Title
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Publication Date
2010

Peer reviewed|Thesis/dissertation

Host factor regulation of *Toxoplasma gondii* growth and differentiation

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A dissertation submitted in partial satisfaction of the

requirements of the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2010

Abstract

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Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a wide range of mammalian and avian hosts, including up to one third of the human population. Immunocompetent individuals clear acute infection with the parasite, however chronic infection is always established and persists for the life of the infected host. Reactivation of chronic infection in immunocompromised individuals, and vertical transmission from mother to fetus during acute infection can cause devastating neural pathology. Acute and chronic infection are associated with two distinct forms of the parasite: tachyzoites, which are fast replicating and responsible for acute infection and bradyzoites, which are slow replicating and establish chronic tissue cysts. During the course of infection, tachyzoites differentiate into bradyzoites. While tachyzoites can infect and replicate within virtually any nucleated cell, bradyzoite cysts typically only develop within neural and muscle tissue. Despite the critical importance of bradyzoite development to *T. gondii* pathogenesis, the factors responsible for this tissue tropism are unclear. Specifically, there are few defined molecular characteristics of the host cell that have been shown to regulate parasite growth and differentiation as bradyzoites.

Using an optimized *in vitro* system of bradyzoite induction, we have identified several new mechanisms by which *T. gondii* stage conversion is regulated *in vitro*. First, we have shown that enhancement of host cell glycolysis can support continued tachyzoite growth under metabolic stress conditions and thus inhibit bradyzoite conversion in a cell-intrinsic manner. Second, we have shown that cell lines that are intrinsically resistant to conversion, either basally or due to the induction of glycolysis, surprisingly release soluble mediators that inhibit conversion in *trans*. Finally, we have defined a new metabolic function for host Akt in *T. gondii* differentiation. These results suggest two new hypotheses as to how growth and differentiation of *T. gondii* may be regulated *in vivo*. One, the preferential encystment seen in highly glycolytic tissues may be a result of the ability of these tissues to sustain enhanced tachyzoite growth and increased parasite load, and if conversion from tachyzoites to bradyzoites is spontaneously occurring at some level *in vivo*, increased parasite load may lead to a higher incidence of cyst development in these tissues. Two, cells may broadly be releasing inhibitory mediators, making many tissues inhospitable to conversion, and thus restricting cyst development to particular tissues. While the *in vivo* significance of these hypotheses remains to be investigated, they

provide a new metabolic framework within which the mechanisms that regulate tachyzoite to bradyzoite conversion *in vivo* can be investigated.

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Acknowledgements

First and foremost, I'd like to thank my advisor, Bill Sha, for teaching me what it means to be a scientist-to think through problems clearly, to not give up until every bit of information from each experiment is extracted, and to see the bright side of every confusing piece of data. I'd like to thank Ellen Robey for her support throughout the years, for her enthusiasm for the Toxo project, and for teaching me to be as precise as possible in the description of my data. I'd like to thank Tony Iavarone in the QB3 mass spectrometry facility for his tremendous help completing a crucial part of my project. I'd like to thank members of the Sha lab both past and present and the 4th floor for their help-Eric V. for getting me started, Eric L.,Sonia, Kevin, Danielle, Derek and Veronica for lending a helping hand and for helpful discussion. I'd like to thank my family for their unconditional love always. To my parents, thank you for encouraging me to go as far as I can and for never doubting I would get there. To my sisters, thank you for reminding me what is important in life and for being my best friends. Last but not least, I'd like to thank Mason, whose continuous presence in my life for the past nine years has been a bright light through dark times. Without your love and support this whole process would have been infinitely harder. Thank you so much, I love you with my whole heart.

CHAPTER 1

Introduction

Pathogenesis of T. gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite of the phylum Apicomplexa, which includes other human pathogens such as the causative agent of malaria. *T. gondii* is one of the most widespread parasites in nature and can infect a wide range of warmblooded and avian hosts, with approximately one third of the world's human population infected, although prevalence varies widely across different populations (Tenter et. al. 2000). Although acute infection with the parasite is readily cleared by a functional immune system, chronic infection is always established and persists for the life of the infected host. Reactivation of chronic infection in immunocompromised hosts can cause serious disease, and if left untreated can lead to fatal toxoplasmic encephalitis. Congenital *T. gondii* infection in neonates resulting from vertical transmission following acute infection of the expectant mother can cause severe neurological birth defects, including blindness and mental retardation (Black and Boothroyd, 2000, Coppens and Joiner, 2001).

T. gondii has both sexual and asexual life cycles. Sporozoites develop during the sexual life cycle which takes place exclusively in cats. The asexual life cycle occurs within all intermediate hosts and involves conversion between two distinct life forms: tachyzoites and bradyzoites. Tachyzoites are the fast-replicating form that are responsible for the flu-like symptoms, if any, that are experienced during acute infection. During the course of infection, tachyzoites differentiate into bradyzoites, which are the slow-replicating form responsible for the establishment of long-lived tissue cysts that persist during chronic infection. Infection is acquired by the ingestion of either bradyzoite cysts in undercooked meat, or sporozoite cysts shed in cat feces, or as tachyzoites passed vertically from infected mother to unborn fetus. While tachyzoites can infect and replicate within virtually any nucleated cell, bradyzoites typically develop *in vivo* within neural and muscle tissue (Black and Boothroyd, 2000, Coppens and Joiner, 2001, Ferreira da Silva et. al. 2009). Differentiation from tachyzoites to bradyzoites is key to parasite pathogenesis, as it allows for long-term persistence within infected hosts. Reactivation of bradyzoite tissue-cysts is a major cause of pathology of parasite infection and provides the major mode of transmission between intermediate hosts.

The most devastating consequence of parasite infection, either due to reactivation of bradyzoite tissue cysts or congenital transmission, is extensive neural pathology. In immunocompromised hosts, such as AIDS patients and transplant recipients, rupture of reactivated tissue cysts can cause mass lesions in the brain due to dissemination of replicating parasites, resulting in swelling and increased cranial pressure that can be fatal. Due to destruction of brain tissue, bradyzoite reactivation can also lead to dementia (Simpson and Tagliati 1994, Mele et. al. 2002).

In congenital toxoplasmosis, the clinical manifestations vary depending on the trimester in which the infection was acquired. The severity of the disease increases the earlier the infection is acquired, with early infections resulting in scarring of the retina, cerebral calcifications, epilepsy, mental and psychomotor retardation, and encephalitis (Dunn et. al. 1999). If infection is acquired later *in utero*, the newborn may be asymptomatic at birth, but reactivation of bradyzoite cysts, even in immunocompetent individuals, can cause vision problems later in life. Furthermore, there is evidence that ocular toxoplasmosis occurs in

immunocompetent patients infected post natally, with anywhere between 2 to 20% of infected individuals developing the disease due to bradyzoite reactivation at some point in their lifespan, depending on the study (Latkany 2007).

Host-pathogen interactions in bradyzoite development

Despite the critical importance of bradyzoite differentiation to *T. gondii* pathogenesis, little is known about how host biology directs bradyzoite development. Bradyzoite differentiation is thought of primarily as a stress response, as all *in vitro* methods of inducing bradyzoite conversion involve subjecting infected cells to a wide variety of stress conditions. Thus, it has been postulated that stress exterted by the host immune system may induce tachyzoites to convert to bradyzoites (Gross et. al. 1996). This view is supported by the observation that bradyzoites convert back to tachyzoites in immunocompromised hosts, also suggesting that an active immune respone against the parasite maintains the parasites in the bradyzoite form (Montoya and Liesenfeld 2004). It is not clear, however, if immune-related stress directly induces bradyzoite conversion, or if tachyzoites spontaneously convert to bradyzoites at some low-level and the remaining tachyzoites are cleared by the immune system (Gross et. al. 1996).

As mentioned above, while tachyzoites infect and replicate within most cells, bradyzoite differentiation takes place primarialy within neural and muscle tissue. This striking tropism is without adequate explanation. It has been postulated that immune privilege, particularly in the case of neural tissue, could be contributing to parasite persistence in these sites (Jones et. al. 2006). However, immune privilege fails to adequately account for the preferential development of bradyzoites in neural tissue *in utero* within neonates lacking a fully functional immune system.

These dual hypotheses for the involvement of the immune system in regulating *T. gondii* growth and differentiation *in vivo* highlight the lack of clear molecular and cellular understanding that underlie the complex tissue tropism of parasite infection and bradyzoite formation. On one hand, the immune system may actively facilitate conversion of the parasite from the tachyzoite to bradyzoite form. Alternatively, the absence of an effective immune response in immunoprivileged tissues may facilitate growth of the parasite as a tachyzoite prior to bradyzoite conversion, and also allow for bradyzoite persistence in these these tissues. Both of these potential mechanisms could contribute to the observed preferential encystment within neural and muscle tissues. Whatever the actual cause(s) of preferential encystment in neural and muscle tissue, the *in vivo* tropism is clearly not a result of specific receptor mediated entry of parasites into host cells as *T. gondii* can infect virtually any nucleated cell.

Additionally, it is unclear if molecular characteristics distinguish a host cell environment as either permissive or restrictive to bradyzoite conversion. Preferential encystment within neural and muscle tissue could be the result of a particular host cell environment that provides a molecular signal to the parasites to convert. Alternatively, many host cell environments may be hostile for conversion, leaving bradyzoites to develop within cells that lack this hostile environment. Thus, certain host factors may either specifically enhance or inhibit bradyzoite conversion.

There has been limited investigation into the contribution of individual host factors to parasite conversion, and some evidence points towards IFNγ playing a role in directing parasite conversion. IFNγ is the major mediator of resistance to parasite infection *in vivo* (Suzuki et. al. 1988, Scharton-Kersten et. al. 1996), and treatment with the cytokine consistently results in slowed parasite growth *in vitro*. Its impact on parasite conversion is less consistent however, and differential effects may be due to cell type differences (Jones et. al. 1986, Bohne et. al. 1993, Bohne et. al. 1994, Weiss et. al. 1995). One putative mediator of bradyzoite induction downstream of IFNγ treatment, nitric oxide, resulted in slowed parasite replication and bradyzoite antigen expression *in vitro* (Bohne et. al. 1994). However, the *in vivo* significance of this result is unclear, as bradyzoites still develop normal in inducible nitric oxide synthase knock out mice (Silva et. al. 2002). Nonetheless, cell type differences in the ability of IFNγ to induce conversion *in vitro* perhaps points to a dependence on individual host cell characteristics in the facilitation of parasite conversion.

The most direct line of evidence implicating host cell contribution to conversion is the demonstration that bradyzoite conversion can be induced *in vitro* by the expression of human cell division autoantigen 1 gene (CDA-1). Radke *et. al.* (2006) demonstrated that a small molecule could induce bradyzoite conversion *in vitro*, and this induction was found to be dependent upon host cell transcription of the CDA-1 gene. Expression of CDA-1 induced growth arrest of the parasites as well as the host cells, and thus led to bradyzoite conversion. These results are the first to demonstrate that changes in the host cell environment can act directly on the parasite to initiate bradyzoite development.

Host-pathogen interactions in tachyzoite growth

The elucidation of individual host factors that influence parasite differentiation has been hindered by the lack of an *in vitro* system that reproducibly induces robust tachyzoite to bradyzoite conversion. In contrast, much more is known about the interaction of tachyzoites and host cell biology. Many *in vitro* studies have defined changes in the host cell induced by parasite to facilitate its own growth and survival. *T. gondii* resides within its own highly-restrictive parasitophorous vacuole (PV) surrounded by a membrane (PVM) that actively excludes host proteins and prevents fusion with host cell lysosomes (Mordue et. al. 1999), thus preventing its destruction. While resisting fusing with host cell lysosomes, the PVM readily associates with other organelles such as host mitochondria and ER. Upon infection, host mitochondria and ER are rapidly recruited to and tightly associate with the PVM (Sinai et. al. 1997), and while the exact reason for this directed interaction has not been shown, presumably this is done in part to provide the parasite with nutrients/energy. In fact, nutrient acquisition from the host cell is a major consideration of the parasite, and several modifications to the host cell are made explicitly to acquire specific nutrients.

T. gondii survival is dependent upon the scavenging of host cell purines and lipids. *T. gondii*, as many other intrcellular pathogens, is incapable of *de novo* purine synthesis, and thus must acquire them from the host cell (Perrotto et. al. 1971, Schwartzmann and Pfefferkorn, 1982). Parasite purine nucleoside transporters are capable of transporting certain purine stereoisomers that host proteins are not, and through an unknown mechanism, parasite infection allows for the uptake into host cells of those previously excluded stereoisomers (Al Safarjalani

et. al. 2003), stressing the importance of the host cell interaction with parasite purine metabolism. Similarly, *T. gondii* is incapable of synthesizing all their membrane lipids, and restricting lipid access to the parasite results in impaired parasite growth. For example, reduced levels of exogenous cholesterol resulted in reduced parasite growth, whereas the addition of cholesterol stimulated growth (Coppens et. al. 2000). Additionally, interference with normal choline metabolism and phospatidylcholine synthesis has been shown to alter membrane structure and inhibit parasite growth (Gupta et. at. 2005). Furthermore it has been shown that tachyzoites attract and direct host microtublues (Coppens et. al. 2006), and it has been postulated that this provides a direct method of acquiring host cell lipids from endolysosomes (Boyle and Radke 2009).

In addition to actively acquiring nutrients from the host cell to support parasite growth, *T.gondii* also induce specific changes that promote the survival of the infected host cell. Upon infection, host cells are rendered resistant to apoptosis by a variety of stimuli (Nash et. al. 1998, Carmen et. al. 2008), which has been shown to be dependent on NF-κB actvation (Payne et. al. 2003). Furthermore, it has been shown that the NF-κB subunit p65 is phosphorylated upon parasite infection (Molestina et. al. 2005), which results in anti-apoptotic gene expression, such as Bcl-2, and translocation of phosphorylated IκB to the PV membrane. In another study, it was shown that upon infection with *T. gondii,* Akt is phosphorylated, and this activation was required for host cell resistance to apoptosis (Kim et. al. 2006). In addition to preventing apoptosis, *T. gondii* infection can also cause upregulation of HIF1α, a master regulator of cells exposed to low oxygen levels, and it was shown that HIF1α inducible genes are required for parasite growth at physiologic oxygen levels (Spear et. al. 2006). Therefore the parasite actively promotes its own survival and survival of the host cell by altering host gene expression/inducing modification of host proteins. Additionally, these studies illuminate the tremendous benefit of tractable *in vitro* systems, and demonstrate the depth of knowledge that can be acquired if rigorous experiments are designed to test specific effects of parasite upon host, and host upon parasite.

In vitro differentiation of bradyzoites

The reasons for the lack of understanding of the mechanisms which direct bradyzoite development *in vivo* are two-fold. First, experiments *in vivo* aimed at dissecting molecular and cellular mechanisms are impossible to perform without directed hypotheses aimed at testing the function of specific genes/pathways. More general studies of the kinetics of bradyzoite differentiation *in vivo* have been performed using parasites engineered to express luciferase genes in a stage specific manner (Boyle et. al. 2007, Di Cristina et. al. 2008), but these studies have been uninformative as to the mechanisms of bradyzoite development. Secondly, the field has been hindered by the lack of an *in vitro* system that induces robust, reproducible levels of conversion. As mentioned above, much is known about the interaction of tachyzoites with host cell biology, thanks to the relative ease of maintaining parasites *in vitro* in the tachyzoite stage. However, despite efforts aimed at developing a tractable system with which to readily and reproducibly induce tachyzoite to bradyzoite conversion *in vitro*, the systems developed are inadequate. Various stresses, such as change in pH (Soete, et. al. 1994, Weiss et. al. 1995), heat shock (Soete, et. al. 1994), and mitochondrial inhibitors (Bohne et. al. 1994, Tomavo and Boothroyd, 1995), have been shown to induce bradyzoite antigen expression, but were all inefficient and/or the conditions are toxic to the host cell. Thus, rigorous studies that may

identify factors that either enhance or inhibit bradyzoite conversion cannot be conducted under these conditions. The net result of the lack of good *in vivo* and *in vitro* systems with which to study conversion is that while some factors that can influence tachyzoite to bradyzoite conversion are known, we still understand little about the *in vivo* significance of these findings.

Fortunately, a system that does induce robust, reproducible levels of conversion has recently been developed. Dzierszinski *et. al.* (2004) described an optimized system that induces robust conversion by pyrimidine starvation that exhibits little host cell toxicity (the details of this method will be discussed further in a later chapter). Conversion within this system can be modulated up or down, allowing for the identification of factors that may either enhance or inhibit bradyzoite conversion. Use of this system has allowed us to begin studying how host factors regulate *T. gondii* stage conversion.

By utilizing this optimized system, we have investigated the effects of the host cell environment on bradyzoite conversion in several ways. We tested the effects of individual host gene expression and found that two anti-apoptotic genes, Bcl-2 and Akt, have opposite effects on conversion. Additionally, we found that different cell lines in our system were either highly permissive or highly restrictive for conversion, and investigation into the basis for difference led to the discovery of a novel cell-extrinsic mechanism of inhibition of bradyzoite conversion by constitutive release of small molecular weight metabolites. We executed a purification scheme in order to identify these metabolites and determined that lactate is an inhibitory factor released from cells that can inhibit parasite conversion in *trans*. Furthermore, the identification of lactate prompted us to investigate the broader effect of host cell glycolysis on conversion. We found that the enhancement of host cell glycolysis can inhibit bradyzoite conversion in a cell intrinsic and extrinsic manner. These results indicate clear evidence for host cell involvement in the regulation of *T. gondii* differentiation and suggest that not only the particular infected host cell, but also the larger tissue environment can play an important role in supporting parasite growth and differentiation.

Finally, two features of our data may validate the use of *in vitro* systems in studying parasite conversion. The identification of lactate as a cell-extrinisic mediator of inhibition may represent a physiologic link to other *in vitro* methods of bradyzoite induction, such as change in pH and mitochondrial inhibitors, indicating we may have identified a metabolite signal that is representative of the actual *in vivo* mediator of *in vitro* stresses that induce parasite conversion. Additionally, we have developed a new tool with which to study *T. gondii* conversion *in vitro* by the inhibition of host cell autophagy. Inhibtion of autophagy can induce bradyzoite development independent of pyrimidine starvation, and conversion induced by this method is also inhibited by the small molecular weight metabolite(s) released from restrictive cells. This method may prove to be a more physiologically relevant method by which to study parasite conversion, and further demonstrates the potential significance of our identification of cell extrinsic inhibitory mediators.

CHAPTER 2

Host anti-apoptotic genes differentially regulate *T. gondii stage* conversion

Introduction

Dzierszinski *et. al.* (2004) described an optimized culture system for the *in vitro* study of bradyzoite conversion. Human foreskin fibroblasts (HFFs) are infected with parasites of the Prugniaud strain, and bradyzoite conversion was induced by pyrimidine starvation (Bohne et. al. 1997). The cells are then cultured in a minimal medium that lacks sodium bicarbonate at ambient CO_2, and since CO_2 is required for the de novo synthesis of the pyrimidine ring (Pfefferkorn 1978), culture at ambient CO_2 will result in pyrimidine starvation of the parasites. Using these conditions, bradyzoite differentiation is induced as early as 24 hours and can be maintained for up to two weeks with no toxicity to the host cells. While the concern with any *in vitro* system is that the biology is not representative of the *in vivo* setting, we feel confident that using this *in vitro* system, we will elucidate mechanisms that underlie *in vivo* conversion from tachyzoites to bradyzoites. The stress used in this system to induce conversion, CO_2/HCO_3 restriction, has been utilized as standard condition to study ampicomplexan gene regulation (see toxodb.org), and importantly, the bradyzoites differentiated *in vitro* were morphologically similar to those differentiated *in vivo* and harvested from mouse brain, thus providing validity to the use of this system.

