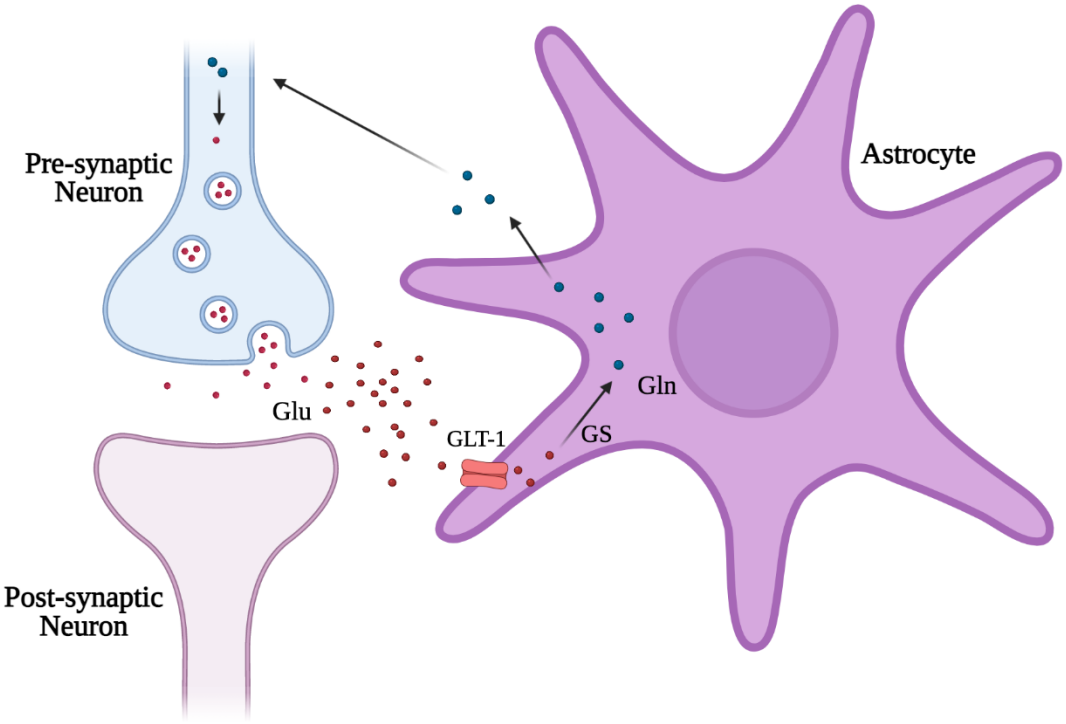

Figure 6. Neuron derived glutamate (Glu) is regulated by astrocytes via GLT-1. Presynaptic neurons release glutamate into the synaptic cleft. Glutamate is then transported into astrocytes through glutamate transporter, where it is converted into glutamine (Gln) via glutamine synthetase (GS). Glutamine is released back into the extracellular space, uptaken by neurons, and converted into glutamate.

Glutamate is an essential excitatory neurotransmitter, which is regulated by astrocytes (24). When homeostatic levels of extracellular glutamate are interrupted, this leads to neurotoxicity and neuronal damage (18). Astrocytes intake glutamate released from neurons via glutamate transporter (GLT-1) mainly, but also glutamate-aspartate

transporter (GLAST), where it is then converted into glutamine via glutamine synthetase (GS) (25) (Figure 6). Glutamine can then be released for neurons to intake and convert back into glutamate. Studies show that during chronic infection, 3 weeks post-infection, there is a defect in the astrocyte's ability to intake glutamate causing a build-up of extracellular glutamate (18). We also know that GLT-1 transcripts are significantly reduced meanwhile GLAST and GS remain unaffected (18). Although we know that levels of glutamate are dysregulated during infection and inflammation of *T. gondii*, the method of the process that leads to uncontrolled glutamate in the CNS is still unclear.

2.6 Astrocytes and Exosomes

Astrocyte derived exosomes have the ability to participate in non-classical signal transduction with neurons (26). Exosomes are a subcategory of small extracellular vesicles (EVs) and 30-100 nanometers in diameter (27). Essentially every cell can secrete exosomes, but components in an exosome vary based on cell type and purpose. Nonetheless, they can contain messenger RNA, microRNA (miRNA), lipids, and proteins (27). Studies show that exosomes are able to selectively target neurons and function as signal carriers in the astrocyte-neuron communication network (28). Moreover, it has been seen that small extracellular vesicles (sEVs) can affect neuron morphology during stress-induced environments (28). Specifically, it was the molecular cargo, miRNA which reduced neuron dendrite complexity through protein expression regulation. It was

seen that miRNA 26a-5p was significantly increased in hippocampal neurons after incubation with sEVs (miRNA 26a-5p is predicted to control morphogenesis and its content was upregulated in stressed sEVs) and increase in the miRNA was correlated with reduced dendritic complexity. (28). Additionally, exosomes derived from neurons can upregulate GLT-1 expression in astrocytes (29). This was proven by isolating neuronal exosomes, characterizing its content, physically placing exosomes on astrocytes, and measuring levels of GLT-2 expression. They found that miRNA124a was significantly high in neuronal exosomes and when treating astrocytes with antisense for miRNA124a GLT-1 expression levels would revert to significantly low amounts as compared to astrocytes treated with neuronal exosomes (29). However, it is unclear how GLT-1 expression in downregulated during infection. Nonetheless we know that neuron astrocyte communication is disrupted during infection. Hence, there is a possibility that exosomes can carry molecular cargo that might influence neurons or other astrocytes. For this reason, the role exosomes play in signal transduction during *T. gondii* infection should be explored.

