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Differential gene expression in murine astrocytes infected with virulent (type I) vs. a virulent (type II) strains of *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is a ubiquitous parasite infecting up to 30% of the population worldwide. Most individuals infected with *T. gondii* harbor a chronic infection that persists for the lifetime of the host with the parasite located predominantly in neural and muscular tissue. In some immune competent hosts however, chronic infection is associated with neurological disorders including schizophrenia, depression, suicidal behavior, headaches and cryptogenic epilepsy. There are three strains of *T. gondii*, designated type I, type II and type III, with evidence indicating type I strains are more virulent in humans, causing more severe clinical outcomes. Astrocytes are the predominant glial cell in the brain and can serve as a host cell for tachyzoite and bradyzoite stages in the brain. In this study we have used a transcriptional approach to dissect the host response to virulent type I RH strain vs. a virulent type II Me49 strain, in murine astrocytes. The results showed the type I strain induces a distinctly different host cell response in astrocytes inducing larger changes in gene expression levels and affecting different host cell pathways as compared to the type II strain. Type I vs. type II strains showed some common responses that may represent the 'core' response to parasite infection with host immune response genes the dominant pathway affected by both strains. However *Toxoplasma* strains show clear differences in modulation of some of these core host responses and importantly in some pathways related to pathogenesis of the nervous system. Neurobiological processes significantly affected by type I strain infection included effects on neurodevelopmental processes and nerve impulse transmission, including many gene homologues associated with Schizophrenia. The strain-specific effects could help explain the different clinical outcomes of *Toxoplasma* infections in humans and specifically, the distinct neurological complications such as Schizophrenia that occurs in the latent infection in some immune competent individuals.

Keywords: Toxoplasmic Encephalitis; Cerebral Toxoplasmosis; Astrocytes; Neuropsychiatric

Introduction

Toxoplasma gondii is a ubiquitous parasite infecting up to 30% of the population worldwide. Most individuals infected with *T. gondii* harbor a chronic infection that persists for the lifetime of the host in which the parasite resides in cysts that are located predominantly in neural and muscular tissue. Infection with *T. gondii* is typically asymptomatic due to an effective host immune response, dependent upon the cytokine IFNγ [1,2]. In immunosuppressed hosts, such as AIDS patients, the infection reactivates from the cyst stage in the brain resulting in severe, potentially fatal, necrotizing encephalitis [3]. Persistent neurological deficits are often present in HIV+ patients, who survived an episode of Toxoplasmic Encephalitis (TE) indicating the parasite may cause persistent effects on the central nervous system [4,5]. Additionally, in some immune competent hosts, chronic infection may be associated with neurological disorders such as schizophrenia, depression, suicidal behavior, headaches and cryptogenic epilepsy [6-14]. Thus while the chronic infection is typically asymptomatic in immune competant individuals, increasing evidence indicates the parasite has impacts on the brain in chronic Toxoplasmosis at least in some immune competent individuals. There are three canonical strains of *T. gondii*, designated type I, type II and type III, with the three strains sharing approximately 98% identity [15]. The strains show strong phenotypic differences, with the most dominant difference being virulence. In mice, type I strains are virulent (LD100 of 1) while type II and type III strains are avirulent (LD50>100 and 1000-10,000 respectively). Evidence indicates type I strains are also more virulent in humans [16,17]. For example in humans, type I strains are associated with more severe clinical outcomes in Congenital Toxoplasmosis and a higher prevalence of type I strains are associated with Ocular Toxoplasmosis and in immune compromised patients, in some locations [18-23]. Virulence is also associated with a faster rate of parasite dissemination in the host, an increased rate of parasite replication, and differential effects on the host immune response, including reduced dendritic cell responses which can lead decreased activation of $CD8^+$ T cell responses and a greater stimulation of inflammatory mediators which can lead to over stimulation of Th1 immune response leading to enhanced mortality in mice [24-26]. Transcriptional analyses of *T. gondii* interactions with its host cells have found the parasite infection induces a strong transcriptional response in the host cell [27,28]. The transcription factors NF-kB, which activates anti-apoptotic genes, and STAT3 and STAT6, which subvert pro-inflammatory cytokine