We have employed this system to explore how host gene expression regulates tachyzoite to bradyzoite conversion. Induction of conversion is detected using a transgenic Prugniaud strain of parasites with an inducible bradyzoite-specific YFP marker (a gift from D. Roos, U. Penn). This parasite strain is transgenic for a constitutive RFP marker under transcriptional control of the gra8 promoter (a housekeeping gene, Carey et. al. 2000) and a bradyzoite-specific YFP marker under transcriptional control of the bag1 promoter (a bradyzoite-specific gene, Bohne et. al. 1995). RFP fluorescence can be used as a marker for all infected host cells, and YFP fluorescence is used to identify those host cells containing parasites that have converted to bradyzoites. These parasites are hereafter referred to as Pru gra8-RFP bag1-YFP. After 2-3 days of culture in bradyzoite inducing conditions, parasite growth and differentiation within host cells is quantitatively measured as RFP and YFP fluorescence by flow cytometry.

Using this system, we have investigated a potential role in the regulation of conversion of host genes in three categories: p47 GTPases, NF-κB family members, and anti-apoptotic genes. Genes in these families have all been shown to impact upon parasite biology. The p47 GTPases, activated downstream of IFNγ, the most important regualtor of immunity against *T. gondii in vivo* (Suzuki et. al. 1988, Scharton-Kersten et. al. 1996), have been shown to accumulate at the PV membrane (Butcher et. al. 2005, Martens et. al. 2005) and are important in controlling infection *in vivo* (Taylor et. al. 2000, Collazo et. al. 2001). We tested 3 genes in this family: IGTP, LRG-47 and GTPI. IGTP and LRG-47 deficient mice both rapidly succumb to *T. gondii* infection. GTPI has not been shown to have any known function in controlling *T. gondii* infection, but is closely related to IGTP and LRG-47 (Taylor et. al. 2004).

Upon infection, host cells are rendered resistant to apoptosis by a variety of stimuli (Nash et. al. 1998, Carmen et. al. 2008), which has been shown to be dependent on NF-κB activation (Payne et. al. 2003). Furthermore, it has been shown that the NF-kB subunit p65 is phosphorylated upon parasite infection (Molestina and Sinai 2005, Shapira et. al. 2005), which results in anti-apoptotic gene expression, and translocation of phosphorylated IκB to the PV

membrane. Thus we tested both p65 and the related NFkB subunit c-Rel for an effect on conversion.

Additionally, we tested the effect the anti-apoptotic genes Bcl-2 and Akt. Bcl-2 was found to be upregulated as a consequence of NF-kB signaling upon parasite infection (Molestina and Sinai 2005). In another study, it was shown that upon infection with *T. gondii,* Akt is phosphorylated, and this activation was required for host cell resistance to apoptosis (Kim et. al. 2006). Thus we tested both Bcl-2 and a myristolated dominant-active form of Akt (myrAkt) for an effect on conversion as well.

Out of the genes tested, only the anti-apoptotic genes had any effect on conversion, and surprisingly, they had opposite effects on conversion. Based on our results, it appears that Bcl-2 enhances conversion later during infection, presumably by promoting survival of infected host cells. MyrAkt, on the other hand, inhibits conversion at the earliest time point that conversion can be detected with our assay and thus has a more direct, and potentially more physiologically important, impact upon the regulation of bradyzoite conversion.

Materials and Methods

Cell culture

Parasites of the Prugniad (type II) strain, expressing red fluorescent protein (RFP) under the control of the gra8 promoter and yellow fluorescent protein (YFP) under the control of the bag1 promoter, or YFP under control of the tubulin promoter were provided by D. Roos (University of Pennsylvania, Philadelphia, Pennsylvania). Tachyzoites were maintained by serial passage in human foreskin fibroblasts (HFFs) in Dulbecco's Modified Eagle's Media (DMEM; Mediatech, Inc) containing 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories), 1% penicillin/streptomycin mix (Invitrogen) and 0.1% fungizone (Invitrogen) as previously described (Roos 1994). To purify tachyzoites for use in infection assays, infectcted HFF monolayers were scraped from culture flasks, needle-passed, and filtered using a 3-micron Nucleopore membrane.

Parasite conversion and growth assays

Unless otherwise stated, parasite conversion was induced by culture in minimal essential media (Invitrogen) without $NaHCO_3$, with 25mM HEPES, 2mM L-glutamine (Invitrogen), 1% FBS, and 1% penicillin/streptomycin mix at ambient CO_2 as previously described (Bohne 1997). This media is designated “starvation media” throughout. Host cells and purified parasites are pelleted together in 15mL polypropylene tubes for 10 min at 2500 rpm and then incubated for 30 min at 37°C + 5% CO_2. Cells are resuspended, pelleted at 1500 rpm and washed 1x with normal media to get rid of extracellular parasites. Infected cells are placed into culture conditions in 6 or 12 well plates. After 36-72 hours, infected cells are trypsinized and analyzed via flow cytometry using either a Beckman Coulter FC-500 or a Coutler EPICS XL, and data were plotted using FlowJo software (Tree Star).

Expression plasmids and transduction

All genes were expressed in the MSCV retroviral vector containing an IRES-Thy1.1surface marker protein. Plasmid DNA was transfected into Bosc23 packaging cells, and 48 hours later supernatant was harvested and used to transduce target cells. The Thy1.1 (Thy1) marker was used to purify transduced cells using a biotinylated Thy1.1 antibody (BD Pharmingen) and streptavidin-coated magnetic beads (Miltenyi biotech). Thy1.1 was also used as a marker for transduced cells during infection of mixed cell populations. Infected cells were stained using biotinylated Thy1.1 antibody followed by streptavidin-conjugated PE-Cy5 (BD Pharmingen) to allow for analysis of conversion within transduced and untransduced cell populations.

Results

Bradyzoite conversion is induced by pyrimidine starvation

To demonstrate the efficacy of our assay at inducing conversion specifically in CO_2 starvation conditions, HFFs were infected with Pru gra8-RFP bag1-YFP and cultured for 2 days in starvation media at 37ºC with or without 5% CO_2, then harvested and analyzed for RFP and YFP expression by flow cytometry (Figure 1A). The bag1-YFP marker is induced specifically at atmospheric CO_2, demonstrating that the expression of the YFP marker of conversion is specific to bradyzoite inducing conditions. The percentage of infected cells containing converted parasites is calculated as the percent YFP positive cells divided by the percentage of RFP positive cells. To indicate the extent to which conversion in this system can be modulated, serum starvation is used to further stress the parasites and therefore enhance conversion, and uridine is added to complement pyrimidine starvation and thus inhibit conversion. Uridine is the starting point for the synthesis of all other pyrimidines by the parasite (Asai, et. al. 1982), and will relieve the stress imposed by CO_2/HCO_3 restriction. The fold change in conversion can be calculated by dividing the percent conversion of the experimental condition by the percent conversion in the control. The fold change with uridine was 0.5, or a 2-fold reduction in conversion (Figure 1A).

Bcl-2 and myrAkt have opposite effects on conversion

To examine the role of host gene function in conversion, HFFs were transduced with retroviral vectors expressing the indicated genes, along with an empty vector control. Transduced cells were infected with Pru gra8-RFP bag1-YFP parasites, and cultured for 2-3 days in bradyzoite conversion conditions. Infected cells were then harvested and analyzed by flow cytometry and the percent conversion is calculated. Fold change in conversion in cells expressing each gene is determined by comparison to empty vector control (Figure 1B). The NF-κB family members p65 and c-Rel, and the p47 GTPases IGTP, LRG-47 and GTPI all had no impact on bradyzoite conversion. The anti-apoptotic genes Bcl-2 and myr-Akt had opposite effects on conversion, with Bcl-2 expression resulting in a 1.6-fold increase in conversion, and myr-Akt expression resulting in a 2-fold decrease in conversion.

To determine the nature of the effects of Bcl-2 and myrAkt on conversion, we did a time course, assessing conversion within Bcl-2, myrAkt, or empty vector expressing cells at 26, 36, 46 and 66 hours post-infection (Figure 2). At 36 hours, comparable levels of conversion were observed in empty vector as well as in Bcl-2 expressing cells (9.4% and 8.6%, respectively). Conversely, at this early time point, a reduction in conversion was already evident within myrAkt expressing cells, with 4.7% infected cells having undergone conversion. As the infection progressed, some enhancement of conversion became more progressively evident with Bcl-2 expression, with the greatest enhancement seen at 66 hours post-infection. The level of inhibition seen with myrAkt expression remains constant across the time course, with a 0.4-0.5 fold change in conversion at all 3 time points. This data suggested that myrAkt has a direct effect on inhibiting parasite conversion, whereas the effect of Bcl-2 on enhancement of conversion may be an indirect consequence of host cell survival.

Bcl-2 and z-VAD-fmk enhance survival of infected host cells

Considering the induction of conversion takes place under "stress" conditions of minimal media, low serum and ambient CO_2, this data suggested that the effect of Bcl-2 on enhancement of conversion may be due to the prolonged survival of infected host cells, with the effect occuring later when host cells have been exposed to stress conditions for longer. To determine if the prevention of host cell death by another means also enhanced conversion similarly as Bcl-2, the pan caspase inhibitor z-VAD-fmk was tested for an effect on parasite conversion. Z-VAD did indeed enhance parasite conversion, although to a lesser extent as Bcl-2, and the effect was additive with Bcl-2 (Figure 1B).

To determine if Bcl-2 and z-VAD result in the prolonged survival of infected host cells, individual host cells were monitored throughout the course of infection by microscopy. HFFs expressing Bcl-2 or empty vector, with or without z-VAD, were infected and scored 18-24 hours later for infection with a single parasite and were selected and marked using a Nikon object marker. The same cells were scored on day 2 and day 3 post-infection for YFP positive or negative parasites, or for host cell lysis by the parasites. The percentages of host cells in all 3 categories were approximately similar across all conditions on day 2 post-infection (Figure 3). By day 3, the anti-apoptotic conditions all had fewer lysed cells than the empty vector control, and had higher percentages of cells containing YFP positive parasites as well. The percentage of cells containing YFP negative parasites was approximately the same across all conditions on both day 2 and day 3. The Bcl-2 expressing cells, with or without z-VAD, exhibited the lowest frequency of lysis, and this corresponded to an increase in YFP positive parasites, compared to control cells. Control cells with z-VAD also exhibited a decrease in host cell lysis as well as an increase in YFP positive parasites, although to a lesser extent than cells expressing Bcl-2. These data suggest that the decrease in host cell lysis by day 3 of infection allowed for the increase in conversion observed within Bcl-2 and z-VAD treated cells.

Bcl-2 expression in 3T3s enhances conversion and decreases incidence of secondary infection

The decrease in host cell death and the subsequent increase in conversion with Bcl-2 expression were even more apparent with NIH3T3 cells. Microscopic observation of infected

cell cultures suggested that 3T3s were more sensitive to the stress of converting conditions than HFFs, as evidenced by a higher incidence of host cell death/lysis in the 3T3s under similar cell density as the HFFs. Not surprisingly, Bcl-2 expression resulted in even great enhancement of conversion within 3T3s than in the HFFs (Figure 4A). Bcl-2 expression resulted in a 3.3 fold enhancement of conversion over control cells; z-VAD treatment resulted in 1.8 fold enhancement and Bcl-2 expression with z-VAD treatment resulted in 4.7 fold enhancement of conversion. The decrease in lysis of infected 3T3s expressing Bcl-2 was apparent based on the decreased incidence of secondary infection. Over a 3 day period, the percentage of infected cells increased, due to host cell lysis and the subsequent secondary infection of neighboring cells. The rate of secondary infection increased more dramatically in the control 3T3 cells than in the Bcl-2 expressing 3T3s, suggesting Bcl-2 prevented the death of infected host cells, thus leading to a lower percentage of infection (Figure 4B). Coincident with a lower total level of infection, Bcl-2 expression also resulted in a higher percentage of RFP high cells. Even at 37 hours post-infection, a distinct population of RFP high cells is apparent within Bcl-2 expressing cells, which corresponded to cells containing higher numbers of parasites (Figure 4B). This suggests that even early on in infection, Bcl-2 expression was promoting the survival of cells containing larger parasitophorous vacuoles, which could then lead to a higher rate of conversion within these cells.

Discussion

Little is known about how individual host gene expression influences the preference of the parasite to convert within particular host cells *in vivo*. Using an optimized *in vitro* system that results in robust induction of bradyzoite development, we demontrated that the host anti-apoptotic gene Bcl-2 can enhance bradyzoite conversion *in vitro*. By contrast, the host anti-apoptotic gene myrAkt inhibits conversion, and at an earlier time point, perhaps implying a more direct role in the regulation of conversion.

While initially the opposite effects of Bcl-2 and myrAkt seem puzzling, these genes actually play very different roles in host cell biology which may be contributing to their differential effects on conversion. Bcl-2 is thought to act solely on the inhibition of apoptosis, and does so by preventing cytochrome *c* release from the mitochondria (Kelekar et. al. 1998, Verma et. al. 2006). Akt promotes cell survival by facilitating nutrient uptake, which leads to a more distal inhibition of apoptosis than the direct effect of Bcl-2 on mitochondrial integrity (Plas and Thompson, 2002). Additionally, Akt has many different roles in addition to its role in promoting cell survival, such as in the regulation of cell growth and proliferation, and glucose metabolism (Manning and Cantyley, 2007). Experiments discussing the mechanism by which myrAkt inhibits conversion as related to its role in glucose metabolism will be discussed in a later chapter.

In addition to Bcl-2 expression, the prevention of apoptosis by the caspase inhibitor z-VAD-fmk also results in enhanced bradyzoite conversion. Our data demonstrate that prolonged survival of host cells under stress conditions can result in the enhancement of bradyzoite conversion, especially in the case of 3T3 cells that are particularly sensitive to the stress conditions used in our system to induce conversion. While the enhancement of conversion mediated by the inhibition of apoptosis may be specific to the particular *in vitro* stress conditions

used in our assay, parasite conversion *in vivo* does occur within long-lived cells of muscle tissue and brain, perhaps implying host cell resistance to apoptosis is a factor in tissue cyst persistence.

Although the prevention of host cell death seems to be the principle mechanism behind the enhancement of conversion mediated by Bcl-2, its expression resulted in a greater enhancement of conversion than z-VAD treatment, and the effects of Bcl-2 and z-VAD were additive, particularly within the 3T3 cells. However, this could be due to Bcl-2 being a more potent inhibitor of apoptosis than z-VAD. Rigorous comparison of the ability of Bcl-2 expression and z-VAD to prevent apoptosis in our system was not performed. Nonetheless, our data is consistent with Bcl-2 preventing death of infected host cells, as evidenced by a reduction in the lysis of infected host cells coincident with an increase in parasite conversion within HFFs, and a reduction in secondary infection of 3T3 cells, implying prolonged survival of infected host cells.

Interestingly, none of the host defense genes tested had any impact upon conversion in our system. NF-κB signaling is critical for the initiation of innate immune responses, and the p47 GTPases are important mediators of innate defense against the parasite *in vivo*. The absence of effect of any of these genes on conversion suggests the regulation of bradyzoite conversion by immune host factors is largely cell-extrinsic and restricted to immune cells. Along those lines, the following chapter will discuss the identification of novel cell extrinsic factors released from non-immune cells that can inhibit bradyzoite conversion.

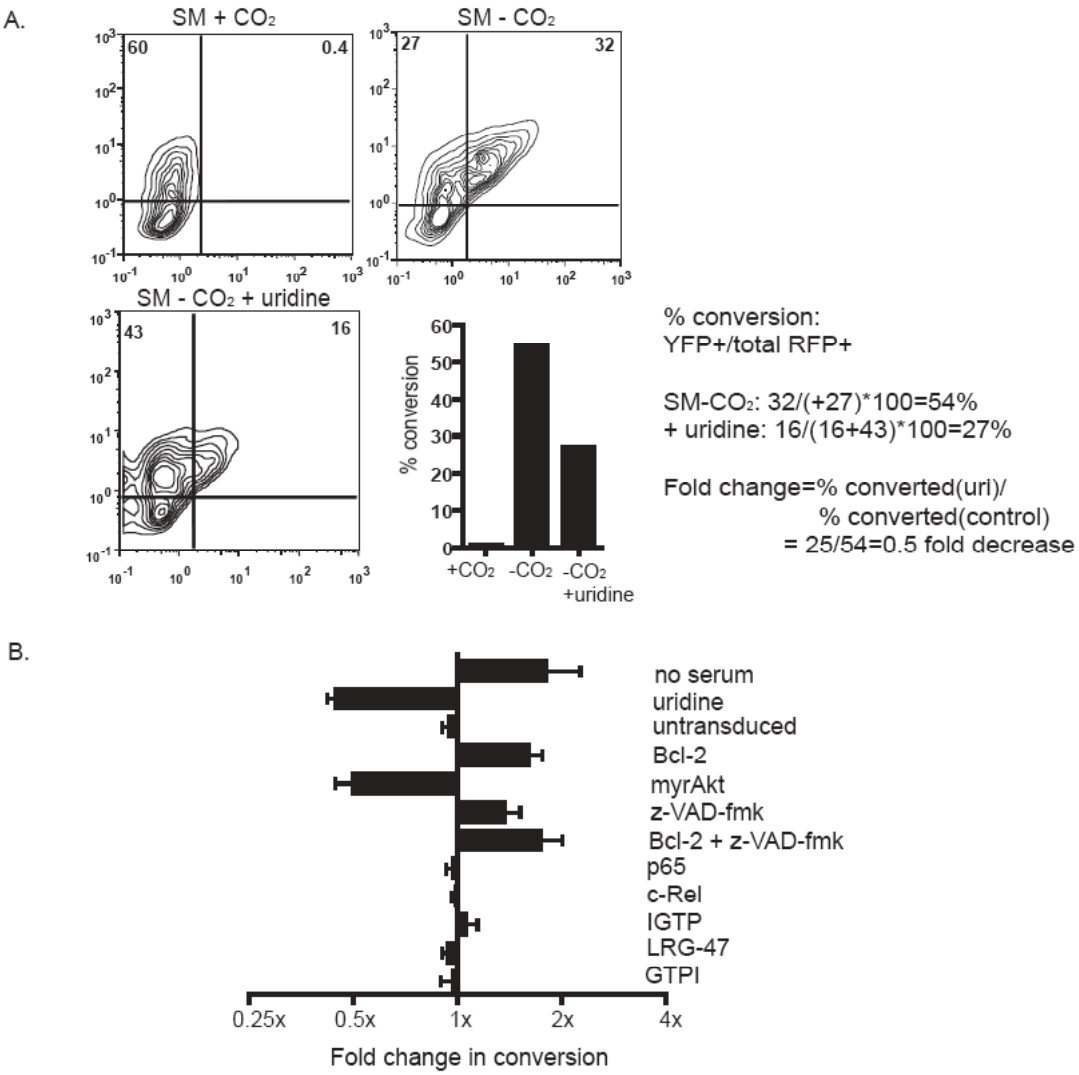


Figure 1. Host apoptotic genes differentially regulate *T. gondii* stage conversion.

(A). HFFs infected with Pru gra8-RFP bag1-YFP parasites were cultured in starvation media at 5% or ambient CO_2. Infected host cells were analyzed by flow cytometry for RFP and YFP fluorescence 48 hours post-infection.

(B). HFFs transduced with retroviral vectors containing the indicated host genes were infected with Pru gra8-RFP bag1-YFP and analyzed by flow cytometry 48-72 hours post infection. The fold change in conversion was calculated by comparison to control cells expressing empty vector.