2.7 Summary of questions and hypothesis

Before exploring the role of exosomes during infection of *T. gondii*, a simplified experiment was conducted to examine total EVs and their potential to induce changes in naïve cells. Small extracellular vesicles are functional packages of cellular communication. There are three subclasses of EVs based on size and origin. Exosomes

come from internal budding of vesicles and are 30-100 nm (20). Mircovesicles are produced by external budding from plasma membranes and are 100 to 1,000 nm (30). Apoptotic bodies are derived as a result of programmed cell death as blebs and are 1,000 to 5,000 nm (30). Studies have shown that EVs participate in the proinflammatory by directly activating the production of cytokines (30). Additionally, when comparing EV concentrations between healthy individuals and Toxoplasmosis patients, there was a significant increase in EVs released in infected patients (31). These findings lead to the investigation, if EVs produced by *T. gondii* infected HFFs could be involved with the immune response.

For this experiment, EVs derived from *T. gondii* infected human foreskin fibroblasts (HFFs) were placed on naive HFFs. Hypothesized that infected HFF-derived EVs would alter naive HFF cell viability and cytokine production. During infection, parasites replicate and form cyst in cells that cause cell lysis as they are released. Furthermore, innate cytokine IL-6 is released when there is an infection to drive an acute inflammatory response. If infection with T. gondii alters EV production in fibroblast, then it is expected that cell death and IL-6 production in naïve cells will be significantly higher than control.

Three groups were set up, naïve HFFs, infected HFFs and media only in T-175 flasks. There were two biological replicates for each group. HFF cells were infected with *T. gondii*. Naïve HFFs will serve as a baseline control to compare with infected HFFs. Media only group will serve as a negative control. Once cells were confluent and infected

with *T. gondii* the media from all groups was removed and replaced with EV media. The EV media provides an environment to promote the release of EVs. After 34 hours samples were collected and EVs were isolated from each group. Total protein content of EV enriched fraction was quantified via bicinchoninic acid (BCA) assay.

Naïve HFFs were plated on a 96-well plate and treated with EVs. There were five different treatment groups with four biological replicates each (EVs from naïve HFFs, EVs from infected HFFs, EVs from media only, PBS, and LPS). PBS group served as a negative control for EV treatment. Lipopolysaccharides (LPS) group served as a positive control to measure IL-6. EV treatment on naïve cells was observed after 24 hours. Supernatant was collected after corresponding time points and measured for IL-6 via enzyme linked immunosorbent assay (ELISA). Additionally, HFF cells were inspected for viability visually by staining with trypan blue.

3. Methods

3.1 Cell culture

HFFs were cultured in HFF media which consists of 1% penicillin/streptomycin, 10% FBS (fetal bovine serum) 1% L-glutamine, 1mL/L gentamicin and to volume with high-glucose DMEM (Dulbecco's modification of Eagles medium) and maintained in incubator at 37 °C. Parasites were maintained and grown in confluent HFF flasks using

parasite media which consists of 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1mL/L gentamicin and to volume with high-glucose DMEM. Prior to experiments, cells were rinsed with DMEM three times and fed with EV-depleted FBS containing media (0.5% FBS, DEMEM, and 1% pen/strep) for 34 hours. This EV media will create a stressful environment that should induce cells to release EVs. It is critical that cells were placed in EV media at the same time across both experiments to ensure there is no variability for exposure (Table 1, Table 2).

Table 1. EV media feeding times for experiment 1.

Naive HFFs	**Infected HFFs**	**Media**
10:12 am	10: 45 am	11:08 am
10: 26 am	10:58 am	11:16 am

Table 2. EV media feeding times for experiment 2.

Naive HFFs	**Infected HFFs**	**Media**
10:38 am	10:15 am	11:07 am
10:50 am	10:26 am	11:16 am

3.2 EV isolation

Conditioned media was obtained from naïve HFFs, infected HFFs, and control after 34 hours of culture and cells were removed by centrifugation at 300xg for 10 min at 4°C. This first centrifugation was run at a slower speed to remove heavier molecules such as floating cells, larger apoptotic bodies, and debris. The cell-free supernatant was collected and further centrifuged at 16,500xg for 20 minutes at 4°C to remove microvesicles and apoptotic bodies. Lastly, this supernatant was obtained and centrifuged at 100,000xg for 3 hours at 4°C. The pelleted EVs were washed once with PBS. A JA 30.5 Ti rotor, Beckamn Coulter, was used for both 20 min and 3-hour centrifugations. The final EV pellet was suspended in PBS and stored at -80°C for further experiments. EV protein concentration was measured using BCA protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

3.3 Bicinchoninic Acid (BCA) assay

Bicinchoninic acid or BCA assay is a common assay performed in order to measure total protein concentration. The BCA assay consists of two steps. For the first step, cupric (Cu 2+) is reduced to cuprous cation (Cu+) by protein in an alkaline environment which creates a blue colored complex (Figure 7). One cupric ion binds to peptides to form a blue colored complex. The intensity of the color is directly proportional to the number of peptide bonds participating in the reaction. In the second step, cuprous cation chelates

with bicinchoninic acid, BCA reagent is a highly sensitive and selective colorimetric detection, to form a copper-BCA complex that produces the color purple (Figure 7). The copper-BCA complex has an absorbance of 562 nm. Additionally, a darker color is associated with higher concentration of cuprous which means higher amount of protein in the sample. Prior to measuring samples, a calibration curve (standard) was prepared using a known protein concentration, bovine serum albumin (BSA) through a series of dilutions. Samples were measured on a Nanodrop Spectrometer (Thermofisher Scientific).