production, are activated within minutes after invasion. Infection also induces the host cell transcription factor, hypoxia inducible factor 1 (HIF1α), which affects transcription of glycolytic enzymes, glucose transporters, transferrin receptor and vascular endothelial growth factor receptor, and has been shown to promote growth of *T. gondii* in fibroblasts perhaps by acquisition of host glucose and iron uptake [29,30]. Other host transcription factors affected by the parasite include Serum Response Factor, shown to regulate immediate early gene expression in *Toxoplasma*-infected cells and early growth response 2 (EGR2), which has been suggested to help host cells survive the stress of the infection [30,31]. Activation of host cell genes 12-14 hrs after invasion, include genes that modulate host cell glucose, melavonate and iron pathways and likely are important to parasite growth [27]. Modulation of host cell transcription in part, is due to the secretion of parasite rhoptry proteins, such as ROP16 into the host cell, and recent evidence suggests other parasite-secreted kinases are also likely involved in modulation and regulation of host cell signaling pathways [32-34].

Recently several transcriptional studies have been done addressing the strain-specific effects of *T. gondii* in host cells. Transcriptional studies in neuro epithelial cells and macrophages found the three lineages of *Toxoplasma* induced distinct host transcriptional responses suggesting the three parasite strains employ different strategies to subvert the host defense mechanisms [35,36]. Similarly an *in vivo* study in mice also found differential gene expression in mice infected with type I, II and III strains [37]. In the *in vivo* study although a differential host response with the three major genotypic strains was found, a set of genes was found affected by all three strains indicating a common gene expression response to *T. gondii* infection in mice.

Astrocytes are the predominant glial cell in the brain and can serve as a host cell for both the tachyzoites and the bradyzoite/cyst stage in the brain [38,39]. Astrocytes can also present endogenous parasite antigens via MHC I pathway and *in vivo* studies in mice have found astrocytes are critical to control replication of the parasite in the brain, thus indicating astrocytes are also an important immune effector cell for the parasite in the brain [40,41]. In this study we have used a transcriptional approach to dissect the host response to virulent type I RH strain, vs. avirulent type II Me49 strain, in murine astrocytes. We found type I strain induces a distinctly different host cell response in gene expression levels and host cell pathways in astrocytes as compared to the type II strain. Our data further reveal that the astrocyte transcriptional response to type I strain involves effects on neurobiological pathways, including genes involved in neuro development and nerve impulse transmission. The strain-specific effects could help explain the different clinical outcomes of *Toxoplasma* infections in humans and specifically, the distinct neurological complications such as Schizophrenia that occurs in the latent infection in some immune competent individuals.

Materials and Methods

Parasite culture and harvest of parasites

Tachyzoites from *T. gondii* strains, RH (ATCC 50940) and Me49 (ATCC 50611), were maintained by *in vitro* culture in human foreskin fibroblasts (HFFs; ATCC CRL-1634) grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotic/antimycotic solution according to established protocols [42]. Parasites were harvested from infected HFF monolayers, passage through a 27 gauge needle, filtered through a 3 μm polycarbonate filter, centrifuged at 600×g for 10 minutes, resuspended in PBS, and used for infection of murine astrocytes cultures as described below.