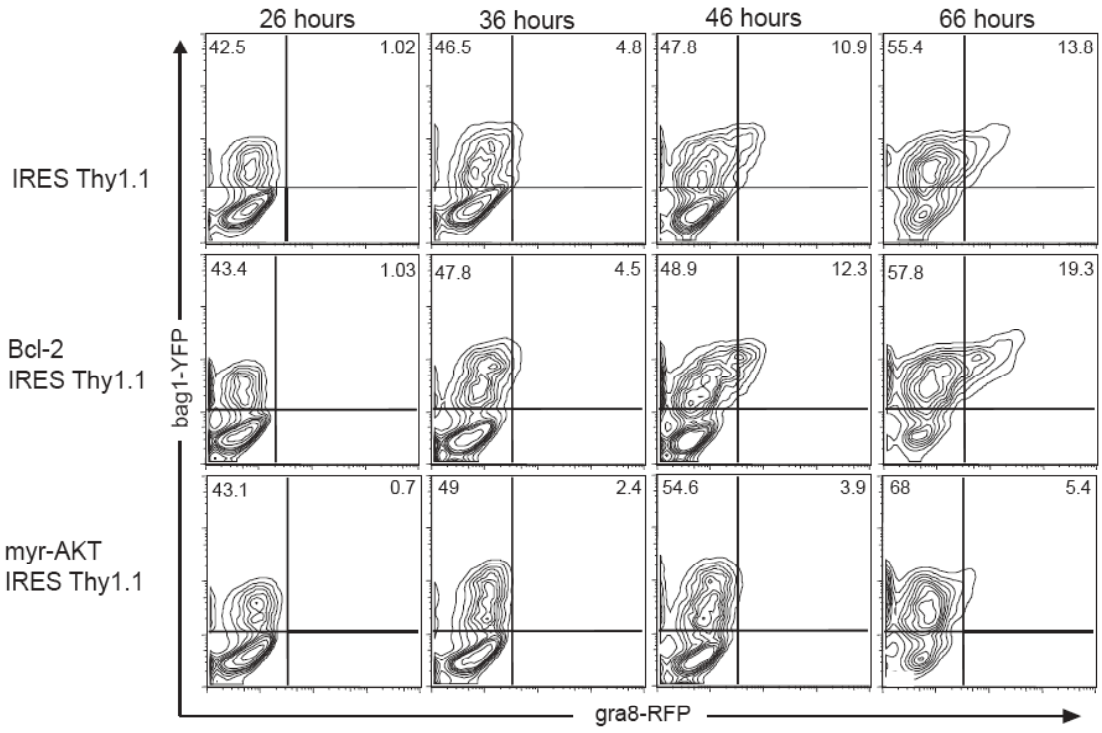


Figure 2. Bcl-2 enhances parasite conversion late in infection, whereas myr-Akt inhibits conversion early.

HFFs transduced with Bcl-2, myr-Akt, or empty vector were infected with Pru gra8-RFP bag1-YFP parasites and cultured in converting conditions for the indicated amount of time. Infected cells were harvested and analyzed via flow cytometry.

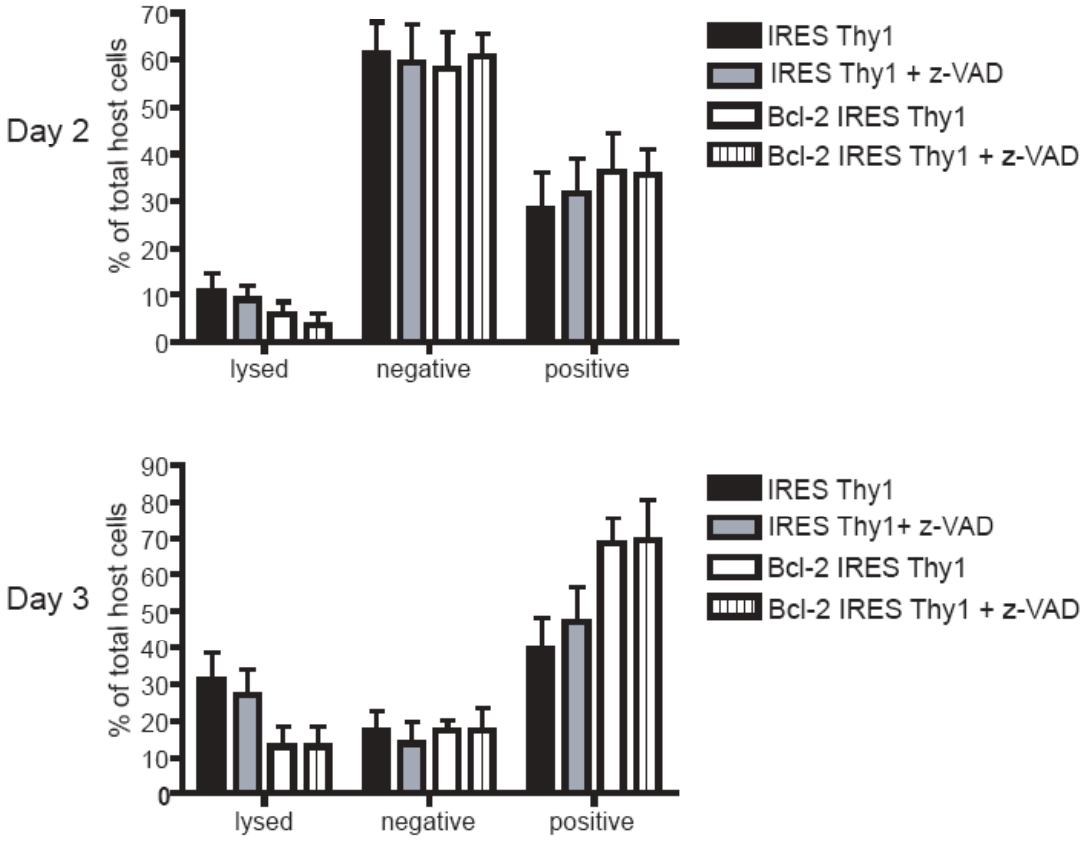


Figure 3. Bcl-2 expression results in increased YFP+ parasites coincident with a decrease in lysed host cells.

HFFs transduced with Bcl-2 or empty vector were infected with Pru gra8-RFP bag1-YFP parasites and cultured in converting conditions for 3 days with or without 50 μM z-VAD-fmk. Individual infected cells containing single parasitophorous vacuoles were selected by microscope 18-24 hours after infection and circled using a Nikon object marker. Host cells were then scored on day 2 and again on day 3 for lysis, YFP- or YFP + parasites. Bars indicate the percentage of host cells either lysed or containing YFP- or YFP+ parasites, calculated from the average of 4 experiments, +/- standard deviation.

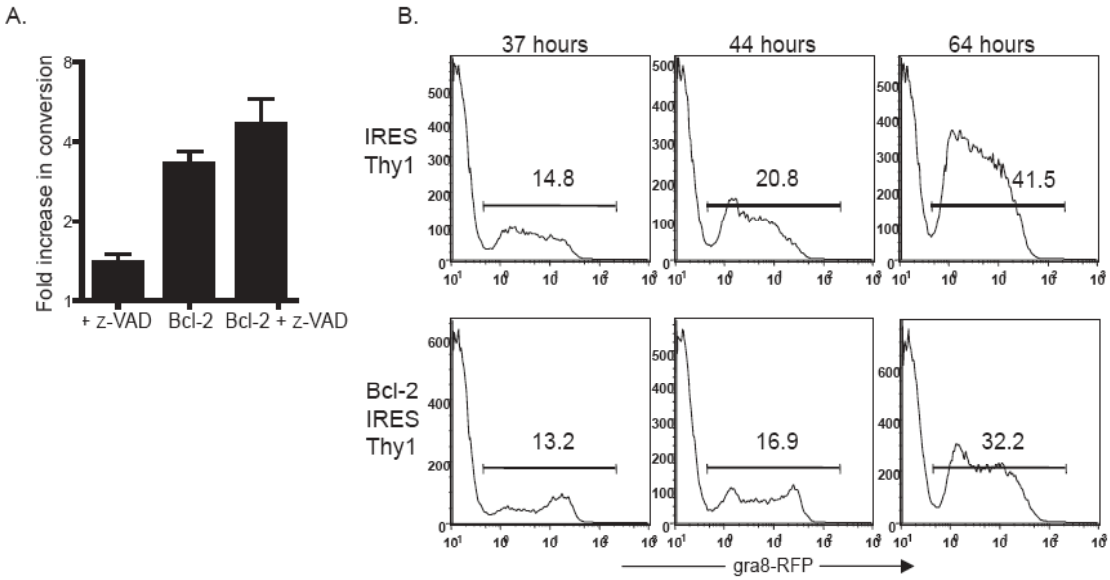


Figure 4. Bcl-2 expression enhances conversion within 3T3 cells and decreases incidence of secondary infection under converting conditions.

(A). Fold change in conversion within 3T3 cells expressing Bcl-2, treated with z-VAD-fmk, or both. Bars indicate the fold change in coversion versus control cells calculated from 3 separate experiments, +/- standard deviation.

(B). The percentage of infected host cells increased more dramatically over time in 3T3 control cells than in 3T3 cells expressing Bcl-2, concurrent with a loss in RFP high cells representing host cells containing large parasitophorous vacuoles.

CHAPTER 3

Identification of a cell extrinisic inhibitor of *T. gondii* stage conversion

Introduction

The extent to which the host cell environment contributes to the regulation of bradyzoite conversion remains unclear. Depsite varying levels of conversion observed within different cell types *in vitro*, the fact that most cell types used have permitted some level of conversion has led to the conclusion that the particular cell type does not contribute greatly to bradyzoite development (Lindsay et. al. 1991, McHugh et. al. 1994, Dubey et. al. 1998), at least in an *in vitro* setting. However, there has not been rigorous investigation into the differential ability of cell lines to facilitate bradyzoite conversion. It is well established that bradyzoites develop *in vivo* with a clear tropism for brain and muscle tissue (Coppens and Joiner, 2001). Additionally, parasites spontaneously convert to bradyzoites *in vitro* within astrocyte, neural and muscle cell lines (Luder et. al. 1999, Ferreira-da-Silva et. al. 2009), thus suggesting that the particular molecular environment of certain host cells helps drive bradyzoite conversion.

In our system, we observed that some cell lines are vastly more permissive for bradyzoite conversion than others. Investigation into the basis for this difference led to the discovery of a novel cell extrinsic mechanism of inhibition of bradyzoite conversion that is mediated by constitutive release of soluble factors from uninfected, non-immune cells that by themselves are also highly resistant to parasite conversion. We focused on two pairs of cells lines: fibroblast cell lines HFF and 3T3, and kidney cell lines Vero and 293T, which are either highly permissive or highly resistant to bradyzoite conversion. We found that when co-cultured, resistant cells potently inhibited parasite conversion *in trans*, and that inhibition was mediated by soluble factor(s) released by resistant cell lines. The soluble factor(s) not only inhibited the induction of bag1, but also promoted continued growth of the parasite as a tachyzoite without an effect on host cell cycle progression. Additionally, these soluble factor(s) also inhibited conversion induced by inhibition of host cell autophagy, a metabolic stress distinct from pyrimidine starvation. A purification scheme for the identification of these soluble factor(s) was executed, and lactic acid was identified as a factor concentrated in inhibitory supernatant fractions. Finally, lactate was demonstrated to be an inhibitory component of the 293T supernatant.

Materials and Methods

Supernatant collection and treatments

293T and NIH3T3 cells were incubated at a concentration of $3x10^7$ per ml in Hank's Balanced Salt Solution (HBSS; Invitrogen) for 6 hours in a 12 well tissue culture dish after which supernatants were collected and stored at 4°C. Supernatants were subjected to various treatments as followed: 3kD cutoff columns (Amicon): spun through the columns at 3700 rcf until less than 50µl volume remained. Boiling: boiled in 1.5ml eppendorf tubes for 10 min, along with a media control, and allowed to cool before addition to cultures. Proteinase K and trypsin: incubated with 0.1mg/ml proteinase K (Invitrogen) or 0.025% trypsin (Invitrogen) for 1 hour at 37°C, then spun through a 3kD cut off column to retain the enzymes. 1kD dialysis: dialyzed using 1kD dialysis tubing (SpectraPor) for 24 hours at 4°C into 1 liter starvation media. For inhibitor treatment experiment, 293T cells were plated at $2x10^7$ per $15cm^2$ dish and incubated with or without sodium oxamate for 48 hours. Phloretin was added to cells 4 hours prior to harvest.

After 48 hours, cells were trypsinized and washed extensively then incubated in HBSS as described above.

Chemicals

The following chemicals were all purchased from Sigma and were used at the following concentrations: 3-methyl adenine (5mM), uridine (0.1mM), thymine (0.4mM), thymidine (0.4mM), adenosine (0.5mM), choline (0.5mM), alanine (10mM), ammonium chloride (0.75mM), nicotinic acid (0.4mM), creatinine (0.5mM), sodium oxamate (20mM), and phloretin (40μM).

Reversed phase columns

293T supernatant + 0.05% TFA (acid) or 0.05% TEA (base) was passed over a C18 sep-pak column (Waters), or Oasis hydrophilic-lipophilic balance columns (Waters), washed with 0.05% TFA or TEA, then eluted with 50% acetonitrile/50% water (elution 1), and 100% acetonitrile (elution 2). Fractions are then lyophilized, resuspended in deionized water.

HiTrap ion exchange columns

0.5 ml 293T supernatant is diluted up to 5 ml in 20mM Tris pH 9 (anion) or 20mM acetic acid pH 4 (cation) and passed over HiTrap (GE healthcare) anion or cation exchange columns. Columns are washed with Tris or acetic acid buffer and then eluted with 0.5M NaCl in Tris or acetic acid buffer. Fractions are lyophilized and resuspended in acetonitrile twice to deplete fractions of buffer components and NaCl.

Oasis ion exchange columns

Supernatant was passed over columns at neutral pH, or acidified with 20μl concentrated HCl per ml for MCX and WAX columns, or 20μl concentrated NH_4OH per ml for MAX and WCX columns. Columns were washed with 5% HCl (MCX and WAX, W1), or 5% NH_4OH (MAX and WCX, W1) then 100% methanol (W2), and eluted with 5% HCl in water (MAX and WCX) or 5% NH_4OH in water (MCX and WAX).

HILIC columns

Hydrophilic interaction chromatography (HILIC) Macro-spin SPE columns were purchased from the Nest group, Inc (Southborough, MA). Columns are wet with methanol, rinsed with distilled water, conditioned with 0.2M sodium acetate 0.3M sodium phosphate buffer for 2 hours, and rinsed with 90% acetonitrile (Fisher)/10% 30mM ammonium formate (Fisher). 500 μl supernatant in 30mM ammonium formate is diluted to 90% acetonitrile then spun over column in 500μl aliqouts. Columns were washed twice with 90% acetonitrile/10% 30mM ammonium formate, and retained compounds were eluted using a 10% step-wise gradient of acetonitrile/ammonium formate, 250μl per elution. Column fractions were lyophlized and resuspended in distilled water.

Mass Spectrometry

Supernatant fractions were analyzed using an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA) that was connected in-line with an Agilent 1200 series autosampler and quaternary pump (Santa Clara, CA). Sample solutions contained in autosampler vials sealed with septa caps were loaded into the autosampler compartment prior to analysis. An injection volume of 75 μL was used for each sample. The injected sample aliquot was pumped to the ESI probe of the mass spectrometer for a period of 8 min using a flow of 99.9% acetonitrile/0.1% formic acid (v/v) delivered at a flow rate of 50 μL/min. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-contamination between samples. The connection between the autosampler and the ESI probe of the mass spectrometer was made using PEEK tubing (0.005" i.d. _ 1/16" o.d., Western Analytical, Lake Elsinore, CA). External mass calibration was performed prior to analysis using the standard LTQ calibration mixture containing caffeine, the peptide MRFA, and Ultramark 1621 dissolved in 51% acetonitrile/25% methanol/23% water/1% acetic acid solution (v/v). The ESI source parameters were as follows: ion transfer capillary temperature 275 ºC, normalized sheath gas (nitrogen) flow rate 25%, ESI voltage 2.0 kV, ion transfer capillary voltage 20 V, and tube lens voltage 45 V. Mass spectra were recorded in the positive ion mode over the range m/z = 80 to 1500 using the Orbitrap mass analyzer, in profile format, with a full MS automatic gain control target setting of 5 _ 10^5 charges and a resolution setting of 6 _ 10^4 (at m/z = 400, full width at half-maximum height). Mass spectra were processed using Xcalibur software (version 4.1, Thermo).

Lactate measurement

Samples for lactate measurements were collected in MEM without phenol red or $NaHCO_3$ (Sigma), with 25mM HEPES, 2mM L-glutamine, 1% penicillin/streptomycin mix and 1% FBS. For "normal media" conditions, $NaHCO_3$, glucose, and FBS were added to final concentrations of 3.7 g/L, 4 g/L, and 10%, respectively. Lactate measurement was performed enzymatically as previously described (Gutmann and Wahlefeld, 1974). Briefly, samples (25 µl) were incubated in glycine/hydrazine hydrate buffer pH 9 + 10µl 2N NaOH with 250 µl 2mg/ml NAD^+ (Sigma) and 1.13 units/ml lactic dehydrogenase (Sigma) for 40 min at 37°C in a 96 well plate. Lactate concentration was observed as an increase in the formation of NADH, which was measured by absorbance at 340nM.

Results

Resistant 3T3s and 293Ts inhibit conversion in permissive HFFs and Veros by the release of soluble mediators

To examine cell type differences in conversion induced in our *in vitro* system, pairs of fibroblast cell lines (HFF and 3T3) and kidney cell lines (Vero and 293T) were infected with Pru gra8-RFP bag1-YFP parasites and cultured in bradyzoite converting conditions for 48 hours then analyzed by flow cytometry for RFP and YFP expression (Figure 1A). HFF and Vero cells

permited a high level of bradyzoite conversion (30% and 52%, respectively) whereas 3T3 and 293T cells were highly resistant to conversion, resulting in only 2.8 and 2.3% conversion, respectively.

To determine the molecular basis for the apparent cell intrinsic difference in the ability to facilitate bradyzoite conversion, infected permissive cells were co-cultured with increasing numbers of uninfected resistant or permissive cells (Figure 1B). Conversion within the infected permissive cells alone (black bar) was not affected with the addition of uninfected permissive cells (hatched bars) but suprisingly was inhibited by the addition of uninfected resistant cells (white bars). The same pattern was observed for the pairs of fibroblast and kidney cell lines. These data demonstrate that the resistant cells were able to inhibit conversion within permissive cells *in trans*, and the level of *trans* inhibition increased with the number of resistant cells added. Similarly, when resistant cells were plated at increasing cell density, the result was even further resistance to conversion, whearas the density of permissive cells had no effect upon conversion (Figure 1C). Furthermore, when permissive and resistant cells were plated together at ratios ranging from 90% permissive/10% resistant, to 10% permissive/90% resistant, the resulting level of conversion within permissive cells mirrored the level of conversion within the resistant cells (Figure 1D), suggesting the level of cell-extrinsic inhibition of conversion within permissive cells depended on the level of cell-intrinsic conversion within resistant cells. These data suggest the cell-extrinsic inhibitory effect mediated by 3T3 and 293T cells is consistent with a titratable inhibitor that regulates both cell-intrinsic and cell-extrinsic levels of conversion. To test if these inhibitory factor(s) are soluble, supernatants from 3T3 and 293T cells were added to converting cultures of infected HFF and Vero cells, and the supernatants were sufficient to mediate inhibition of conversion within permissive cells (Figure 2A). This, to our knowledge, is the first demonstration of soluble factor(s) released from uninfected, non-immune cells that are capable of inhibiting bradyzoite conversion.

293T supernatants enhance parasite growth in converting conditions without affecting host cell cycle progression

Since bag1 induction is an early event in bradyzoite development, with expression detected within 24 hours of bradyzoite induction (Bohne et. al. 1997), we determined the ability of the supernatant to promote continued parasite growth as another measure of inhibition of conversion. Using another transgenic strain of Pruniaud parasites that express YFP under control of the tubulin promoter (a gift from D. Roos, U. Penn), we examined the effect of 293T supernatant on parasite growth. Flow cytometric analysis of cells infected with this strain of parasites yields sharp peaks of fluorescence that correspond to cells containing 1, 2, 4, etc parasites per cell, and the relative growth of the parasites can measured as the percentage of host cells containing different numbers of parasites. Infected cells were cultured in normal media conditions (DMEM + 5% FBS + 5% CO_2) and in bradyzoite-inducing conditions, with and without 293T supernatant. Cells were harvested at 40 hours post-infection and analyzed for YFP expression by flow cytomtery. Under normal media conditions, the addition of 293T supernatant did not affect growth of the parasites (Figure 2B). Under bradyzoite-inducing conditions, however, the addition of 293T supernatant resulted in enhanced parasite growth. By comparing the percentage of host cells in each peak with and without the addition of supernatant, we observed a reduction in the percentage of cells containing fewer parasites (1, 2) and an increase

in the percentage of cells containing higher parasite numbers (8, 16) with the addition of 293T supernatant (Figure 2B). The lack of bag1 reporter induction and enhanced proliferation demonstrate that inhibitory supernatants maintained continued growth as a tachyzoite under metabolic stress induced by CO_2/HCO_3^- restriction.