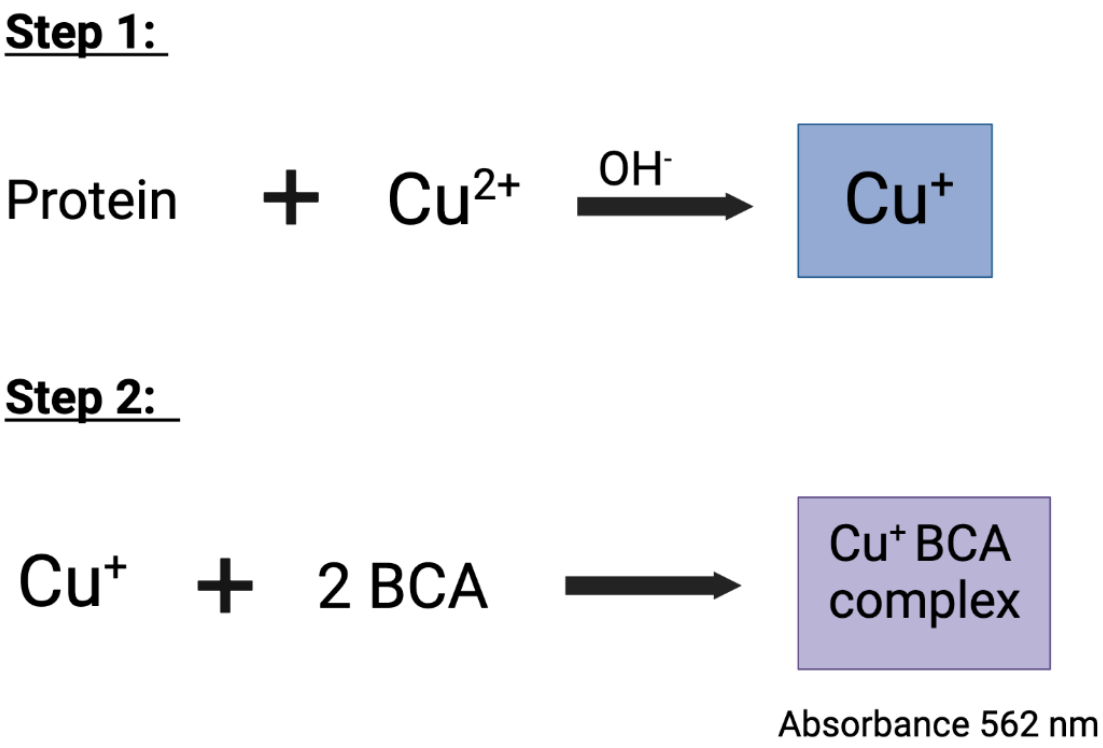

Figure 7. BCA principal concepts.

3.4 Protein Quantification

Equilibrated all the reagents, unknowns, and protein standards at room temperature. Prepared fresh stock reagent for all standards and unknown samples to be measured at a 50:1 ratio of reagents A and B, respectively. Loaded all samples into PCR strip for a 1:1 sample to stock reagent ratio; this is the lowest dilution for a micro-assay. Samples were incubated 60°C for 5 minutes, then loaded one at a time into the Nanodrop spectrometer.

3.5 EV treatment on naive HFFs

HFFs were cultured on a 96-well plate for 24 hours at 37°C before EV treatment. Cells were seeded at a density of 50,000 cells per well. After 24 hours, HFF media was removed from plate and replaced with EV treatment media (10% EVs and 90% EV media). There were 5 different treatment groups, naïve EVs, infected EVs, media EVs, PBS, and LPS. PBS was used as a control for EV treatment and LPS was used as a positive control for measuring IL-6. Supernatant was collected from each well plate after 24hrs and stored in -80°C for further experiments. HFF cells were stained with a dilution of trypan blue (1:1 HFF media, trypan blue) and observed for cell viability under a tissue culture microscope. Images were collected for all wells. To quantify data 10 regions of infection (ROI) were randomly selected in the image and the number of dead cells (blue nuclei) were counted.

3.6 ELISA of EV treated HFFs

Supernatant from all treatments was collected and tested for production of IL-6 via ELISA using a commercial ELISA kit according to the manufacturer's instructions (R&D systems DuoSet hIL-6). A 96-well plate was coated with capture antibodies and incubated overnight at room temperature. Wells were washed three times with Wash Buffer (Thermo Scientific, Wellwash Versa). Reagent Diluent was added to each well and incubated at room temperature for 2 hrs. Wash step was repeated again. Standards and samples were added to wells, covered with an adhesive strip, and incubated for 2 hrs. at room temperature. Detection antibody was added, covered in a new adhesive strip, and incubated for 2 hours at room temperature. Wash step was repeated again. Streptavidin-HRP was added, the plate was covered, protected from light, and incubated for 20 min at room temperature. Wash step was repeated. Substrate solution was added and incubated for 20 min at room temperature. Stop solution was added. Optical density was measured immediately after using a spectrophotometer (Thermo Scientific Varioskan Lux).

4. Does parasite infection lead to changes in extracellular vesicle production

4.1 Introduction and background to Toxoplasma and extracellular vesicles

EVs produced by Toxoplasma infected mice are correlated with activation of the immune response (29). A study showed an increase in cytokine production in splenocytes after stimulation with *T. gondii* derived EVs. There was an upregulation of IFN-gamma, TNF-alpha, and IL-17 all important for controlling parasite infection (30). Consequently, we analyzed the production of EVs from infected HFFs. We also quantified production of IL-6 and cell death after treatment with infected EVs.

4.2. Results

To determine the health of naive versus infected cells, cell viability was measured by Automated Cell Counter (Thermo Fisher Scientific). This demonstrated that both naive and infected cells had low viability, less than 50% viable (Figure 8). Interestingly, the infected group of cells were healthier than the naive group of cells. Additionally, there were more cells in the infected group compared to naive (Table 3). These results are important to consider when analyzing the amount of EVs produced from each group.