Primary Murine Astrocyte Cell Culture

Murine astrocytes from C57BL/6×SV129 mice were cultivated from the brains of neonatal (less than 24 h old) mice as per previously published protocols [43]. Briefly, murine pups were sacrificed, the brains removed from the cranium, the forebrain dissected and the meninges removed. The tissue was minced and incubated in 0.25% trypsin for 5 min at 37°C. After 5 min, the trypsin was inactivated with a solution containing DNase and soybean trypsinase inhibitors, and the tissue was further disrupted by trituration in a 20-ml pipette. The dissociated cells were filtered through a 74 μm Nitex mesh, centrifuged at 200 x g, suspended in growth medium at a concentration of 10^6 cells/ml, and plated onto poly-L-lysine (PLL) coated dishes. Astrocytes were maintained in endotoxin-free minimal essential medium (BRL-GIBCO) supplemented with MEM vitamins, MEM amino acids, glutamine (20 mM) and 20% FBS (BRL-GIBCO). The growth medium (S-20) was changed every 3 days. After 7 days *in vitro*, a confluent monolayer of 1×10^4 cells/cm^2 of astrocytes was reached. By this method, cells were found to be>95% astrocytes, as judged by positive staining for glial fibrillary acidic protein. Cultures contained <5% microglia, as identified by staining with the lectin, BS1-B4 (Sigma L-2895). Astrocyte monolayers were dissociated in trypsin-EDTA, placed onto PLL-coated plates at 10^4 cells/cm, and cultured for 7 days in minimal essential medium, supplemented with MEM vitamins, MEM amino acids, glutamine (20 mM) and 10% FBS (S-10 media). Then astrocytes were then infected with *T. gondii* as described below.

Infection of Astrocytes

Astrocytes were infected with freshly lysed tachyzoites harvested from HFF cells, suspended in S-10 media at a multiplicity of infection (MOI) of 1:5 or with S-10 only (mock-infected), incubated in 5% CO2 at 37°C for 1 hr to allow infection, followed by washing of any remaining extracellular parasites as per standard protocols. By this method astrocytes were found to be infected by at least 50% or greater. All experiments were conducted in triplicate, with experiments conducted on two separate occasions. Cells pellets harvested at 2, 6 and 24 h post infections (p.i.) from infected and mock-infected cells and RNA processed for microarray analysis as specified below.

RNA Extraction and Microarray Analysis

Total RNA was isolated from infected and mock infected murine astrocytes using QIAGEN RNeasy kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. RNA quality was assessed and only RNA samples of high quality (absorbance at 260/280 greater than 1.8 and absence of signs of RNA degradation) were included in the study. For host gene expression profiling, Affymetrix Mouse Genome 430 2.0, which covers the entire mouse genome was used. Target cRNA for microarray analysis was prepared following the manufacturer's protocol (http://www.affymetrix.com) and according to previously published methods [43]. The microarray hybridization was carried out at the Montana State University Functional Genomics Core Facility (http://montana.edu/index.php?page=functional).

Data Normalization and statistical analysis

Microarray data was normalized using the robust multichip algorithm with GC-content correction (GC-RMA) method implemented in FlexArray v1.4.1. A one-way analysis of variance (ANOVA) was used to detect genes with statistically significant expression levels between *T. gondii*-infected astrocytes and mock-infected controls at each time point. Gene transcripts were considered to be differentially expressed when there was a 2-fold change (up or down) from the uninfected controls, and the ANOVA P-value was set at ≤ 0.05.

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis of differentially regulated genes at 2, 6 and 24 h p.i. for type I and type II infected astrocytes was performed using a custom implementation of the algorithm for discovering motifs, as described in Eden et al. [42]. Functional annotations for mouse genes were collected both from Affy metrix probe annotations and from human orthologs for gene ontology (GO) functions and relationship to schizophrenia, respectively. Mouse to human orthology information was retrieved at MGI (ftp://ftp.informatics.jax.org). Schizophrenia annotations were retrieved from the Schizophrenia Gene Resource (SZGR) (http://bioinfo.mc.vanderbilt.edu/SZGR). *P*-values assessing the null hypothesis that a given functional category (either GO function or schizophrenia phenotype) was enriched in the subset of the most up (or down)-regulated genes were further corrected for multiple testing using the Benjamini-Hochberg method.