We then wanted to determine if the effect of the supernatant on parasite growth was specific to the parasites, or if host cell growth was also affected. One of the few host genes that has been identified as having an affect on bradyzoite conversion is human cell-division autoantigen-1, which inhibits both the growth of the parasite and host cell, thus leading to bradyzoite conversion (Radke et. al. 2006). To examine if enhanced host cell growth was perhaps leading to inhibition of bradyzoite conversion, we asked if adding 293T supernatant to uninfected HFFs cultured in converting conditions resulted in enhancement of host cell cycle progression. 10% FBS was added as a control for enhanced cell cycle progression. We found that while 10% FBS did result in enhanced cell cycle progression, there was no difference between media alone or 293T supernatant (Figure 2C). Thus, the inhibitory factor(s) in the supernatant specifically promoted growth of the parasites as tachyzoites under metabolic stress, without any effect on host cell growth.

Inhibitory factor(s) are consistent with small molecular weight metabolites

We then wanted to determine the identity of these novel inhibitory factor(s). We performed initial characterizations to determine the size of the inhibitory factor(s), and whether or not the stability of the factor(s) was consistent with a protein. Supernatant was spun over a 3kD cut off column, and dialyzed through 1kD cut off dialysis tubing. The full inhibitory activity was in the flow through of the column, and was lost in dialysis (Figure 3A). Supernatants were subjected to various treatments that would destroy the activity of proteins: incubation with trypsin, proteinase k, and boiling for 10 minutes. When compared to the activity of untreated control supernatants, none of these treatments resulted in any loss of activity (Figure 3A). Thus, the characteristics of the inhibitory factor(s) were not consistant with proteins, but rather with small molecular weight metabolites.

Reversal of conversion induced by pyrimidine starvation is specific to uridine

Because the *in vitro* system used to induce bradyzoite conversion is based upon metabolic stress, we sought to examine whether cross-talk between different metabolic pathways might be able to non-specifically reverse conversion induced by CO_2/HCO_3^- restriction. We tested the ability of various metabolites to inhibit conversion, including some for which the parasite has been shown to be auxotrophic (adenosine, Perrotto et. al. 1971, Schwartzman and Pfefferkorn, 1982 and choline, Gupta et. al. 2005). We found that the ability to reverse conversion induced by pyrimidine starvation was specific to uridine (Figure 3B), which is the starting point for synthesis of other pyrimidines (Asai, et. al. 1982). In fact, the data suggested that the effect of adding exogenous metabolites tended to enhance rather than inhibit conversion, as three of the metabolites tested resulted in enhancement of conversion, presumably by adding additional stress to the parasites. These results alleviated our specificty concerns regarding inhibition of conversion in our system, but it did not rule out that the inhibitory effect we were studying was specific to conversion induced by pyrimidine starvation.

Induction of conversion by inhibtion of host cell autophagy is inhibited by 293T supernatant

An inhibitory effect on conversion specific to induction by CO_2/HCO_3^- restriction would perhaps be interesting, but somewhat less relevant to the pathogenesis of *T. gondii in vivo*. We attempted to test the ability of the 293T supernatant to inhibit conversion by other published methods, such as alkaline pH and heat shock, but these methods did not result in robust conversion when adapted to our system (data not shown).

In the absence of another another system with which to test our supernatant, we looked for another more physiologic metabolic stress that may also induce conversion. Recently, Wang *et. al*. (2009) have shown that host cell autophagy is induced upon infection with *T. gondii*, and that parasite growth is inhibited within autophagy-deficient cells at physiologically relevant amino acid levels. We postulated that inhibition of host cell autophagy might lead to an amino acid deficiency that could act as a metabolic stress resulting in parasite conversion to bradyzoites. Indeed, treatment of infected cells with 3-methyl adenine (3-MA), an autophagy inhibitor, resulted in induction of bradyzoite conversion under normal media conditions (DMEM + 5% FBS + 5% CO_2). Additionally, the addition of 293T supernatant inhibited conversion induced by 3-MA (Figure 4). Identification of autophagy inhibition as an inducer of bradyzoite conversion provides a new tool with which to study bradyzoite conversion *in vitro,* and demonstrates that *trans* inhibition by 293T supernatants overrides conversion mediated by two distinct metabolic stresses.

Biochemical purification and identification by mass spectrometry reveals lactic acid as unique to inhibitory column fractions

We then proceeded towards identifying the factor(s) that promote continued growth as a tachyzoite under metabolic stress. The strategy was to identify components of inhibitory supernatants by mass spectrometry of "purified" fractions from column chromatography. The starting point was supernatant collected from washed 293T cells in Hank's balanced salt solution (HBSS) without serum and run over a 3kD cutoff column. 293T supernatant was passed over standard C18 reversed phase columns and Oasis® brand hydrophilic-lipophilic balance reversed phase columns, however no retention was detected based on the presence of inhibitory activity exclusively in the column flow through and not in the elutions (Figure 5A). We then attempted to purify inhibitory factor(s) on ion exchange columns, both from GE and Waters Oasis® brand, however no rentention was detected on these columns as well (Figure 5B).

Our final attempt at purifying the inhibitory factor(s) utilized hydrophilic interaction chromatography (HILIC) columns. 293T supernatant was passed over HILIC columns, and inhibitory activity was detected in fraction 2 (Figure 6A), although much inhibitory activity remains in the flow through. This purification was performed several times, and although we consistently detected inhibitory activity in elution 2, variable levels of retention were achieved as evidenced by variable levels of activity remaining in the flow through. Nonetheless, mass spectrometry was performed on the active fraction, as well as surrounding negative fractions. Mass spectrometry revealed a major peak at 135.003 m/z unique to active fractions, corresponding to $C_3H_6O_3$ (Figure 6B). While there are several different chemical compounds

with this formula, a disodium adduct was consistent with a strong acid, making lactic acid (or lactate) the best candidate.

Lactate is an inhibitory component of 293T supernatant

We then sought to validate lactic acid (lactate) as an inhibitory component of 293T supernatant by depletion of lactate from the supernatant and determining if this resulted in a loss of inhibitory activity on conversion. Lactate release from 293T cells was prevented by two classes of inhibitors: sodium oxamate (NaOx) is a lactate dehydrogenase (LDH) inhibitor and thus inhibits the conversion of pyruvate to lactate, and phloretin is a monocarboxylate transporter (MCT) inhibitor thus inhibits lactate transport out of the cell. To demonstrate the efficacy of the inhibitors, 293T cells were cultured in normal media and starvation media conditions with and without the inhibitors, and supernatants are collected and assayed for lactate concentration 24 hours later. Both NaOx and phloretin treatment resulted in a decrease in lactate release from the cells in both media conditions (Figure 7A).

Next, 293T cells were treated with NaOx, phloretin, or left untreated as a control. Prior to harvest, inhibitors were washed out and cells were then incubated in HBSS as before for supernatant collection. Supernatants were added as a series of two-fold dilutions to infected Vero cells in converting conditions, and conversion was assessed at 48 hours post-infection (Figure 7B). At low dilution, the inhibitor treated and control supernatants equally inhibited conversion, however as they were diluted, the level of inhibition observed with the inhibitor treated supernatants was less than that observed with the control supernatant. By calculating the difference in the supernatant dilution required for 15% conversion where the curves were linear, it was determined that the NaOx ($p<0.002$) and phloretin ($p<0.005$) supernatant were respectively 69% and 67% as effective at inhibiting conversion as the control supernatant. These results suggest that lactate is at least partially responsible for the inhibitory effect of the 293T supernatant.

Discussion

The molecular mechanisms for varaible efficiency of bradyzoite conversion within different cell types have remained largely undescribed. Using an optimized *in vitro* system that results in robust induction of bradyzoite development, we have classified commonly used cell lines as either highly permissive or highly restrictive for *T. gondii* conversion. When co-cultured with restrictive cells, conversion was potently inhibited within permissive cells, and supernatants from restrictive cells were sufficent to mediate this inhibition. In addition to inhibiting bag1 reporter induction, inhibitory supernatants also promoted the continued growth of the parasites as a tachyzoite under metabolic stress. This is the first evidence for the existence of soluble factor(s) released from uninfected, non-immune cells that can potently inhibit bradyzoite conversion.

Initial characterizations of these inhibitory factor(s) revealed they were consistent with small molecular weight metabolites, and biochemical purification and subsequent mass spectrometric analysis identified lactic acid as an abundant compound unique to active column fractions. The identification of lactic acid as a candidate for the inhibitory factor in 293T

supernatants was consistent with the characterization data demonstrating the active component was small and non-protein in nature, and blocking lactate release from 293T cells revealed that lactate can account for at least some of the inhibitory activity of the 293T supernatant. The following chapter will describe experiments that further test the effect of lactate on conversion, and, as lactate is a product of glycolysis, also address the broader impact of host cell glycolysis on conversion.

In the process of validating the physiological significance of cell extrinsic inhibition by 293T and 3T3 cells, we have developed a new tool with which to study bradyzoite conversion *in vitro*. Wang *et. al.* (2009) demonstrated that infection with *T. gondii* results in the induction of host cell autophagy and that parasite growth is deficient within autophagy deficient cells. Their data suggests that the parasites may actively direct nutrient-containing autophagosomes to the parasitophorous vacuole, further suggesting that nutrient acquitision is a critical barrier to survival within host cells of these large, metabolically demanding parasites. Futhermore, our data suggest a potential role for metabolic stress in the induction of parasite conversion in a real physiological setting. Further experiments must be done in order to explore the role autophagy may play in the regulation of parasite conversion *in vivo*.

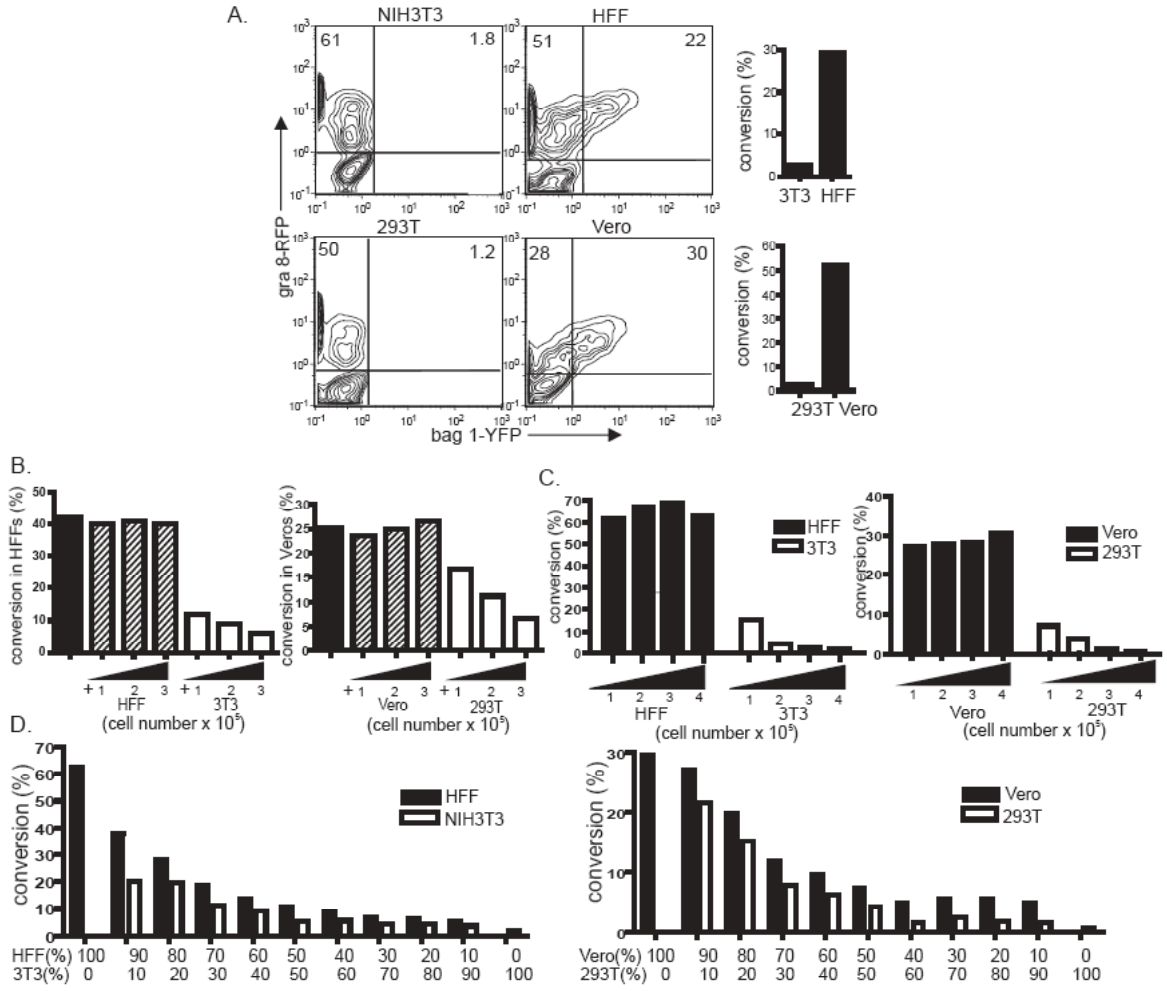


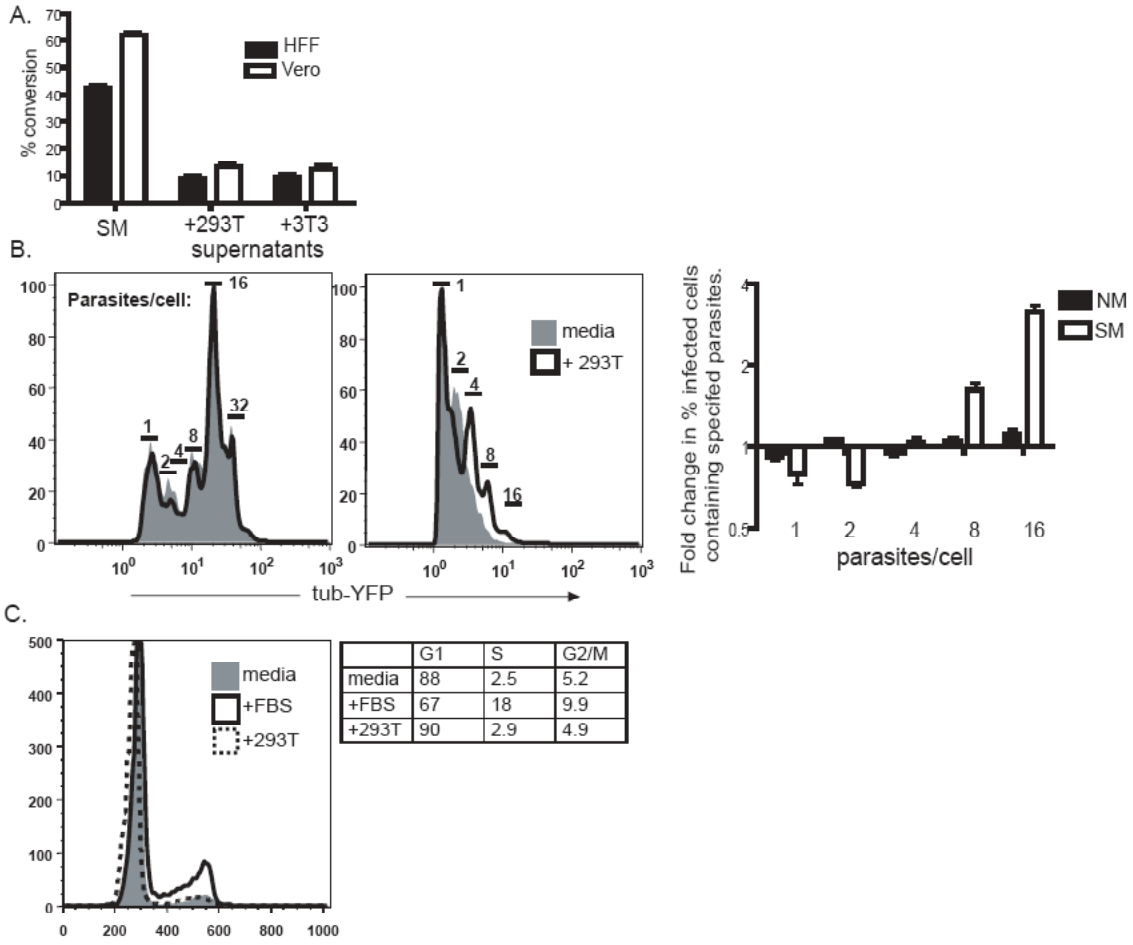
Figure 1. Resistance to conversion is mediated by a titratable soluble factor.

(A). NIH3T3 and 293T cells displayed a resistant phenotype to conversion, whereas HFF and Vero cells were highly permissive for conversion.

(B). Co-culture of resistant cells with permissive cells results in inhibition of conversion within permissive cells. $1x10^5$ infected HFF IRES Thy1 cells were cultured alone (black bar), or with the addition of 1, 2, or $3x10^5$ uninfected wild type HFFs (hatched bars) or 3T3s (white bars). After 48 hour culture in converting conditions, cells were harvested and stained for Thy1, and conversion is analyzed within Thy1 positive cells. The same experiment wass performed with Vero and 293T cells.

(C). Increasing cell density results in a lower rate of conversion within resistant cells, but does not affect conversion within permissive cells. Infected cells were plated at 1, 2, 3, or $4x10^5$ per well, and conversion was assessed at 48 hours post-infection.

(D). The extent of cell extrinsic inhibition of conversion depends upon the level of cell intrinsic conversion within resistant cells. Infected resistant cells transduced with IRES Thy1 and wild type infected permissive cell pairs were mixed at various ratios, from 90% permissive/10% resistant to 10% permissive/90% resistant, with a total number of $4x10^5$ cells per well. Cells were harvested and stained for Thy1, and conversion was assessed within both Thy1 negative (resistant) and Thy1 positive (permissive) cells.



	G1	S	G2/M
media	88	2.5	5.2
+FBS	67	18	9.9
+293T	90	2.9	4.9

Figure 2. Cell supernatants from resistant cells mediate trans inhibition of parasite conversion and promote parasite growth as a tachyzoite without promoting host cell cycle progression.

(A). Supernatants collected from 293T and 3T3 cells inhibit parasite conversion in permissive cells. Supernatants were collected from 293T or 3T3 cells and added to converting cultures of HFF or Vero cells. Conversion wass assessed by flow cytometry 48 hours post-infection. Bars represent the percent conversion calculated from triplicate samples, +/- standard deviation

(B). 293T supernatant promotes continued growth as a tachyzoite under bradyzoite inducing conditions. HFFs infected with Pru tub-YFP_2 parasites were cultured in normal media (DMEM + 5% FBS + 5% CO_2, NM) or starvation media (SM) conditions with (heavy black line) and without (shaded line) 293T supernatant. Bars represent the fold change in the number of parasites per cell with the addition of 293T supernatant in normal media and starvation media calculated from triplicate samples, +/- standard deviation.

(C). 293T supernatant does not promote host cell cycle progression under converting conditions. HFFs are cultured in converting conditions for 2 days, then 293T supernatant or 10% FBS is added, and cells are harvested 2 days later and stained with propidium iodide and analyzed by flow cytometry for DNA content. Watson Pragmatic analysis is used to determine the percentage of cells in G1, S, and G2/M phases.