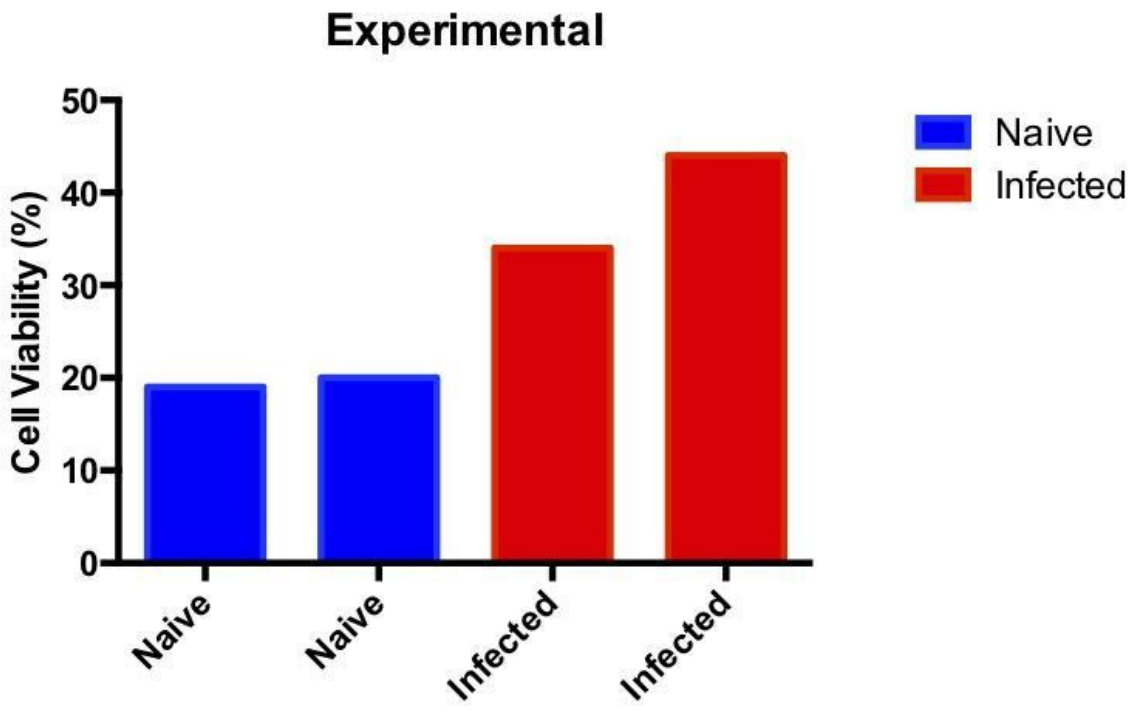

Figure 8. Cell viability (measured in terms of percent) of HFF cells, both naive and infected, was accessed after 34 hour treatment with EV media using an automated cell counter.

Table 3. Cell count of naive and infected groups of HFFs after 34 hr treatment with EV media using an automated cell counter.

Naive	**Infected**
7.68×10^5 cells	9×10^6 cells
3.114×10^6 cells	1.44×10^7 cells

Cell count and viability were relatively low in both naïve and infected HFFs. In naïve group, the first sample contained $7.68x10^5$ live cells with 51% viability and second sample contained $3.114x10^6$ live cells with 57% viability (Figure 8, Table 3). In the infected group, the first sample contained $9x10^6$ live cells with 38% viability and the second sample contained $1.44x10^7$ live cells with 60% viability (Figure 8, Table 2). Cell count measured via Automated Cell Counter, Thermo Fisher Scientific.

To quantify the protein content in the EV enriched fraction performed a BCA assay on the fraction. Unfortunately, this demonstrated that there was not enough protein content in the experimental group to quantify. However, the control group was able to be quantified as expected, which proves technique for EV isolation was not a factor for low concentration.

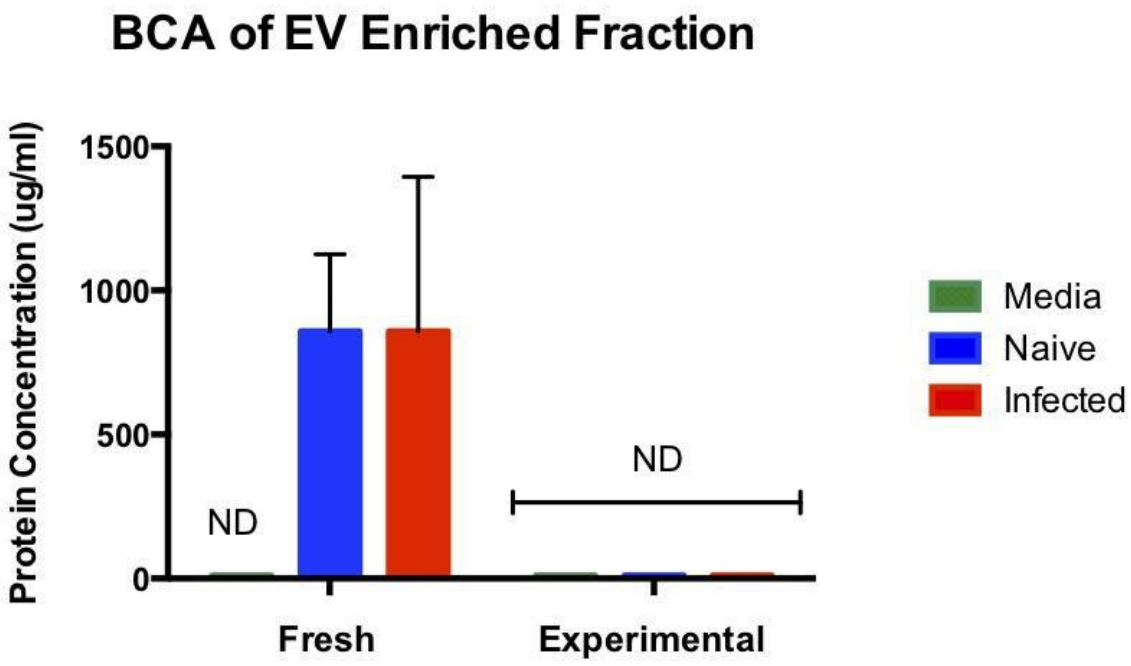

Figure 9. Bicinchoninic acid assay (BCA) of total protein concentration in EV enriched fraction for fresh media control compared to experimental group.