Validation of the relative gene expression values using RT-PCR

Microarray data were validated by reverse transcriptase PCR (RT-PCR) using the QAIGEN/SA Biosciences RT2 Profiler PCR Array for Mouse Chemokine's and Receptors (PAMM-188 022Z). Gene expression of these genes was determined for astrocytes mock-infected or infected with RH strain or Me49 strain and cells harvested at 24 hours post-infection. Total RNA was isolated from each treatment group and then used in RT-PCR assay using SYBR green following the manufacturers protocol (www.sabioscience.com/rt_pcr_product/PAMM-022Z.html).

Results

Astrocyte Transcriptional Response to infection with Type I vs. Type II strains of *T. gondii*

Astrocyte transcriptional response to infection with type I vs. type II strain of *T. gondii* was assessed by transcriptional profiling at 2 h, 6 h and 24 h post-infection (p.i.) to explore host responses correlating to establishment of infection, beginning of parasite replication and active parasite replication respectively. Both strains induced significant host transcriptional changes to infection, affecting approximately 2-3% of the expressed host cell genes and affecting multiple host cell pathways by 24 h p.i (Table 1). The transcriptional response to infection with type I strain was more robust, with a larger number of host genes affected early after infection (2 h and 6 h p.i.) and in most instances the type I strain induced larger fold changes in host cell gene expressions, than did the type II strain (see Supplemental Table S1 and Supplemental Table S2).

Additionally, approximately half of the differentially regulated genes in type I infected astrocytes were down regulated, in contrast to type II infection, where the majority of differentially regulated genes were up-regulated.

Parasite Strain	No. of Genes Affected[1] 2h p.i. 6h p.i. 24h p.i.	% Host Cell Transcripts affected[2]	No. of Host Pathways Affected[3]
Type I	310 681 1095 (139↑ 171€) (314↑ 367€) (449↑ 646€)	2.4	28
Type II	92 240 1238 (56↑ 36€) (216↑ 24€) (934↑ 304€)	2.7	20

Table 1 – Astrocyte Transcriptional Response to Infection with type I vs. type II strains of T. gondi; i1Significant genes affected at p< 0.01; number of genes significantly up (↑) or down (€) regulated indicated in parentheses; 2Out of 45100 transcripts at 24h p.i.; 3GO-SLIMS (n = 72) analysis at 24h p.i.; adjusted FDR P< 0.01.

Kinetic Analysis of Differential Response to type 211 I vs. type II *Toxoplasma* strains in Astrocytes

The astrocyte host cell response to infection with type I vs. type II strains at 2, 6 and 24 h p.i. was further explored via Venn analysis. Only 419 (18.5%), 473 (21.2%) and 252 genes (11.3%) of the top 5% of the genes affected by each strain, were in common at 2, 6 and 24 h p.i. respectively (Figure 1).

This analysis indicates type I vs. type II infections induce qualitatively distinct astrocyte host cell responses throughout the first 24 h post-infection, including during the establishment of infection (2 h p.i.), the beginning of parasite replication (6 h p.i.) and during active parasite replication (24 h p.i.).

Host Cell Pathways Modulated by type I vs. type II *Toxoplasma* strains in Astrocytes

Gene Set Enrichment Analysis (GSEA) was done to further explore host cell pathways differentially modulated by infection with type I vs. type II strains at 2 h, 6 h and 24 h p.i., using GO-SLIMS pathway classification (number of categories=72) which provides a broad overview of biological processes. A large number of processes were affected by both type I and type II strains over 24 h of infection, with type I strain significantly affecting 28 processes and type II strain affecting 20 processes by 24 h p.i. (Figure 2).