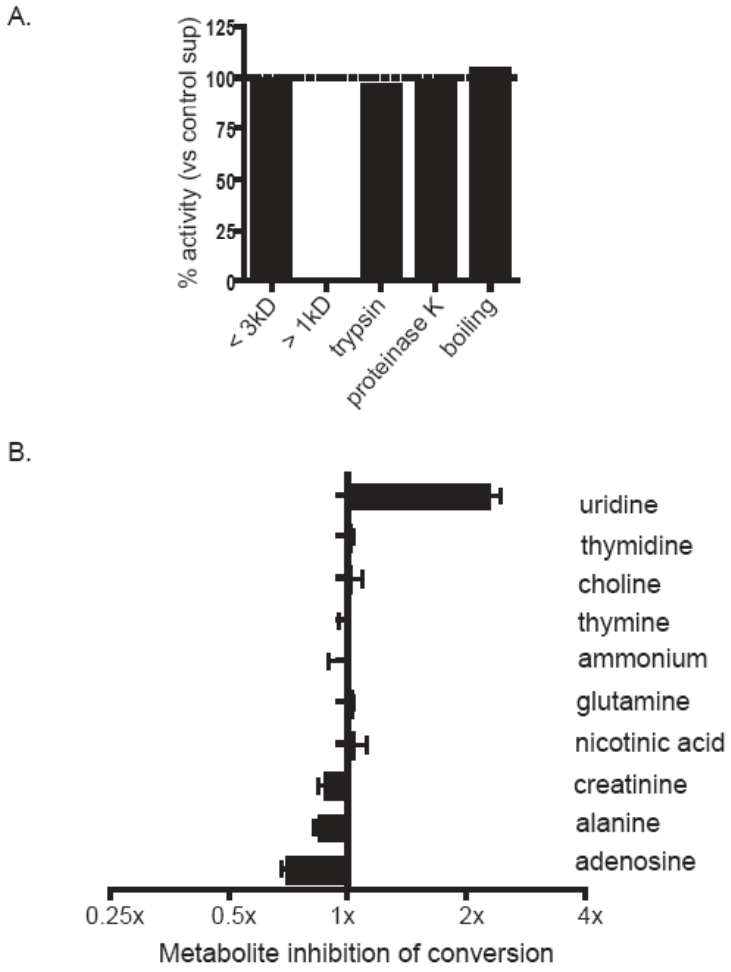


Figure 3. Inhibitory factor(s) are consistent with small molecular weight metabolites, and the reversal of conversion induced by CO_2/HCO_3^- restriction is specific to the complementation of pyrimidine synthesis by uridine.

(A). 293T supernatants were subjected to various treatments and tested for inhibitory activity. Bars indicate the percent inhibitory activity vs. control supernatant.

(B). Complementation of pyrimidine starvation by uridine specifically inhibits parasite conversion induced by CO_2/HCO_3^- restriction. Metabolites were added to infected HFFs in converting conditions. Bars indicate the fold inhibition of conversion calculated from triplicate samples versus media alone, +/- standard deviation.

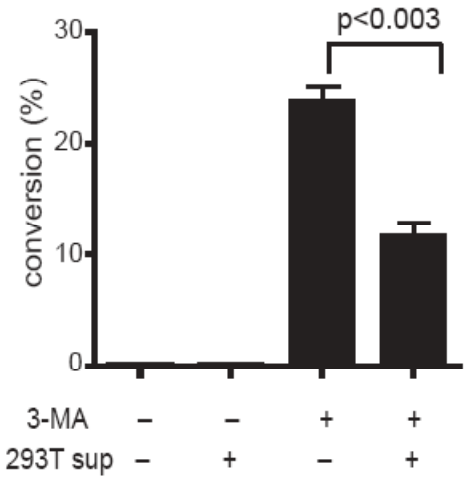


Figure 4. Inhibition of autophagy by 3-MA induces conversion, and conversion induced by 3-MA is inhibited by 293T supernatants.

Infected Vero cells were cultured in DMEM + 5% FBS + 5% CO_2 with or without 3-methyl adenine (5mM) and 293T supernatant. Bars indicate the mean percent conversion calculated from triplicate samples, +/- standard deviation. Student's *t*-test, $p<0.003$.

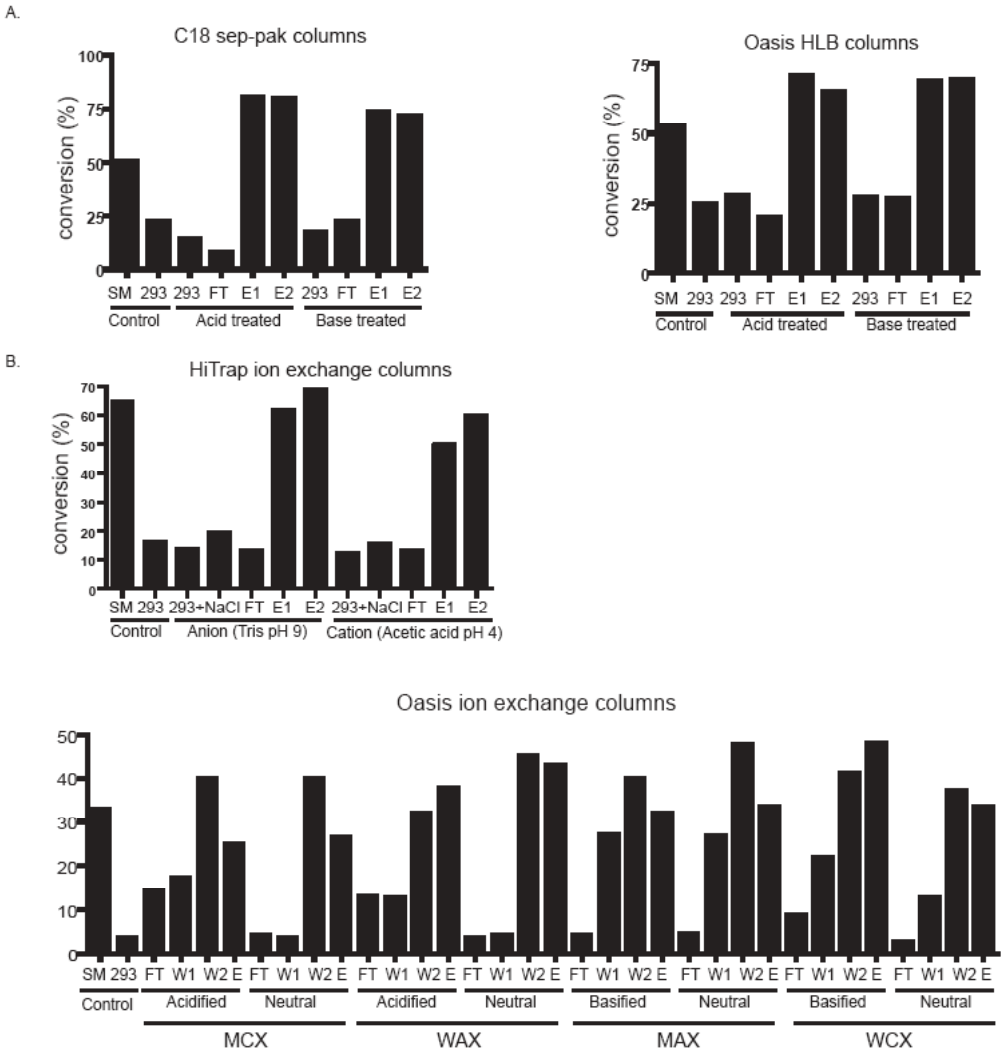


Figure 5. Inhibitory factor does not bind reversed phase or ion exchange columns

(A). Inhibitory factor does not bind to reversed phase columns. 293T supernatant was passed over C18 sep-pak and Oasis® hydrophilic-lipophilic balance (HLB) sorbent columns at acidic, basic, and neutral pH. Flow through, washes and elutions are lyophilized and resuspended in deionized water, then added to infected HFFs in converting conditions to test for inhibitory activity on conversion. Buffer alone controls are also tested for an effect on conversion. Bars indicate the percent conversion in each well in the presence of the indicated column fraction or control.

(B). Inhibitory factor does not bind to ion exchange columns. 293T supernatant is passed over GE anion and cation exchange columns, and Oasis® strong and weak anion (MAX and WAX, respectively) and strong and weak cation (MCX and WCX, respectively) exchange columns. Fractions are tested for inhibitory activity on conversion as described in (A).

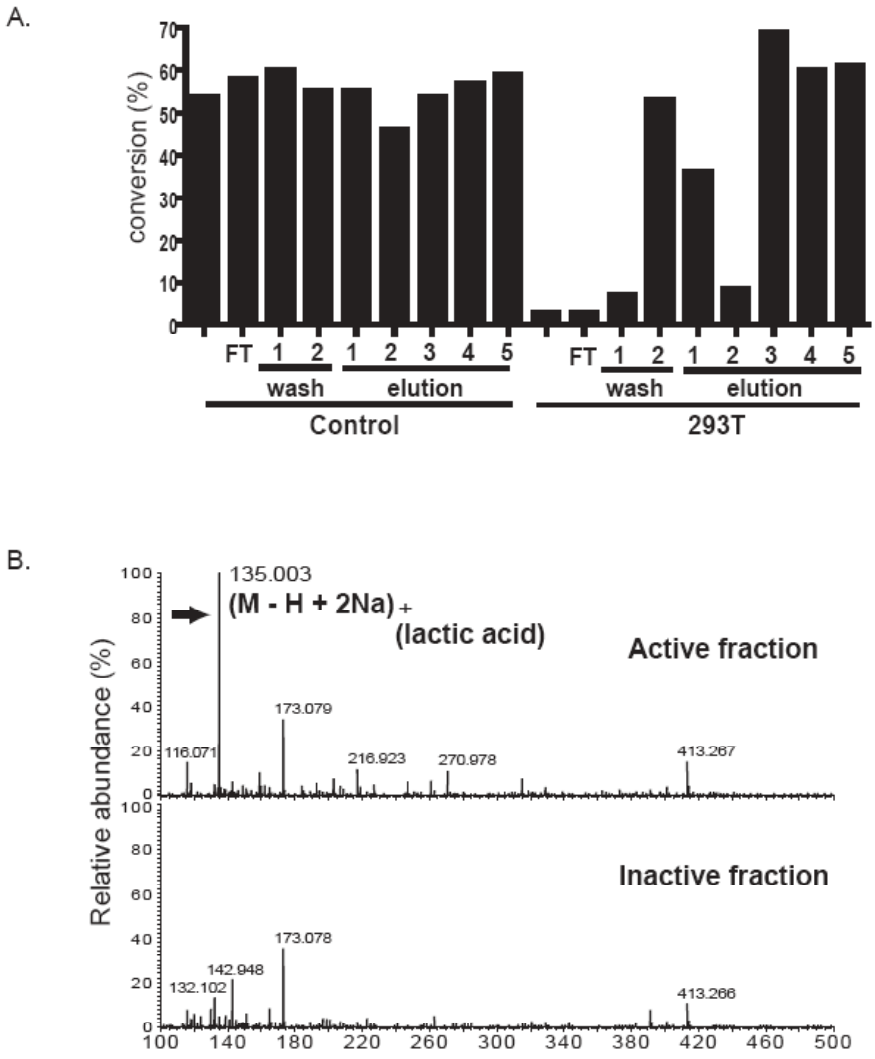


Figure 6. HILIC columns bind inhibitory factor, and mass spectrometry reveals peak unique to active fractions corresponding to lactic acid.

(A). HILIC columns demonstrate partial retention of inhibitory factor. 293T supernatant and buffer control are passed over HILIC columns and flow through, washes, and elution fractions are tested for inhibitory activity on conversion as described in Figure 5A. Plot shown is representative of 4 individual experiments.

(B). Mass spectra measured for active (top spectrum) and inactive (bottom spectrum) fractions of 293T supernatant. The singly charged positive ion at m/z = 135.003 (top spectrum) corresponds to the $(M - H + 2Na)^+$ ion of $M = C_3H_6O_3$ (lactic acid).

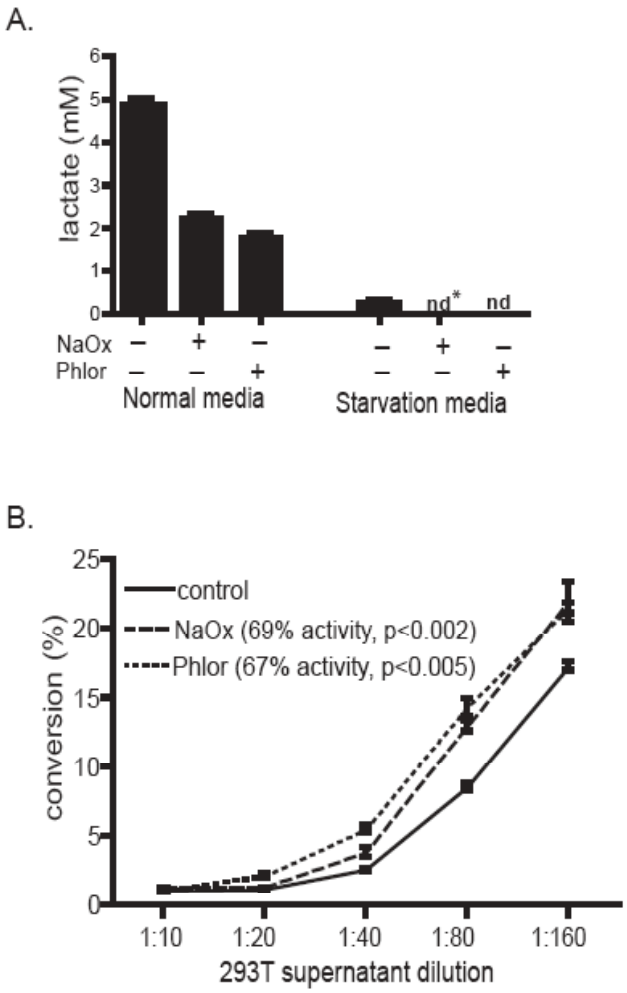


Figure 7. Lactate depletion decreases inhibitory activity of 293T supernatant.

(A). Sodium oxamate and phloretin inhibit lactate release from 293T cells. 293T cells were cultured in normal media and starvation media with and without sodium oxamate (NaOx) and phloretin (phlor) for 24 hours, then supernatant was collected and the lactate concentration determined. Bars indicate the concentration of lactate in each supernatant calculated from triplicate samples, +/- standard deviation. *nd = not detected.

(B). Depletion of lactate decreases inhibitory activity of 293T supernatant. Control, sodium oxamate (NaOx) treated, and phloretin (phlor) treated supernatants were assessed for inhibitory effect on conversion within Vero cells. Points are averages of triplicate samples +/- standard deviation and indicate the percent conversion with the addition of supernatant at the particular dilution. The relative activity of the inhibitor treated supernatants was calculated by comparison to control supernatant at 15% conversion where dilution curves are linear. P values calculated using the student's *t*-test are 0.002 and 0.005 for NaOx and phloretin supernatants, respectively.

CHAPTER 4

Host cell glycolysis exerts cell-extrinsic inhibition of *T. gondii* stage conversion

Introduction

Glycolysis is one of the most highly conserved pathways by which one molar equivalent of glucose is converted to two molar equivalents of pyruvate through a series of 10 enzymatic steps that take place in the cytosol. Two molar equivalents each of ATP and NADH are also generated per molar equivalent of glucose. Pyruvate and NADH are then used as substrates in the oxygen-dependent process of mitochondrial respiration for the total oxidation of glucose to CO_2 and H_2O, resulting in the generation of 36 total molecules of ATP per molecule of glucose. In limiting oxygen conditions, glucose is not converted entirely to CO_2 but instead to two molecules of lactate. Pyruvate is reduced to lactate, in the process regenerating the two molecules of NAD^+ that were reduced to NADH during glycolysis. This allows for the continued, although much less efficient, production of ATP in limiting oxygen conditions, such as during intense exercise. Since anaerobic generation of ATP by glycolysis is much less efficient than mitochondrial respiration, lactate has historically been considered a "waste product" of glycolysis. However, in recent years the biology of lactate has been appreciated to be interesting and complex.

It has now been shown that lactate is continuously producted even under fully aeorbic conditions (Brooks 1986, Richardson et. al. 1998). It is now appreciated that lactate produced by highly glycolytic cells is actively shuttled into oxidative cells and oxidized to pyruvate thus providing energy by feeding into the citric acid cycle (Brooks 2002). Furthermore, lactate has been proposed to act as a pseudo-hormone, acting as a metabolic signal at the whole-organism level to regulate metabolism across many tissues, either as an oxidizable substrate or as a gluconeogenic precursor (Brooks 2002, Philip et. al. 2005). Along those lines, lactate has been shown to regulate the gene expression of monocarboxylate transporters (MCT), channels through which lactate crosses the cellular membrane, and the expression of cytochrome *c* oxidase (COX), part of the mitochondrial lactate oxidation complex. Increasing lactate concentrations *in vitro* led to the transcription of MCT and COX genes (Hashimoto et. al. 2007). Thus lactate is not simply a waste product but rather is actively utilized as an energy source and can regulate the expression of genes involved its own biology.

The identification of lactate as an inhibitory factor of parasite conversion is intriguing based upon its connection to known *in vitro* stimulators of conversion and other aspects of parasite biology. Lactate regulates both intracellular and extracellular pH, which has been implicated as a stress that can induce bradyzoite conversion. Mitochondrial inhibitors have also been shown to induce conversion, and lactate is converted by LDH to pyruvate – the key metabolite utilized by the mitchondria. Removal of pyruvate is equivalent to inhibiting mitochondrial respiration using mitochondrial inhibitors. Additionally, upon infection host cell mitochondria are rapidly recruited to the PV membrane (Sinai et. al. 1997), presumably for energy acquisition. This provides another potential interplay between the parasites with lactate, particularly as a source of energy. Finally, lactate metabolism by the parasites themselves appears to be developmentally regulated, as different LDH genes are expressed in tachzoites and bradyzoites (Yang and Parmley, 1997). Thus, there are many potential points of interaction between parasite biology and lactate metabolism.

We explored the ability of lactate to inhibit conversion, as well as the broader role of host cell glycolysis in *trans* inhibition of bradyzoite conversion. We found that while lactate can indeed inhibit parasite conversion within Vero cells, it did not inhibit conversion within HFFs and depletion of lactate did not fully reverse the inhibitory effect of 293T supernatant. Therefore lactate cannot be the only inhibitory factor in the 293T supernatant.

However, the identification of lactate led us to consider the broader role of host cell glycolysis in *trans* inhibition of bradyzoite conversion. We found that the enhancement of host cell glycolysis by the addition of glucose and by the expression of myrAkt resulted in both cell-intrinsic and cell-extrinsic inhibition of parasite conversion. This finding represents a novel mechanism by which bradyzoite differentiation may be regulated *in vivo*; that the parasites may not be sensitive solely to the particular intracellular environment of the host cell, but also to the larger metabolic tissue environment.

Materials and Methods

Chemicals

Lactate was used either as lactic acid (Fisher) neutralized with 10N sodium hydroxide or as Lactated Ringer's solution (Baxter Healthcare). Pyruvate (Invitrogen) was used at 5mM. Akt VIII inhibitor (Calbiochem) was used at 100nM. Glucose (Fisher) was added to a final concentration of 4 g/L.

Phospho Akt staining

Cells were fixed and permeabilized with CytoFix/CytoPerm (BD Pharmingen) for 20 min on ice, then washed twice with HBSS + 2% FBS + 0.01% saponin. Cells were incubated with Alexa 647 conjugated anti-phospho-Akt-ser473 (Cell Signaling Technology) in HBSS + 2% FBS + 0.01% saponin for 1 hour at room temperature. Cells were washed twice with HBSS + 2% FBS + 0.01% saponin then analyzed using a Beckman Coulter FC-500, and data were plotted using FlowJo software (TreeStar).