To determine the health of naive versus infected cells after EV treatment, cell viability was measured after 34 hr. treatment with EVs by staining cells with trypan blue and counting dead cells in small regions of infection (blue nuclei). Image analysis demonstrated that there were more dead cells in the EV infected group compared to all other treatments.

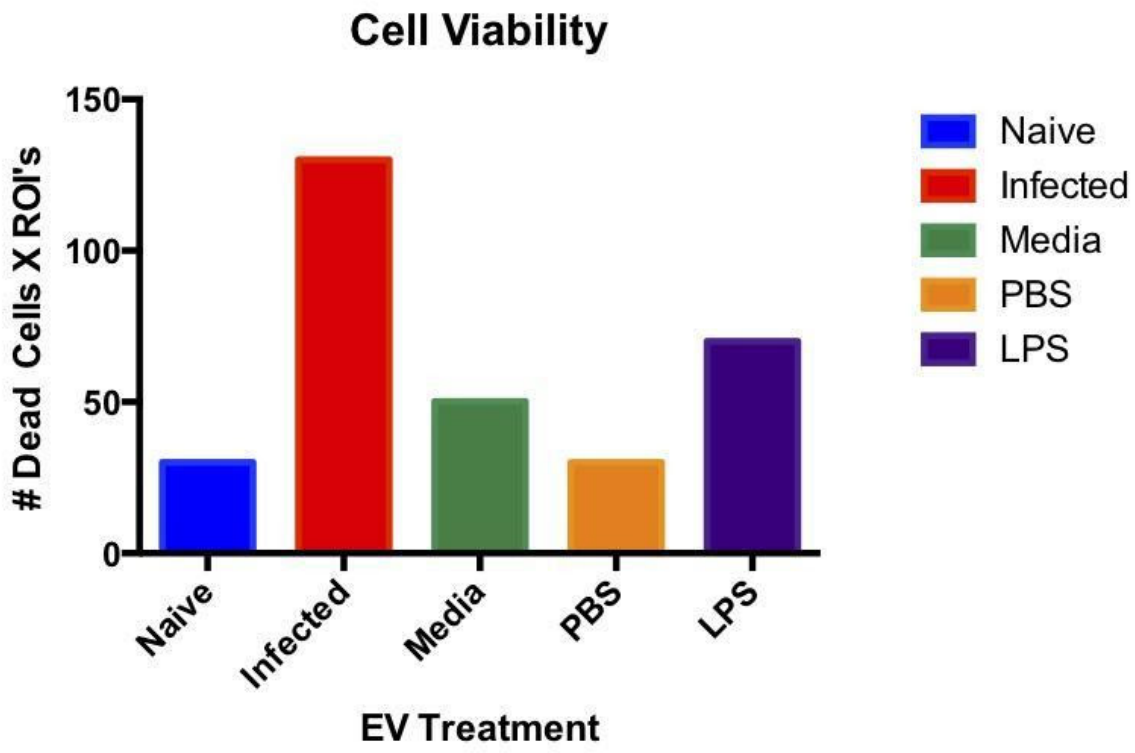

Figure 10. Cell viability of HFFs after 24hr treatment with EVs (naive, infected, media, PBS, and LPS). Ten regions of infection (ROI's) were analyzed for cell death (number of dead cells in ROI's).