Results

Inhibition of conversion by lactate is specific to Vero cells

To determine if lactate can inhibit bradyzoite conversion, lactate was added to infected HFF and Vero cells in converting conditions, and conversion was assessed 48 hours post-infection. Addition of lactate did result in inhibition of bradyzoite conversion within Vero cells at physiologically relevant concentrations, from 1.3 fold inhibition at 1mM to 2.7 fold inhibition at 3mM. However, lactate did not have a strong impact upon conversion within HFFs (Figure 1A). Considering our system of bradyzoite induction utilizes metabolic stress, we postulated that lactate may be acting as a nutrient for the parasite, thus facilitating continued growth. If so, we would expect pyruvate to have a similar effect on conversion. Surprisingly, the addition of pyruvate had the opposite effect, resulting in enhancement of bradyzoite conversion within Vero cells (Figure 1A), thus ruling out the simple nutrient hypothesis.

We then sought to determine if lactate production by the four cell lines correlated with the permissive or resistant phenotype. Cells were cultured in normal media and starvation media conditions, and supernatants were collected and assayed for lactate concentration 24 hours later. All cell lines released high levels of lactate under normal media conditions, and released much less in starvation media conditions (Figure 1B). The level of lactate released in either media condition, however, did not correlate with the permissive or resistant phenotype, i.e. 3T3 and 293T cells did not release significantly higher levels of lactate than HFF and Vero cells. However, the markedly lower levels of lactate release under converting conditions is perhaps consistent with a requirement for reduced glycolysis in the induction of bradyzoite conversion.

Although we have demonstrated that lactate has some inhibitory effect on conversion, it is apparent that lactate is not the sole mediator of the cell extrinsic inhibitory effect on conversion by resistant cell lines 3T3 and 293T. Unlike 3T3 and 293T supernatants, lactate does not inhibit conversion within HFFs, and inhibition of lactate release does not fully reverse the inhibitory effect of 293T supernatant on Vero cells. Clearly, there are other factor(s) released from resistant cells that are also capable of inhibiting bradyzoite conversion. We then became interested in exploring whether or not these factor(s) are, like lactate, regulated by host cell glycolysis. Thus, we asked the broader question of can enhanced host cell glycolysis inhibit parasite conversion in a cell extrinsic manner.

Glucose inhibits conversion concurrent with an increase in host cell glycolysis

We had previously tested the ability of glucose to inhibit conversion, and found that increasing the concentration of glucose in starvation media from 1g/L to 4g/L results in the inhibition of conversion within HFFs. In tissue culture media, glucose at 1g/L corresponds to normal physiological concentrations, while glucose at 4g/L corresponds to a hyperglycemic condition. We had initially assumed that the inhibition of conversion was due to increased availability of glucose as a nutrient to the parasites, thus facilitating continued growth as a tachyzoite. We were puzzled then when, we found that addition of glucose did not inhibit conversion within Vero cells (Figure 2A). If the parasite was able to directly access extracellular glucose as a nutrient, one would expect glucose to inhibit conversion regardless of the cell type. We postulated that the effect of glucose on conversion was not merely due to its use as a nutrient, but rather due to its ability to enhance host cell glycolysis. We then tested the effect of added glucose on glycolysis in HFF and Vero cells as measured by lactate production, and found that additional glucose enhanced lactate production by HFFs, but had no effect on the Vero cells (Figure 2B). The inhibitory effect of glucose conversion correlated with the ability of glucose to enhance glycolysis. Thus, induction of host cell glycolysis appeared to be a requirement for inhibition of parasite conversion by glucose.

Enhancement of glycolysis by glucose in HFFs exerts in cell extrinsic inhibtion of conversion in Veros

We then asked if the inhibitory effect of enhanced glycolysis on conversion was cell-intrinsic or cell-extrinsic in nature. We took advantage of the non-responsiveness of Vero cells to glucose and asked if enhanced glycolysis in the HFFs resulted in cell extrinsic inhibit of conversion within Vero cells. Infected Vero cells were co-cultured with uninfected Veros or

HFFs in 4g/L glucose, or cultured alone in 1g/L or 4g/L glucose as controls. As seen before, culture in 4g/L glucose did not inhibit conversion within Vero cells, nor did co-culture with uninfected Veros (Figure 2C). Conversion was, however, inhibited within Vero cells co cultured with HFFs. This demonstrated that enhanced glycolysis turned "permissive" HFF cells into a source of cell-extrinsic inhibitory factor(s).

MyrAkt expression exerts cell extrinisic inhibition of conversion independent of infection

We now reexamined our previous results of inhibition of conversion by myrAkt expression. We had initially been interested in Akt for its role as an anti-apoptotic gene, however another important function of Akt is in the regulation of glucose metabolism. Akt specifically upregulates glycolysis with a commesurate increase in mitochondrial respiration (Manning and Cantyley, 2007). Intriguingly, myrAkt expression also resulted in cell-extrinsic inhibition of conversion (Figure 3A). Retroviral transduction of HFFs was not 100% efficient, therefore the resulting pool of cells contained both transduced and untransduced wild type cells. In a mixed population of cells, conversion could be assessed separately within transduced and wildtype cells by antibody staining for Thy1, the cell surface marker used for transduction, before flow cytometric analysis of infected cells. When we assessed conversion within mixed populations of HFFs, we found that conversion was inhibited not only within cells expressing myrAkt, but also within neighboring wild type cells (Figure 3A). This cell-extrinsic inhibition of conversion, like the inhibition mediated by 3T3 and 293T cells, did not require parasite infection of myrAkt expressing cells.

The non-requirement for infection was rigorously tested by the use of transwell culture apparatus. Top and bottom chambers of the transwell are separated by a permeable membrane, allowing for free media exchange while the cells in each chamber remain separate. Cells expressing either myrAkt or empty vector were cultured either infected or uninfected in the top chamber of a transwell. Conversion was assessed within infected wildtype HFFs cultured in the bottom chamber of the transwell. Conversion was inhibited within the wildtype HFFs that were exposed to both infected and uninfected myrAkt expressing HFFs (Figure 3B). These results demonstrated that myrAkt expression results in the release of soluble inhibitory factors that are not dependent upon parasite infection.

Effect of myrAkt expression on conversion is specific to Akt, and does not promote parasite egress

To demonstrate specificity, we tested the ability of an Akt inhibitor to reverse the inhibitory effect of myrAkt on conversion. Staining for phospho-Akt indicated a partial inhibition of Akt phosphorylation by Akt VIII inhibitor under converting conditions (Figure 4A). We then tested the effect of Akt VIII inhibitor on conversion within both wild type and myrAkt expressing cells. Inhibitor treatment restored conversion to wild type levels in myrAkt expressing cells, and, additionally, led to enhancement of conversion within wild type cells (Figure 4B), $p<0.0006$. This result points to a potential physiologic role of endogenous Akt in the regulation of bradyzoite conversion.

Nagamune *et. al.* (2008) reported abscisic acid (ABA) controls parasite egress from host cells, and inhibition of ABA synthesis prevents egress and leads to bradyzoite formation *in vitro*. It is possible, then, that induction of parasite egress could lead to a perceived inhibition of bradyzoite conversion, by preventing parasites from persisting long enough within individual host cells to initiate the program of bradyzoite conversion. To determine if myrAkt expression impacted upon parasite egress, individual infected cells were tracked by microscope over time to determine if there was an increase in egress with myrAkt expression. MyrAkt expression did not change the percentage of cells containing either tachyzoites (YFP–) or bradyzoites (YFP+) on day 2 that remained intact on day 3 (Figure 5), demonstrating that myrAkt did not impact egress of tachyzoites or bradyzoites from host cells. Thus myrAkt did not inhibit conversion by preventing parasite persistence within host cells.

MyrAkt expression enhances glycolysis in HFFs and Veros, and also results in cell extrinisic inhibition of conversion by Veros that is enhanced by glucose

We next focused on determining whether the metabolic function of Akt was responsible for inhibition of conversion, and looked at the ability of myrAkt expression to enhance glycolysis in both HFF and Vero cells. Indeed, myrAkt expression resulted in enhanced glycolysis in both cell types as evidenced by increased lactate production (Figure 6A). We then tested the ability of myrAkt expression to inhibit bradyzoite conversion within Vero cells, and found that similarly, myrAkt expression resulted in both cell-intrinsic and cell-extrinsic inhibition of conversion in Vero cells (Figure 4B). Strikingly, myrAkt expression now renders the Vero cells sensitive to glucose, as greater inhibition of conversion is observed in cells expressing myrAkt in 4g/L glucose (2.2 fold inhibition) than in 1g/L glucose (1.7 fold inhibition, $p<0.04$). This indicates that the effect of myrAkt on conversion is tied to its role in glucose metabolism, as conversion in Vero cells was previously unaffected by glucose.

Importantly, the cell extrinsic effect on conversion by myrAkt expression in Vero cells was also enhanced by the addition of glucose, as inhibition of conversion within neighboring wild type cells co-cultured with myrAkt expressing cells in the presence of 4g/L glucose (1.4 fold inhibition) is greater than cell extrinsic inhibition at 1g/L glucose (1.2 fold inhibition, $p<0.05$). Moreover, the total amount of cell extrinsic inhibition by enhanced glycolysis in Vero cells, evident as the difference between conversion in cells alone at 4g/L glucose and in wildtype cells cultured with myrAkt expressing cells at 4g/L glucose, results in a 1.4 fold inhibition of conversion ($p<0.003$). These results allow us to conclude that cell-extrinsic inhibition by myrAkt is indeed tied to its role in glucose metabolism.

In the more highly glycolytic HFFs, the inhibition of conversion by the combination of myrAkt expression and 4g/L glucose resulted in a very large reduction in conversion, 5.4 fold inhibition. These results demonstrate that induction of host cell glycolysis can render permissive HFF and Vero cell lines resistant bradyzoite conversion, and furthermore can induce release of cell extinsic inhibitory factor(s).

Discussion

By exploring the molecular basis for the difference of in the ability of various cell lines to support bradyzoite conversion, we have identified a novel and unexpected role for cell-extrinsic regulation of *T. gondii* stage conversion by host cell glycolysis. Lactate was found to inhibit conversion within Vero cells, and while lactate did not account for the full cell extrinisic inhibitory effect of resistant cell lines 293T and 3T3, it led us to consider the broader role of glycolysis on conversion. By determining the ability of glucose to inhibit conversion within permissive cells, we found that inhibition correlated with lactate release and thus enhanced glycolysis, and furthermore, the inhibition by glycolysis was mediated by cell extrinisic factors, as the enhancement of glycolysis in glucose-sensitive HFFs resulted in the inhibition of conversion in the glucose-insensitive Veros. This result is striking, as it demonstrates that the parasites do not simply utilize glucose as a nutrient, but are dependent upon and thus sense the induction of host cell glycolysis to facilitate their continued growth as tachyzoites under metabolic stress.

The basis for the differential effect of lactate on inhibition conversion within HFFs and Veros are unclear. Likely key to understanding this difference is the finding that the enhancement of conversion with pyruvate is also specific to the Vero cells. Considering that the ratio of pyruvate to lactate is a key regulator of cellular redox, the opposite effects on conversion of pyruvate and lactate in the Vero cells may be due to the redox state of the cell. The effect on conversion of the redox state of the host cell has not been investigated, and this may be indicative of an interesting mechanism by which bradyzoite conversion is regulated. The HFFs may not be able to effectively transport lactate and/or pyruvate into the cell, or the regulation of cellular redox in the HFFs and Veros may be different.

The identification of lactate allowed us to reinterpret data we previously had on the cell-extrinsic inhibition of conversion by myrAkt expression. We had previously shown that myrAkt expression in HFFs resulted in the release of soluble factors from uninfected cells that could inhibit conversion, and expressing myrAkt in the Vero cells allowed us to definitively determine that the inhibitory activity of myrAkt is due to its role in glucose metabolism and glycolysis, based on the additive effect of myrAkt expression and glucose on cell extrinsic inhibition in the previously glucose insensitive Veros. Furthermore, inhibition of endogenous Akt resulted in the enhancement of conversion within HFFs, suggesting that Akt may be playing a role in the physiologic regulation of conversion.

The mechanism by which glycolysis exerts cell-extrinsic inhibition of parasite conversion is independent of 2 previously reported methods of enhancing conversion *in vitro*. Radke *et. al.* (2006) demonstrated that expression of CDA-1 can inhibit parasite growth as well as delay host cell cycle progression, leading to bradyzoite development. Nagamune *et. al.* (2008) demonstrated that inhibition of parasite egress from host cells can lead to the development of bradyzoites. Soluble inhibitory mediators from resistant host cells specifically enhance parasite growth without affecting host cell cycle progression, and do not enhance parasite egress from host cells. Therefore we have discovered a new mechanism by which bradyzoite conversion is regulated.

Although we have identified lactate as one cell-extrinsic inhibitory mediator, clearly lactate is not the only cell extrinsic inhibitory mediator released in conditions of enhanced glycolysis. To that end, we also tested glutamine, alanine and ammonium for an effect on conversion, which have all been shown to be released from different cell types as a consequence of glucose metabolism, and none had any inhibitory effect (Chapter 3, Figure 3B). In fact, the addition of alanine slightly enhanced conversion. Further work needs to be done to identify the other inhibitory factors. Additionally, once other mediators are known, experiments can then be designed to determine how the parasites senses these mediators and what pathways they are affecting within the parasite. Identification of these mediators will potentially provide key insight into a novel regulatory mechanism of bradyzoite differentiation *in vivo*.

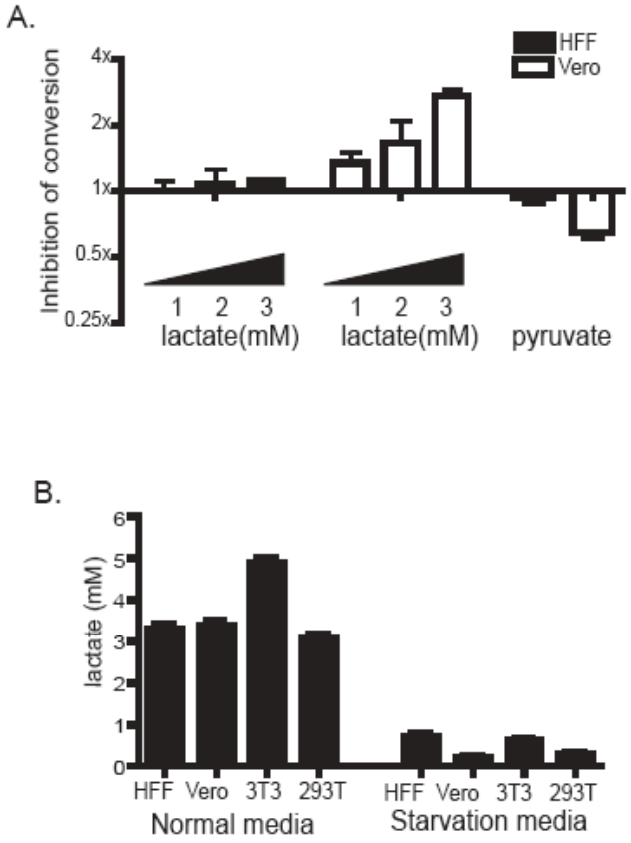


Figure 1. Lactate inhibits conversion within Vero cells, but release of lactate does not correspond to permissive or restrictive phenotype.

(A). Lactate inhibits and pyruvate enhances conversion in Vero cells. Lactate or pyruvate was added to infected HFF or Vero cells in converting conditions and conversion was assessed 48 hours post-infection via flow cytometry. Bars indicate the fold inhibition of conversion calculated versus media alone control from 3 independent experiments, +/- standard deviation.

(B). Lactate release does not correlate with permissive and resistant cells in either normal media or starvation media conditions. HFF, 3T3, Vero and 293T cells were cultured in normal media ($+CO_2$) and in starvation media ($-CO_2$) for 24 hours, then supernatant was collected and the lactate concentration was determined. Bars indicate the concentration of lactate in each supernatant calculated from triplicate samples, +/- standard deviation.

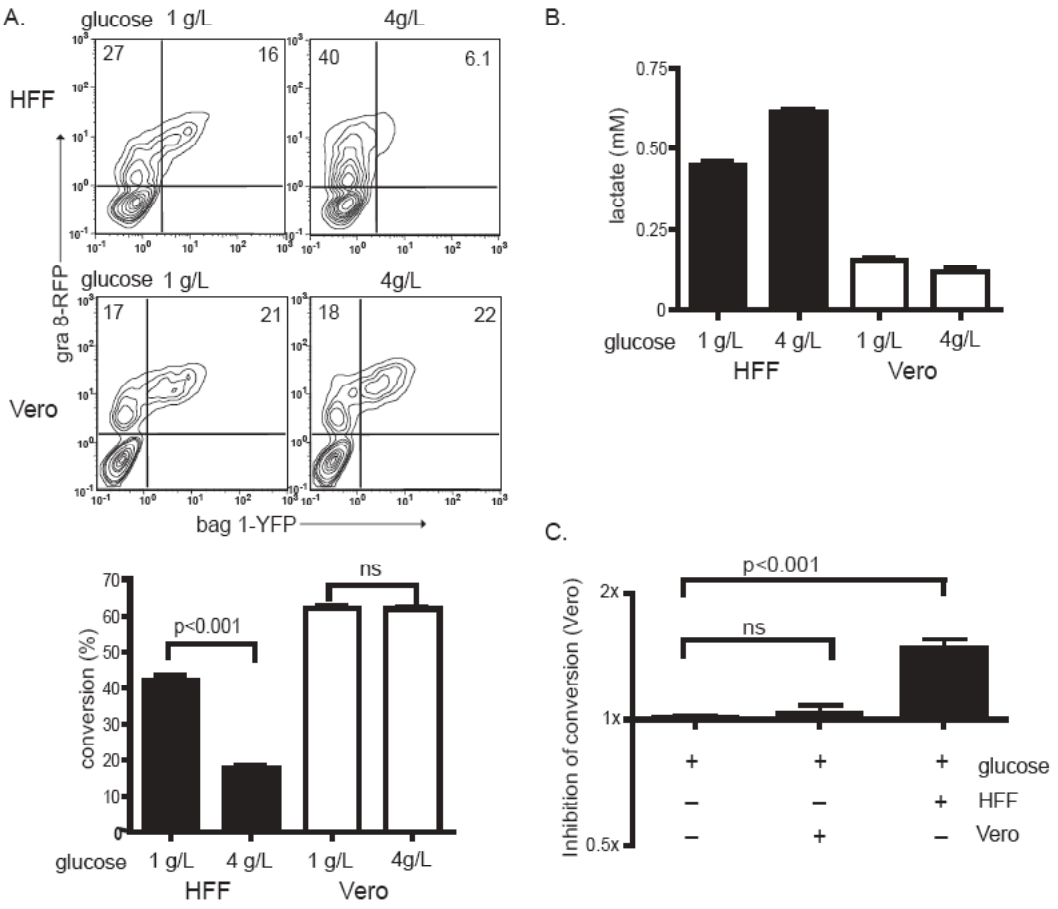


Figure 2. Enhanced glycolysis results in cell extrinsic inhibition of conversion by HFFs.

(A). 4g/L glucose inhibits conversion within HFFs but not Veros. Infected HFF and Vero cells were cultured in converting conditions at 1g/L or 4g/L glucose and conversion was assessed at 48 hours post-infection. Bars represent the mean percent conversion calcuated from triplicate samples, +/- standard deviation. Student's *t*-test, $p<0.001$.

(B). The addition of 4g/L glucose results in enhanced glycolysis by HFF, but not Vero cells. HFF and Vero cells were cultured in starvation media at 1g/L or 4g/L glucose. Supernatants were collected and assayed for lactate concentration at 24 hours. Bars indicate the mean lactate concentration calculated from triplicate samples, +/- standard deviation.

(C). Enhanced glycolysis within HFFs inhibits conversion within Vero cells in a cell-extrinsic manner. Infected Vero cells ($1x10^5$) are cultured in converting conditions at 4g/L glucose alone, or with the addition of $9x10^5$ uninfected Veros or HFFs. Bars represent the mean fold inhibition of conversion versus Vero cells cultured in normal converting conditions (1g/L glucose), calculated from triplicate samples in 2 independent experiments, +/- standard deviation. Student's *t*-test, $p<0.001$.