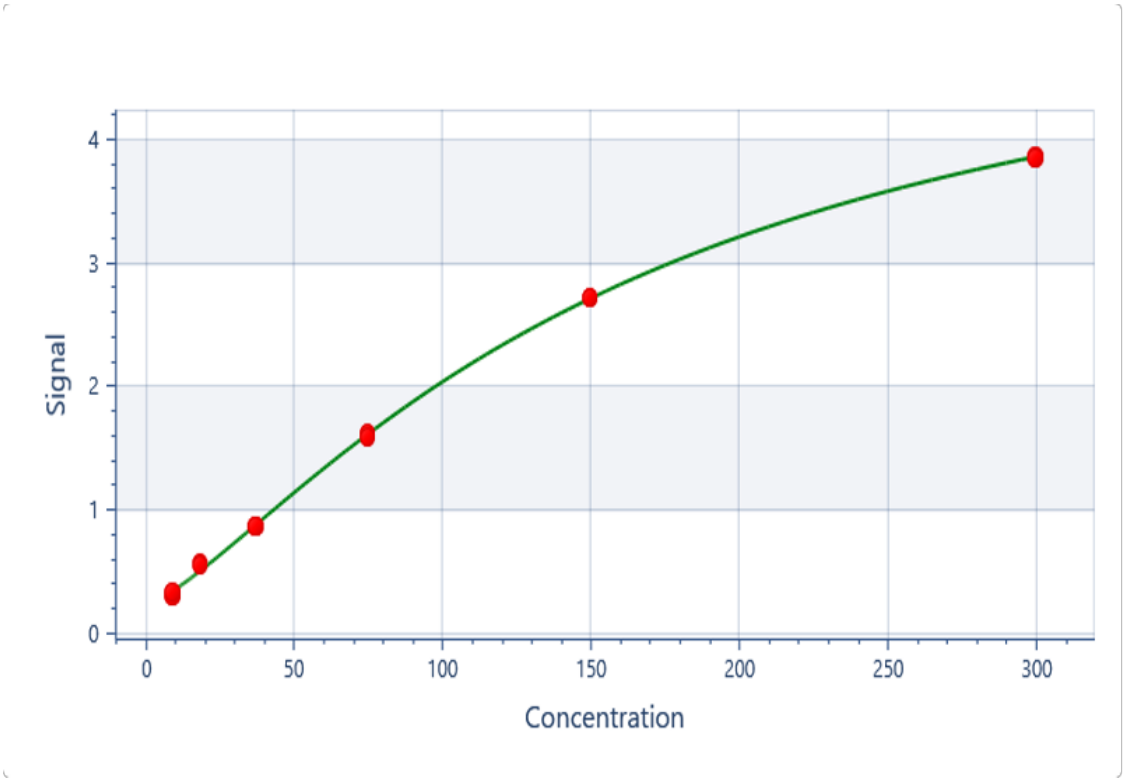

Figure 11. Humana IL-6 standard curve were r^2 = 1.

To determine if fibroblast production of IL-6 was altered in the presence of EVs from infected cells completed enzyme-linked immunosorbent assay on supernatant after 24 hr treatment. Results revealed production of IL-6 was significantly increased in the presence of EVs from infected fibroblasts compared to the naive group. Results were consistent throughout biological replicates and experiments (Figure 12,13).

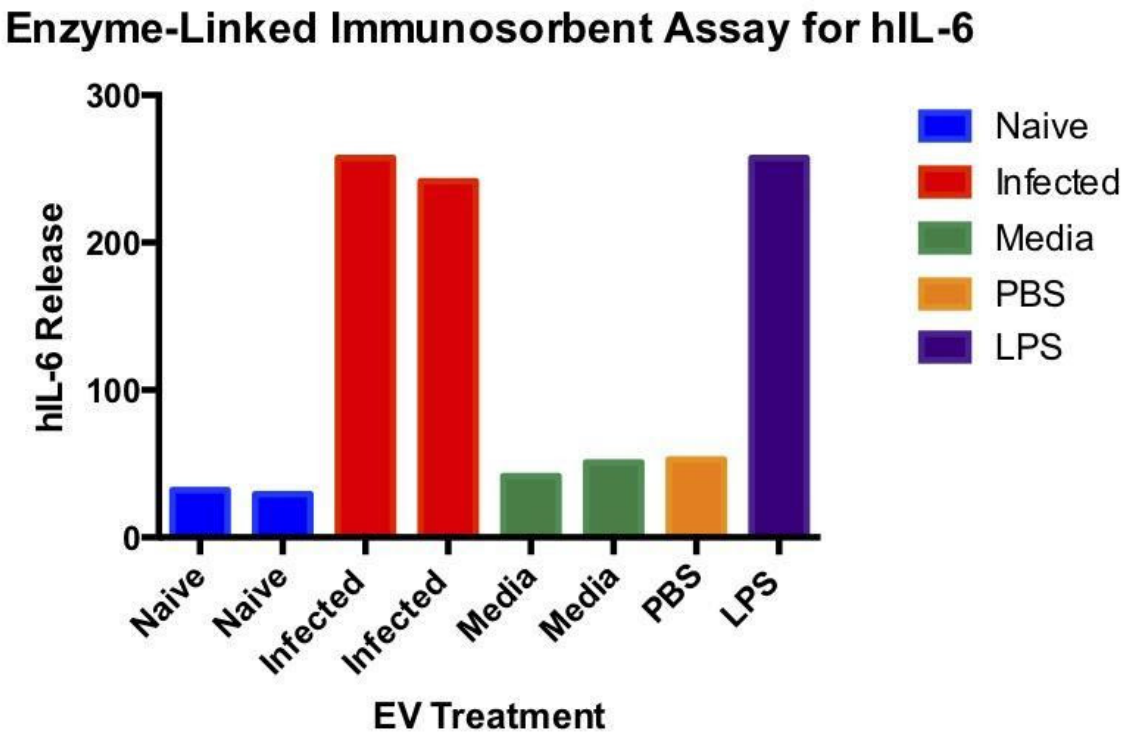

Figure 12. Experiment 1 ELISA for hIL-6 from HFFs after 24 hr. treatment with EVs (naive, infected, media, PBS, and LPS). Biological replicates for naive, infected and media treatments.