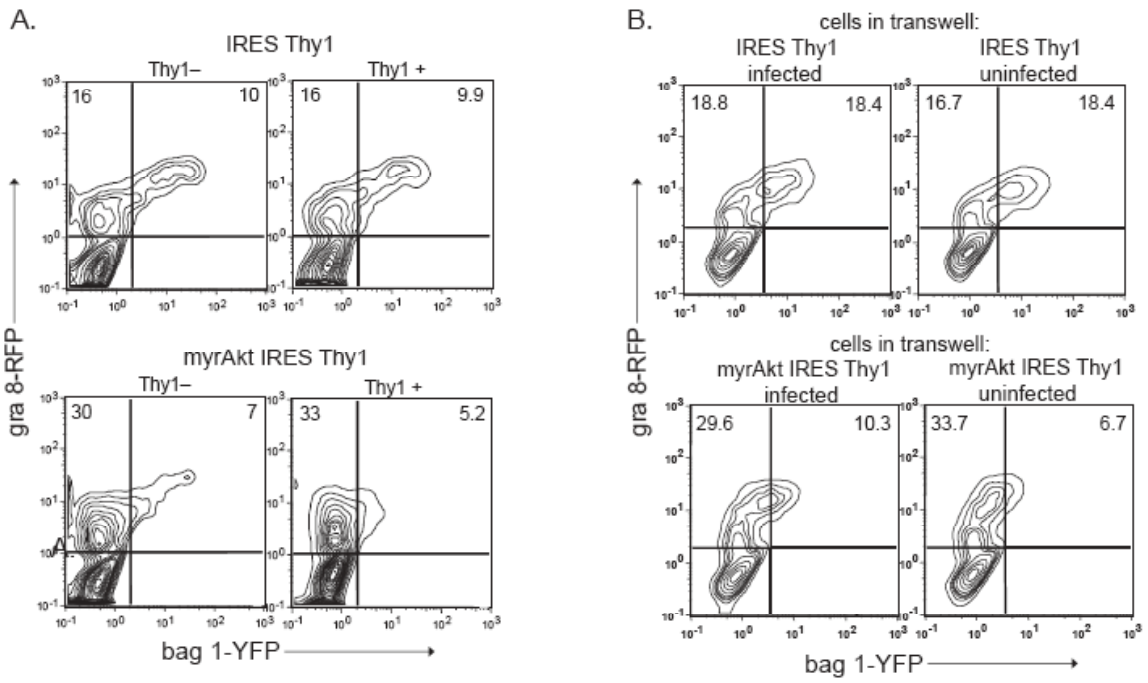


Figure 3. MyrAkt expression mediates cell-extrinisc inhibition of conversion.

(A). MyrAkt expression results in cell-intrinsic as well as cell-extrinsic inhibition of conversion. Bradyzoite conversion is assessed within mixed populations of HFFs that have been retrovirally transduced with myrAKT IRES Thy1 or IRES Thy1 empty vector control. Staining with anti-Thy1 antibody allows for assessment of conversion within transduced and untransduced cell populations.

(B). Cell extrinisic inhibition of converion by MyrAkt expression does not require infection. HFFs exressing myrAkt or empty vector control are cultured either infected or uninfected within a transwell chamber above infected wildtype HFFs. Conversion is assessed within wildtype HFFs. Labels correspond to the cell type within transwell chamber.

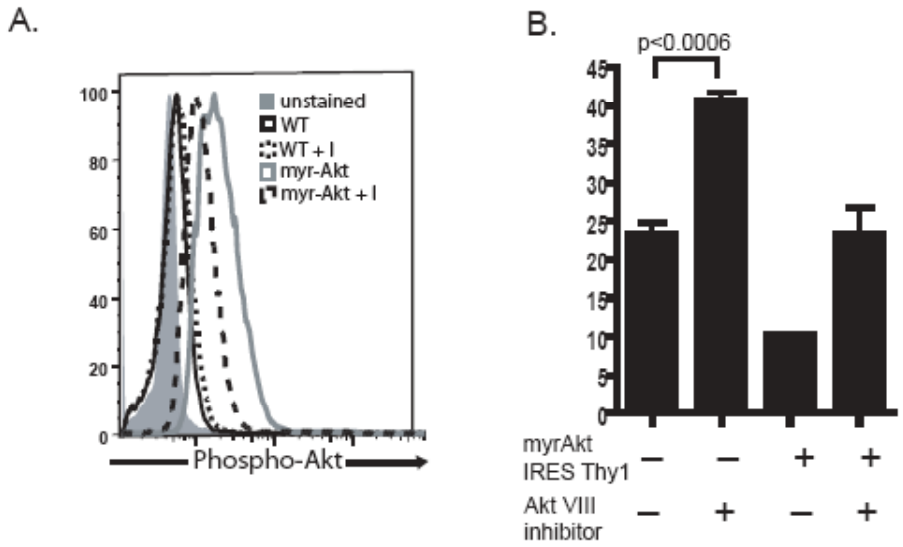


Figure 4. Inhibition of conversion by myrAkt is specific to Akt activation, and inhibition of endogenous Akt enhances conversion

(A). Phospho-Akt staining +/- Akt VIII inhibtor. Wildtype or myrAkt expressing HFFs are cultured in starvation media for 48 hours with and without Akt VIII inhibitor (100nM), then harvested and stained for phospho-Akt.

(B). Akt VIII inhibitor reverses the effect of myrAkt expression on conversion, and also enhances conversion within wildtype HFFs. Akt VIII inhibitor is added to infected wild type HFFs or HFFs expressing myrAkt in converting conditions, and conversion is assessed 48 hours after infection. Bars indicate percent conversion calculated from triplicate samples, +/- standard deviation. Student's *t*-test, $p<0.0006$.

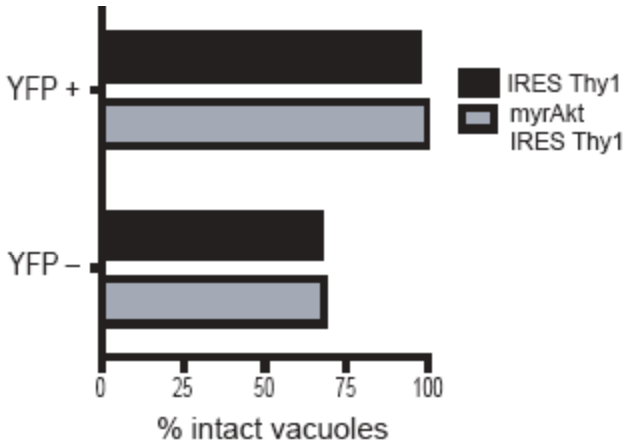


Figure 5. MyrAkt expression does not enhance egress of tachyzoites or bradyzoites.

Singly infected myrAkt expressing (gray bars) or IRES Thy1 control (black bars) cells are selected by microscope on day 2 post-infection and scored for YFP+ and YFP- parasites. Cells are scored again on day 3 as either intact or lysed. Bars represent the percentage of cells that remain intact on day 3 that contained either YFP+ or YFP– parasites on day 2.

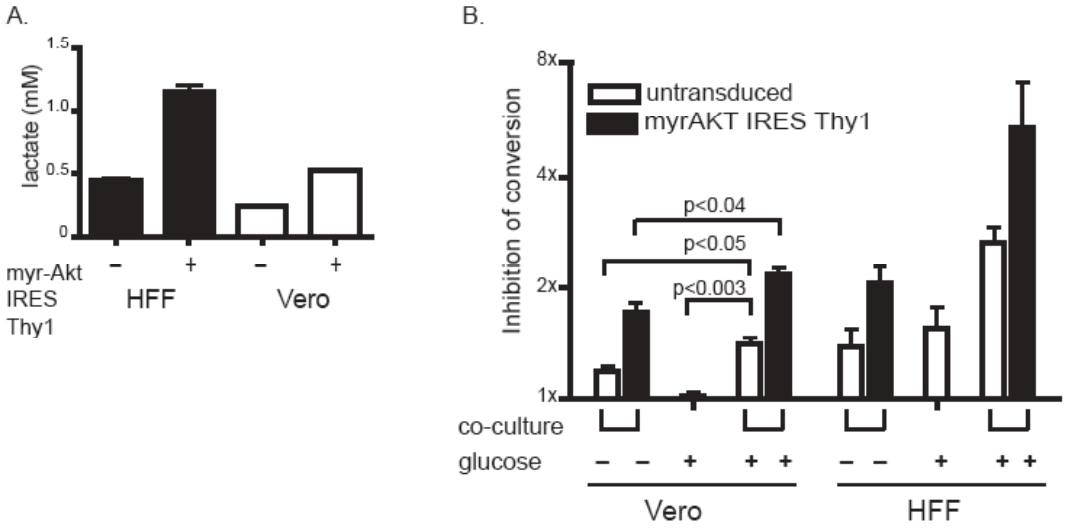


Figure 6. MyrAkt expression results in enhanced glycolysis, and cell-extrinsic inhibition of conversion by HFF and Vero cells.

(A). MyrAkt expression results in enhanced glycolysis in HFF and Vero cells. MyrAkt expressing or wildtype HFF and Vero cells were cultured in starvation media; supernatants are collected and assayed for lactate concentration 24 hours later. Bars represent lacate concentration calculated from triplicate samples +/- standard deviation.

(B). MyrAkt expression results in cell extrinsic inhibition by Vero, as well as HFF cells. Conversion was assessed within myrAkt expressing Vero and HFF cells, as well as in neighboring wild type cells, with or without the addition of 4g/L glucose. Brackets indicate co-culture of myrAkt expressing and wild type cells. Bars indicate inhibition of conversion versus wildtype cells calculated from triplicate samples in 3 independent experiments, +/- standard deviation.

CHAPTER 5

Conclusions and future directions

Using an *in vitro* system of bradyzoite induction, we have identified several new mechanisms by which *T. gondii* stage conversion is regulated. First, we have shown that enhancement of host cell glycolysis can support continued tachyzoite growth under metabolic stress conditions and thus inhibit bradyzoite conversion in a cell-intrinsic manner. Second, we have shown that cell lines that are intrinsically resistant to conversion, either basally or due to the induction of glycolysis, surprisingly release soluble mediators that inhibit conversion in *trans*. Importantly, these soluble mediators are released from uninfected, non-immune cells, suggesting that cell-extrinsic inhibition may be broadly restricting bradyzoite conversion *in vivo*. Finally, we have defined a new function for host Akt in *T. gondii* infection. Previous work had demonstrated that Akt is activated upon infection with *T. gondii* (Kim et. al. 2006), and this activation was important for the resistance to apoptosis observed in infected cells. We have provided evidence that the metabolic function of Akt in upregulating host cell glycolysis may take precedence over its role in host cell survival in the inhibition of *T. gondii* differentiation.

To date, there are no defined cellular and molecular mechanisms that adequately account for the remarkable tissue tropism of *T. gondii* growth and differentiation *in vivo*. The most commonly invoked explanation for the regulation of *in vivo* differentiation involves two alternative, but complementary, views of the overall function of the immune system. On one hand, immune surveillance has been postulated to induce bradyzoite conversion through the release of inflammatory mediators. A weakness of this hypothesis is that the physiological significance of the two mediators identified through *in vitro* studies, IFNγ and nitric oxide, in mediating induction of tissue-specific conversion is unclear. On the other hand, lack of immune surveillance in immunoprivileged sites, like the central nervous system, has been postulated to result in enhanced parasite growth at these sites resulting in tissue specific encystment. A weakness of this immune privilege hypothesis is that it does not account for the tissue-specific encystment in muscle tissue of adults and in neural tissues of immunocompromised fetuses and neonates.

Although we did not have the opportunity to directly test the *in vivo* significance of our findings, our data suggest two new hypotheses as to how growth and differentiation of *T. gondii* may be regulated *in vivo*, not by the immune system, but instead by differential regulation of a common host metabolic pathway. One, cells may broadly be releasing inhibitory mediators, making many tissues inhospitable to conversion, and thus restricting cyst development to particular tissues. In determining the identity of *trans* factor(s) which may play a role in the inhibition of conversion *in vivo*, we were surprised by the identification of lactate as a soluble inhibitory mediator present in 293T supernatants. Lactate is produced in high amounts in glycolytic tissues, such as brain, retina and muscle, which are precisely the tissues in which conversion occurs *in vivo*. The contradictory nature of the identification of lactate may be in part reconciled by the second hypothesis suggested by our data indicating that the parasite is sensitive to the glycolytic state of the overall tissue environment. In this hypothesis, the preferential encystment seen in highly glycolytic tissues may be a result of the ability of these tissues to sustain enhanced tachyzoite growth and increased parasite load, and if conversion of tachyzoites to bradyzoites is spontaneously occurring at some level *in vivo*, increased parasite load may lead to a higher incidence of cyst development in these tissues. The *in vivo* pattern of bradyzoite development may be partially explained by a combination of these two hypotheses, a postulation that is at this point speculative. Nonetheless, our results provide a new framework within which

the metabolic mechanisms that regulate parasite growth and differentiation *in vivo* can be investigated. Each of these hypotheses will now be discussed in more detail.

Glycolysis in host-parasite interactions

Many *in vitro* studies have focused on parasite metabolism and how they acquire and utilize nutrients from the host cell, as the metabolic demands of large apicomplexan parasites are critical features of their growth and survival within host cells. In particular, glycolysis has been implicated as the critical source of energy for *T. gondii* motility (Pomel et. al. 2008, Starnes et. al. 2009). Pomel *et. al.* (2008) demonstrate that strikingly, glycolytic enzymes are repositioned within the parasite depending on whether it is intracellular or extracellular. Glycolytic enzymes are concentrated near the plasma membrane while extracellular, as opposed to distributed uniformly throughout the cytoplasm while inside a host cell. The authors postulate this allows for optimal ATP utilization by those processes especially critical to either extracellular parasites, such as motility, or intracellular parasites, such as growth and replication. The critical importance of glycolysis is demonstrated by the high level of regulation governing the glycolytic enzymes. Furthermore, much work has also been done defining the metabolic differences between the fast-replicating tachyzoite and quasi-dormant bradyzoite stages of the parasite life cycle (Tomavo 2001) and in fact, several studies have demonstrated that *T. gondii* express several glycolytic enzymes in a stage specific manner (Manger et. al. 1998, Dzierszinski et. al. 1999, Yang and Parmley, 1997). Of particular interest considering our identification of lactate as an inhibitory factor of stage conversion is that *T. gondii* has two isoforms of LDH that are differentially expressed in tachyzoites and bradyzoites. The sum of this data points towards a highly regulated role of parasite glycolysis and specifically lactate metabolism in the differentiation of bradyzoites.

Surprisingly less attention has been focused on the role of host cell metabolism in *T. gondii* differentiation. Studies aimed at broadly defining the sum total of host transriptome and proteome changes in response to parasite infection have identified host glycolytic enzymes as upregulated upon infection both at the RNA (Blader et. al. 2001) and protein level (Nelson et. al. 2008), but investigation into the function or benefit of this upregulation has been limited. Interestingly, Spear *et. al.* (2006) demonstrated that HIF1α, the transcription factor that regulates glycolytic enzymes, is upregulated by the parasite in a cell non-autonomous fashion. Additionally, host Akt has been shown to be activated upon infection (Kim et. al. 2006), and while this was thought to serve primarily an anti-apoptotic function, our data suggest that the activation of Akt by parasite infection may actually serve a metabolic function for the parasite. Interestingly, a similar phenomenon is seen with *Plasmodium falciparum* infection, the causative agent of malaria. Erythrocytes infected with *P. falciparum* have dramatically upregulated glycolytic enzymes and utilize glucose at a level 50-100 times higher as uninfected erythrocytes (Roth et. al. 1988), and interestingly, infected erythrocytes released a soluble factor that decreased glycolysis in neighboring, uninfected erythrocytes (Mehta et. al. 2006).

These results indicate that the induction of host cell glycolysis may be advantageous to various intracellular parasites, and the importance to the parasites is perhaps further indicated by the fact that both *P. falciparum* and *T.gondii* can control host cell glycolysis in a cell non-

autonomous fashion. However, the benefit to the parasite is not necessarily obvious. While it is clear that *T. gondii* are dependent upon the host cell for many metabolic processes, it is not known how precisely the parasite acquires energy from the cell. It has been postulated that mitochondrial recruitment to the PVM upon infection serves to provide the parasite with energy, but whether that energy comes in the form of pyruvate, lactate, ATP or another metabolic intermediate is unknown. How exactly the parasite is sensing and utilizing the enhancement of host cell glycolysis to support continued growth as a tachyzoite remains to be determined. Nonetheless, our results have placed host cell metabolism, along with inflammatory mediators and cell cycle regulation, on a short list of host features that impact upon bradyzoite differentiation.

Enhanced glycolysis may support tachyzoite growth

At face value, there appears to be a major discrepancy between our results and the *in vivo* tissue tropism of bradyzoite conversion. As mentioned, cysts development takes place preferentially within highly glycolytic tissues such as muscle and brain, sites of active lactate shuttles between fast and slow twitch skeletal muscle (Brooks 2002), and astrocytes and neurons (Pellerin et. al. 2007). Our data suggests that bradyzoite development should be inhibited within these highly glycolytic tissues. However, conversion *in vivo* can be thought of as a two-step process: a proliferative phase characterized by rapid replication of tachyzoites, followed by an intrinsically programmed phase of slowed parasite growth and initiation of bradyzoite development (Jerome et. al. 1998). Thus, the highly glycolytic status of the tissues in which *T. gondii* preferentially converts *in vivo* may provide an environment that supports rapid tachyzoite expansion, leading to an increased parasite load that ultimately results in higher levels of encystment in the tissue. Therefore the perceived enhancement of bradyzoite development within certain tissues may actually be a function of enhanced tachyzoite growth.

This hypothesis requires the spontaneous conversion of tachyzoites to bradyzoites *in vivo* in a non-specific manner, rather than a specific signal to convert provided by the host environment of neural and/or muscle tissue. The spontaneous conversion of tachyzoites to bradyzoites *in vivo* is perhaps suggested by the results in one *in vivo* imaging study of parasite dissemination and conversion following infection with tachyzoites. In Di Cristina *et. al* (2008), they observed that as early as one day post-infection, there was wide-spread expression of a bradyzoite-specific marker in parasites within abdomen tissues, but this massive switch did not lead to cyst formation within these tissues. This may suggest that tachyzoite to bradyzoite conversion is indeed a spontaneous event, therefore cyst formation and persistence within particular tissues is not necessarily explained by a specific signal to convert provided by these tissues.

This hypothesis of enhanced tachyzoite growth leading to the development of higher numbers of tissue cysts is especially attractive in terms of explaining the high incidence of ocular toxoplasmosis. Symptoms of ocular toxoplasmosis have been recorded in anywhere from 2-20% of infected individuals with acquired toxoplasma infection, and in 70-90% of individuals congenitally infected (Latkany 2007). Symptoms of ocular toxoplasmosis can occur in immunocompetent individuals, with lesions of the retina caused by active parasite replication as tachyzoites. Interestingly, the retina is one of these most highly glycolytic tissue sites and is

constantly performing aerobic glycolysis (Winkler 1989). The retina is also a site with an active lactate shuttle between Müller cells and photoreceptors (Tsacopoulos et. al. 1998). Our data might suggest that the parasite is able to take advantage of the glycolytic state of retinal tissue and preferentially replicate in this site even in immunocompetent individuals.

Additionally, preferential tachyzoite expansion may also explain the predilection for neural tissue during congenital infection. During neonatal toxoplasmosis, parasites are transmitted from mother to fetus as tachyzoites, and as mentioned previously tachyzoites can infect and replicate within virtually any nucleated cell. Disease caused by congenital infection, as in the reactivation of acquired chronic infection, primarily affects the central nervous system. Tachyzoite growth in utero occurs in the absence of a fully functional immune system, thus minimizing the role of immune privilege in tissue-specific pathology. Again, this may be due in part to the parasite taking advantage of the highly glycolytic state and preferentially expanding within neural tissue.