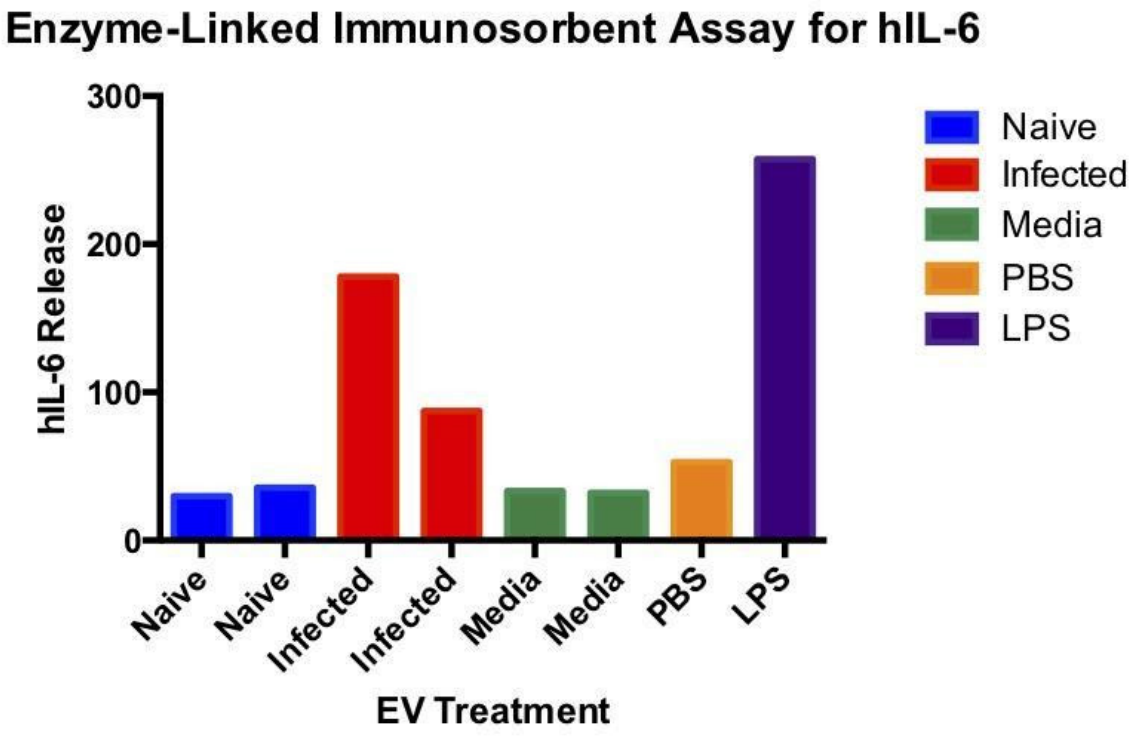

Figure 13. Experiment 2 ELISA for hIL-6 from HFFs after 24 hr treatment with EVs (naive, infected, media, PBS, and LPS). Biological replicates for naive, infected and media treatments.

4.3. Discussion

The main purpose of my thesis was to determine if EVs isolated from *T. gondii* are involved in cellular communication. The role of EVs in regulating immune responses, parasite replication and alterations in neurochemistry during chronic infection in the brain is unknown. Here I attempted to start answering some of these questions by isolating EVs from *T. gondii* infected cells and placing them on naive cells then measuring cell death

and presence of IL-6. The final result demonstrates that EVs derived from infected cells negatively affected cell viability and induced the production of IL-6. I can therefore conclude EVs are directly involved in cell-cell communication during infection with *T. gondii*. More specifically EVs are involved in initiating innate immune response against *Toxoplasma* infection.

Fresh Media group served as a control for EV media and processing. Cells were obtained from flask after EV media treatment and measured for cell count and viability. This was important because it demonstrated that there was a relatively low number of cells, and the viability of these cells were very low (Figure 8). This viability potentially contributes to the below detectable protein concentration in EV enriched fraction (Figure 9). If cell viability was optimized to be 80%, then HFFs might have the metabolic energy to produce more EVs. Optimizing number of cells and viability will be critical for future experiments. Furthermore, treatment of cells in EV media was only 34 hours instead of an optimal 48-72 hours (EV paper). Cells were only in EV media treatment for 34 hours, because prior to EV media cells were infected for 48 hours. If cells were left in EV media treatment for 48 hours, the parasite vacuoles would have burst causing cell lysis. For this reason, EVs were collected earlier than anticipated, which could have also contributed to low protein concentration in EV enriched fraction. In future experiments, we should use EV media when infecting cells with parasites instead of parasite media, therefore providing HFFs with optimal time to produce EVs.

It is important to note that BCA assay measures total protein, not just EV protein receptors. For this reason, the sample was processed for EVs through a series of EV isolation steps prior to quantification. However, there is still a possibility that other proteins not pertaining to EVs were detected. To address this issue, would need to further characterize through western blot or agilent microarray-based RNA profiling (Van Deun). Nevertheless, BCA micro assay had a limited dynamic range of 25-200 ug/mL. Nanodrop was unable to detect protein in between 0-25ug/mL, for this reason all of the samples in the experimental group were not detected (ND). This gray area does not necessarily mean that there is no protein content, it just means it was unable to be quantified. Therefore, when treating naïve HFFs with EVs a percent of EVs will be measured instead of microgram amount.

HFFs were plated in 96-well plate and cultured in HFF media 24 hours prior to EV treatment. For EV treatment, HFF media was removed and replaced with a media that consisted of 10% corresponding EVs and 90% EV media. Supernatant was collected after 24 hr. treatment. HFFs were stained with trypan blue and visually assessed for viability. Results showed that HFFs from naive media and PBS were relatively healthy (Figure 10). However, HFFs from infected and LPS experienced significantly more cell death (Figure 10). Data supports the hypothesis that EVs derived from Toxoplasma infected cells alter cell viability in naive HFFs. Unfortunately, we do not know if cells experienced programmed cell death or necrosis, our results suggest that EVs can affect the viability of cells. Importantly, future studies should investigate EVs derived from

infected astrocytes and place them on naive astrocytes. This could provide additional information regarding the signal transduction that occurs during infection and the role EVs play in the brain.

ELISA results showed that EVs derived from infected treatment had significantly larger concentrations of hIL-6 in supernatant compared to EVs derived from naive and negative controls across both experiments and replicates. (Figure 12, Figure 13). Although there is slight variation between replicates in both experiment 1 and 2, the infected EV treatment was consistently higher in hIL-6 concentration. This supports the hypothesis that EVs derived from Toxoplasma infected HFFs induce HFFs to release IL-6. This suggests that EVs may be involved in proinflammatory response elicited during infection, since IL-6 is an important innate cytokine used in initiating an inflammatory response. Perhaps HFFs can be signaling to neighboring HFFs that there is an infection occurring which requires proinflammatory cytokines. There is also a possibility that EVs were from *T. gondii* and were able to induce IL-6. Nevertheless, these results suggest that EVs may play a role in cell-cell communication during infection. Therefore, it is critical to explore the role of EVs derived from other CNS cells throughout infection such as astrocytes and neurons. Especially since we know astrocytes function is altered during chronic infection (18).

For future directions, it would be interesting to explore if exosomes (subcategory of EVs), derived from Toxoplasma infected astrocytes alter GLT-1 expression in naive astrocytes (Figure 14).

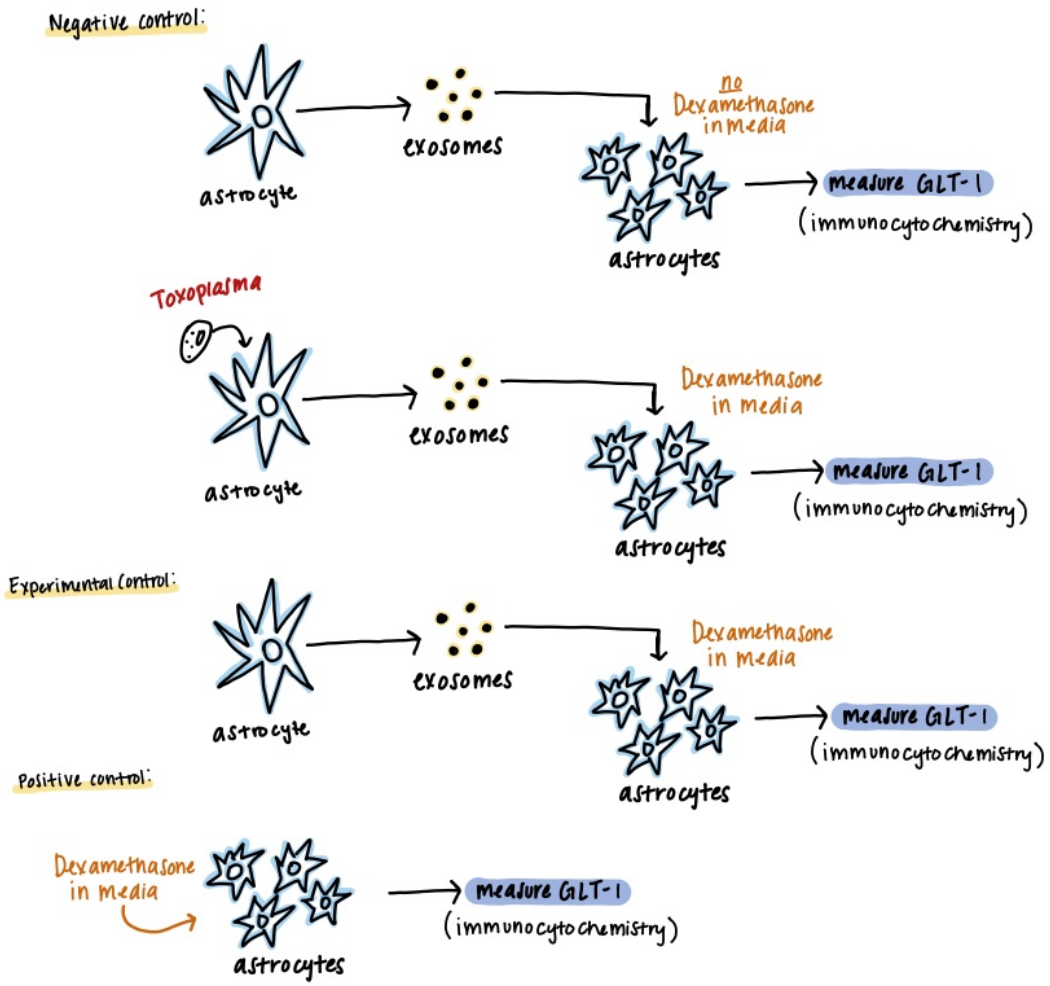

Figure 14. Proposed experimental design for future experiment. Does infection induced exosomes from astrocytes downregulate GLT-1.

5. Conclusions

The main goal of this study was the investigation of total EV production from *T. gondii* infected HFFs and their ability to alter HFF viability and cytokine production. This thesis explored the capability of EVs to be used as communication between cells. There is a chance that EVs isolated from infected cells were *T. gondii* EVs since the parasite can also produce EVs. Nevertheless, naive HFFs are able to distinguish between infected and noninfected EVs. This was seen by the significantly higher concentration of IL-6 in supernatant compared to naive group and controls. Interestingly, cell viability was also affected which was visualized through the number of dead cells in small regions of infection throughout each well. Finally, these data can suggest that interaction between HFFs and *T. gondii* can occur via EVs from infected HFFs. In other words, EVs have huge potential as signaling molecules during infection in the brain and systemically. Future directions include isolating EVs from CNS cells such as astrocytes and neurons to investigate their role in the brain during chronic infection.

6. References

All figures were made using Biorender.com

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A Note on Parasite optimizations.

Table 4. List of optimum parasite volume for infection of HFFs.

Flask	**Parasite volume**	**Time**	**Percent vacuoles**	**Cell lysis**
T-25	50 μL	6 days	65-70%	none
T-25	50 μL	7 days	90%	50%
T-25	100 μL	5 days	65-70%	none
T-25	100 μL	6 days	90%	50%
T-175	8 mL	24 hrs.	10-20%	none
T-175	8 mL	48 hrs.	50%	none
T-175	16 mL	24 hrs.	30-40 %	none
T-175	16 mL	48 hrs.	70%	none

Parasites need to infect cells in order to replicate and form vacuoles. Wilson Lab uses HFFs to continuously grow parasites. HFF media is replaced with parasite media, volume differs based on flask (T-175 = 8mL total volume, T-175 = 30mL total volume) and placed in an incubator at 37°C. When passing parasites there must be at least 70% vacuoles (crest shaped), and relatively no cell lysis. If parasites will be passed, just for

stock purposes 50% cell lysis is acceptable. However, if parasites are being used for an infection there should be no cell lysis in the flask.