Cell-extrinsic regulation of bradyzoite development

The discovery of cell-extrinsic mediators that can inhibit conversion *in vitro* points to an interesting unappreciated potential mechanism of regulation of bradyzoite differentiation *in vivo*. Bradyzoite conversion has been thought of as requiring a signal or switch to initiate *in vivo*, either provided by the immune system through inflammatory mediators, or by a particular cellular environment that is conducive to conversion. Our data suggests that perhaps bradyzoite conversion could potentially occur in many cell types *in vivo*, but that the presence of soluble inhibitory mediators would restrict conversion to only those cells that are either resistant to or sequestered from those mediators. In our system, this would suggest that muscle and/or neural cell lines would perhaps be resistant to inhibition of conversion by the 293T supernatant. To that end, we did test the inhibitory effect on conversion of the 293T supernatant on a mouse astrocyte cell line and found that the supernatant was fully able to inhibit conversion within these cells (data not shown). However, the *in vivo* significance of cell-extrinsic inhibition of conversion should be appreciated at the tissue level. Conversion within individual cell lines may not accurately reflect the complexity of conversion *in vivo*.

The combination of the two hypotheses presented here could suggest an *in vivo* scenario where tachyzoite spread is occurring through the infected host, and preferential expansion is occurring within glycolytic neural/muscle tissues. Stress imparted by the host immune system would place pressure on some parasites to convert to bradyzoites, however this conversion would be inhibited within cell types that are exposed to/not resistant to soluble inhibitory mediators. Greater numbers of tachyzoites present in neural and muscle tissue will undergo bradyzoite conversion, perhaps within an environment where they are not susceptible to inhibitory mediators, thus leading to the persistence of bradyzoite cysts within these tissues. This model is very speculative, but nonetheless our data suggests that tissue tropism of conversion *in vivo* should be considered from the previously unappreciated mechanisms of preferential growth within glycolytic tissues, and restriction by cell extrinsic inhibitory mediators.

Autophagy as a new in vitro tool for studying parasite conversion

Finally, we have developed a new tool with which to study bradyzoite conversion *in vitro* that may be relevant to parasite pathogenesis in a physiologic setting. Parasite dependence upon host cell autophagy for the acquisition of amino acids is one of the most recent of many studies that demonstrate the critical importance of metabolic demands of the parasite to its pathogenesis. We have demonstrated that the inhibition of host cell autophagy can induce bradyzoite conversion, and this new system of induction may prove useful in further *in vitro* studies into the complexity of host-pathogen interactions governing this key feature of *T. gondii* pathogenesis.

ABBREVIATIONS

Abbreviation	Meaning
PVM	Parasitophorous vacuole membrane
DMEM	Dulbecco's modified Eagle's medium
MEM	Minimum Essential Medium
SM	Starvation media
FBS	Fetal bovine serum
RFP	Red fluorescent protein
YFP	Yellow fluorescent protein
HFF	Human foreskin fibroblast
MSCV	Murine stem cell virus
IRES	Internal ribosomal entry site
MyrAkt	Myristolated Akt
HBSS	Hank's balanced salt solution
3-MA	3-methyl adenine
HILIC	Hydrophilic interaction chromatography
ESI	Electrospray ionization
MCT	monocarboxylate transporter
ATP	Adenosine triphosphate
LDH	Lactate dehydrogenase

REFERENCES

Al Safarjalani, O. N., F. N. M. Naguib, et al. (2003). "Uptake of Nitrobenzylthioinosine and Purine beta-L-Nucleosides by Intracellular Toxoplasma gondii" Antimicrob. Agents Chemother. 47(10): 3247-3251.

Asai, T., W. J. O'Sullivan, et al. (1983). "Enzymes of the de novo pyrimidine biosynthetic pathway in Toxoplasma gondii." Molecular and Biochemical Parasitology 7(2): 89-100.

Black, M. W. and J. C. Boothroyd (2000). "Lytic Cycle of Toxoplasma gondii" Microbiol. Mol. Biol. Rev. 64(3): 607-623.

Blader, I. J., I. D. Manger, et al. (2001). "Microarray Analysis Reveals Previously Unknown Changes in Toxoplasma gondii-infected Human Cells." J. Biol. Chem 76: 24223-24231.

Bohne, W., U. Gross, et al. (1995). "Cloning and characterization of a bradyzoite-specifically expressed gene (hsp30/bag1) of Toxoplasma gondii, related to genes encoding small heat-shock proteins of plants." Molecular Microbiology 16(6): 1221-1230.

Bohne, W., J. Heesemann, et al. (1993). "Induction of bradyzoite-specific Toxoplasma gondii antigens in gamma interferon-treated mouse macrophages." Infect. Immun. 61(3): 1141-1145.

Bohne, W., J. Heesemann, et al. (1994). "Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion." Infect. Immun. 62(5): 1761-1767.

Bohne, W. and D. S. Roos (1997). "Stage-specific expression of a selectable marker in Toxoplasma gondii permits selective inhibition of either tachyzoites or bradyzoites." Molecular and Biochemical Parasitology 88(1-2): 115-126.

Bohne, W., A. Wirsing, et al. (1997). "Bradyzoite-specific gene expression in Toxoplasma gondii requires minimal genomic elements." Molecular and Biochemical Parasitology 85(1): 89-98.

Boyle, J. P. and J. R. Radke (2009). "A history of studies that examine the interactions of Toxoplasma with its host cell: Emphasis on in vitro models." International Journal for Parasitology Toxoplasma Centennial Issue 39(8): 903-914.

Boyle, J. P., J. P. J. Saeij, et al. (2007). "Toxoplasma gondii: Inconsistent dissemination patterns following oral infection in mice." Experimental Parasitology 116(3): 302-305.

Brooks, G. (1986). "Lactate production under fully aerobic conditions: the lactate shuttle during rest and exercise." Fed Proc. 45(13): 2924-9.

Brooks, G. A. (2002). "Lactate shuttles in nature." Biochem. Soc. Trans. 30(2): 258-264.

Butcher, B. A., R. I. Greene, et al. (2005). "p47 GTPases Regulate Toxoplasma gondii Survival in Activated Macrophages." Infect. Immun. 73(6):3278-3286.

Carey, K. L., C. G. Donahue, et al. (2000). "Identification and molecular characterization of GRA8, a novel, proline-rich, dense granule protein of Toxoplasma gondii." Molecular and Biochemical Parasitology 105(1): 25-37.

Carmen, J. C., R. C. Southard, et al. (2008). "The complexity of signaling in host-pathogen interactions revealed by the Toxoplasma gondii-dependent modulation of JNK phosphorylation." Experimental Cell Research 314(20): 3724-3736.

Collazo, C. M., * George S. Yap, et al. (2001). "Inactivation of Lrg-47 and Irg-47 Reveals a Family of Interferon-gamma Inducible Genes with Essential, Pathogen-Specific Roles in Resistance to Infection." J Exp Med 194: 181-188.

Coppens, I., J. D. Dunn, et al. (2006). "Toxoplasma gondii Sequesters Lysosomes from Mammalian Hosts in the Vacuolar Space." Cell 125(2): 261-274.

Coppens, I. and K. A. Joiner (2001). "Parasite-host cell interactions in toxoplasmosis: new avenues for intervention?" Expert Reviews in Molecular Medicine 3: 1-20.

Coppens, I., A. P. Sinai, et al. (2000). "Toxoplasma gondii Exploits Host Low-Density Lipoprotein Receptor-Mediated Endocytosis for Cholesterol Acquisition." J Cell Biol 149: 167-180.

Di Cristina, M., D. Marocco, et al. (2008). "Temporal and Spatial Distribution of Toxoplasma gondii Differentiation into Bradyzoites and Tissue Cyst Formation In Vivo." Infect. Immun. 76(8): 3491-3501.

Dubey, J. P., D. S. Lindsay, et al. (1998). "Structures of Toxoplasma gondii Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts." Clin. Microbiol. Rev. 11(2): 267-299.

Dunn, D., M. Wallon, et al. (1999). "Mother-to-child transmission of toxoplasmosis: risk estimates for clinical counselling." The Lancet 353(9167): 1829-1833.

Dzierszinski, F., M. Nishi, et al. (2004). "Dynamics of Toxoplasma gondii Differentiation." Eukaryotic Cell 3(4): 992-1003.

Dzierszinski, F., O. Popescu, et al. (1999). "The Protozoan Parasite Toxoplasma gondii Expresses Two Functional Plant-like Glycolytic Enzymes: Implications for evolutionary origins of Apicomplexans." J. Biol. Chem 274: 24888-24895.

Ferreira da Silva, M. d. F., H. S. Barbosa, et al. (2008). "Stress-related and spontaneous stage differentiation of Toxoplasma gondii." Molecular BioSystems 4(8): 824-834.

Ferreira da Silva, M. d. F., A. C. Tak·cs, et al. (2009). "Primary skeletal muscle cells trigger spontaneous Toxoplasma gondii tachyzoite-to-bradyzoite conversion at higher rates than fibroblasts." International Journal of Medical Microbiology 299(5): 381-388.

Gross, U., W. Bohne, et al. (1996). "Developmental differentiation between tachyzoites and bradyzoites of Toxoplasma gondii." Parasitology Today 12(1): 30-33.

Gupta, N., M. M. Zahn, et al. (2005). "Selective Disruption of Phosphatidylcholine Metabolism of the Intracellular Parasite Toxoplasma gondii Arrests Its Growth." J. Biol. Chem 280: 16345-16353.

Gutmann, I. and A. Wahlefeld (1974). L-(+)-lactate determination with lactate dehydrogenase and NAD. Methods of Enzymatic Analysis. New York, NY, USA, Bergmeyer, H.U. eds.: 1464-1472.

Hashimoto, T., R. Hussien, et al. (2007). "Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis." FASEB J. 21(10): 2602-2612.

Jerome, M. E., J. R. Radke, et al. (1998). "Toxoplasma Gondii Bradyzoites Form Spontaneously during Sporozoite-Initiated Development." Infect. Immun. 66(10): 4838-4844.

Jones, L. A., J. Alexander, et al. (2006). "Ocular toxoplasmosis: in the storm of the eye." Parasite Immunology 28(12): 635-642.

Jones, T. C., K. A. Bienz, et al. (1986). "In vitro cultivation of Toxoplasma gondii cysts in astrocytes in the presence of gamma interferon." Infect. Immun. 51(1): 147-156.
Kelekar, A. and C. B. Thompson (1998). "Bcl-2-family proteins: the role of the BH3 domain in apoptosis." Trends in Cell Biology 8(8): 324-330.
Kim, L. and E. Y. Denkers (2006). "Toxoplasma gondii triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis." J Cell Sci 119(10): 2119-2126.
Latkany, P. (2007). Ocular disease due to Toxoplasma gondii. Toxoplasma gondii the model apicomplexan: perspectives and methods. W. LM and K. Kim.
Lindsay, D. S., J. P. Dubey, et al. (1991). "Examination of Tissue Cyst Formation by Toxoplasma gondii in Cell Cultures Using Bradyzoites, Tachyzoites, and Sporozoites." The Journal of Parasitology 77(1): 126-132.
Luder, C. G. K., M. Giraldo-Vel·squez, et al. (1999). "Toxoplasma gondii in Primary Rat CNS Cells: Differential Contribution of Neurons, Astrocytes, and Microglial Cells for the Intracerebral Development and Stage Differentiation." Experimental Parasitology 93(1): 23-32.
Manger, I. D., A. Hehl, et al. (1998). "Expressed Sequence Tag Analysis of the Bradyzoite Stage of Toxoplasma gondii: Identification of Developmentally Regulated Genes." Infect. Immun. 66(4): 1632-1637.
Manning, B. D. and L. C. Cantley (2007). "AKT/PKB Signaling: Navigating Downstream." Cell 129(7): 1261-1274.
Martens, S., I. Parvanova, et al. (2005). "Disruption of Toxoplasma gondii Parasitophorous Vacuoles by the Mouse p47-Resistance GTPases." PLoS Pathog 1(3): e24.
McHugh, T. D., R. E. Holliman, et al. (1994). "The in vitro model of tissue cyst formation in Toxoplasma gondii." Parasitology Today 10(7): 281-285.
Mehta, M., H. Sonawat, et al. (2006). "Glycolysis in Plasmodium falciparum results in modulation of host enzyme activities." J Vector Borne Dis. 43(3): 95-103.
Mele, A., P. Paterson, et al. (2002). "Toxoplasmosis in bone marrow transplantation: a report of two cases and systematic review of the literature." Bone Marrow Transpl. 29: 691-698.
Molestina, R. E., T. M. Payne, et al. (2003). "Activation of NF-{kappa}B by Toxoplasma gondii correlates with increased expression of antiapoptotic genes and localization of phosphorylated I{kappa}B to the parasitophorous vacuole membrane." J Cell Sci 116(21): 4359-4371.
Montoya, J. and O. Liesenfeld (2004). "Toxoplasmosis." The Lancet 363(9425): 1965-1976.
Mordue, D., D. Naishadh, et al. (1999). "Invasion by Toxoplasma gondii Establishes a Moving Junction That Selectively Excludes Host Cell Plasma Membrane Proteins on the Basis of Their Membrane Anchoring." J. Exp Med 190: 1783-1792.
Nagamune, K., L. M. Hicks, et al. (2008). "Abscisic acid controls calcium-dependent egress and development in Toxoplasma gondii." 451(7175): 207-210.

Nash, P. B., M. B. Purner, et al. (1998). "Toxoplasma gondii-infected cells are resistant to multiple inducers of apoptosis." Journal of Immunology 160(4): 1824-1830.

Nelson, M. M., A. R. Jones, et al. (2008). "Modulation of the Host Cell Proteome by the Intracellular Apicomplexan Parasite Toxoplasma gondii." Infect. Immun. 76(2): 828-844.
Payne, T. M., R. E. Molestina, et al. (2003). "Inhibition of caspase activation and a requirement for NF-{kappa}B function in the Toxoplasma gondii-mediated blockade of host apoptosis." J Cell Sci 116(21): 4345-4358.
Pellerin, L., A.-K. Bouzier-Sore, et al. (2007). "Activity-dependent regulation of energy metabolism by astrocytes: An update." Glia 55(12): 1251-1262.
Perrotto, J., D. B. Keister, et al. (1971). "Incorporation of precursors into Toxoplasma DNA." J. of Protozool. 18: 470-473.
Pfefferkorn, E. R. (1978). "Toxoplasma gondii: the enzymatic defect of a mutant resistant to 5-fluorodeoxyuridine." Experimental Parasitology 44: 26-35.
Philp, A., A. L. Macdonald, et al. (2005). "Lactate - a signal coordinating cell and systemic function." J Exp Biol 208(24): 4561-4575.
Plas, D. R. and C. B. Thompson (2002). "Cell metabolism in the regulation of programmed cell death." Trends in Endocrinology and Metabolism 13(2): 75-78.
Pomel, S., F. C. Y. Luk, et al. (2008). "Host Cell Egress and Invasion Induce Marked Relocations of Glycolytic Enzymes in <italic>Toxoplasma gondii</italic> Tachyzoites." PLoS Pathog 4(10): e1000188.
Radke, J. R., R. G. Donald, et al. (2006). "Changes in the Expression of Human Cell Division Autoantigen-1 Influence Toxoplasma gondii Growth and Development." PLoS Pathog 2(10): e105.
Richardson, R. S., E. A. Noyszewski, et al. (1998). "Lactate efflux from exercising human skeletal muscle: role of intracellular PO2." J Appl Physiol 85(2): 627-634.
Roth, E. J., M. Calvin, et al. (1988). "The enzymes of the glycolytic pathway in erythrocytes infected with Plasmodium falciparum malaria parasites." Blood 72(6): 1922-1925.
Scharton-Kersten, T., Wynn TA, et al. (1996). "In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection." Journal of Immunology 157(9): 4045-54.
Schwartzman, J. D. and E. R. Pfefferkorn (1982). "Toxoplasma gondii: Purine synthesis and salvage in mutant host cells and parasites." Experimental Parasitology 53(1): 77-86.
Shapira, S., O. S. Harb, et al. (2005). "Initiation and termination of NF-{kappa}B signaling by the intracellular protozoan parasite Toxoplasma gondii" J Cell Sci 118(15): 3501-3508.
Silva, N. M., W. L. Tafuri, et al. (2002). "Toxoplasma gondii: in vivo expression of BAG-5 and cyst formation is independent of TNF p55 receptor and inducible nitric oxide synthase functions." Microbes and Infection 4(3): 261-270.
Simpson, D. M. and M. Tagliati (1994). "Neurologic Manifestations of HIV Infection." Ann Intern Med 121: 769-785.
Sinai, A., P. Webster, et al. (1997). "Association of host cell endoplasmic reticulum and mitochondria with the Toxoplasma gondii parasitophorous vacuole membrane: a high affinity interaction." J Cell Sci 110(17): 2117-2128.
Soete, M., D. Camus, et al. (1994). "Experimental Induction of Bradyzoite-Specific Antigen Expression and Cyst Formation by the RH Strain of Toxoplasma gondii in Vitro." Experimental Parasitology 78(4): 361-370.

Soete, M., B. Fortier, et al. (1993). "Toxoplasma gondii : Kinetics of Bradyzoite-Tachyzoite Interconversion in vitro." Experimental Parasitology 76(3): 259-264.
Spear, W., D. Chan, et al. (2006). "The host cell transcription factor hypoxia-inducible factor 1 is required for Toxoplasma gondii growth and survival at physiological oxygen levels." Cellular Microbiology 8(2): 339-352.
Starnes, G. L., M. Coincon, et al. (2009). "Aldolase Is Essential for Energy Production and Bridging Adhesin-Actin Cytoskeletal Interactions during Parasite Invasion of Host Cells." Cell Host & Microbe 5(4): 353-364.
Suzuki, Y., Orellana MA, et al. (1988). "Interferon-gamma: the major mediator of resistance against Toxoplasma gondii." Science 240(4851): 516-518.
Taylor, G. A., * Carmen M. Collazo, et al. (2000). "Pathogen-specific loss of host resistance in mice lacking the IFN-g inducible gene IGTP." PNAS 97(2): 751-755.
Taylor, G. A., C. G. Feng, et al. (2004). "p47 GTPases: regulators of immunity to intracellular pathogens." 4(2): 100-109.
Tenter, A. M., A. R. Heckeroth, et al. (2000). "Toxoplasma gondii: from animals to humans." International Journal for Parasitology 30(12-13): 1217-1258.
Tomavo, S. (2001). "The differential expression of multiple isoenzyme forms during stage conversion of Toxoplasma gondii: an adaptive developmental strategy." International Journal for Parasitology 31(10): 1023-1031.
Tomavo, S. and J. C. Boothroyd (1995). "Interconnection between organellar functions, development and drug resistance in the protozoan parasite, Toxoplasma gondii." International Journal for ParasitologyAustralian Society for Parasitology Proceedings of the Scientific Meeting 25(11): 1293-1299.
Tsacopoulos, M., C. L. Poitry-Yamate, et al. (1998). "Trafficking of Molecules and Metabolic Signals in the Retina." Progress in Retinal and Eye Research 17(3): 429-442.
Verma, Y., G. Gangenahalli, et al. (2006). "Cell death regulation by B-cell lymphoma protein." Apoptosis 11(4): 459-471.
Wang, Y., L. M. Weiss, et al. (2009). "Host cell autophagy is induced by Toxoplasma gondii and contributes to parasite growth." J. Biol. Chem 284: 1694-1701.
Weiss LM, Laplace D, et al. (1995). "A cell culture system for study of the development of Toxoplasma gondii bradyzoites." J Eukaryot Microbiol. 42(2): 150-157.
Winkler, BS (1989). "Retinal aerobic glycolysis revisted." Invest.Ophthalmol. Vis. Sci. 30(6) 1023-
Yang, S. and S. F. Parmley (1997). "Toxoplasma gondii expresses two distinct lactate dehydrogenase homologous genes during its life cycle in intermediate hosts." Gene 184(1): 1-12.