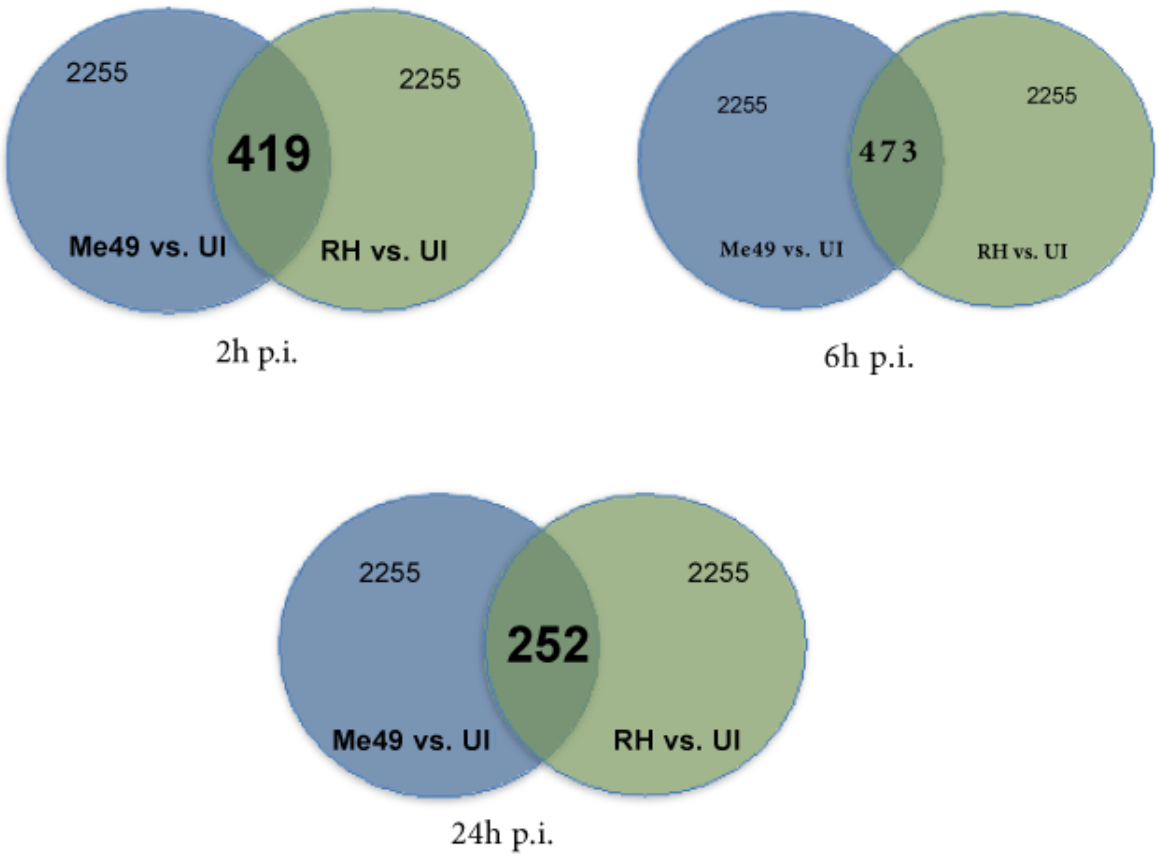

Figure 1: Venn Analysis of Differential Astrocyte Host Response to Infection with RH vs. Me49 strains of *Toxoplasma*. Comparison between top 5% of astrocyte genes affected (n=2255) by infection with RH and Me49 parasites at 2, 6 and 24 h post-infection.

However the biological processes most significantly affected differed dramatically between the type I and type II strain infection in astrocytes with significant temporal differences found between infection at 2, 6 and 24 h p.i. (Figure 2A vs. Figure 2B). Type I strain induced an immediate and robust host cell transcriptional response, affecting a large number of biological processes within 2h p.i., with effects on these biological pathways persisting throughout 6 h and 24 h p.i (Figure 2A). The most significant host cell processes affected were immune system processes, response to stress and signal 235 transduction. However, infection with the type I strain also had highly significant impacts on a broad range of host cell processes including cell death, locomotion, cell motility, cell proliferation, cell adhesion and neurobiological processes, most of which persisted throughout the 24 h course of infection. Beginning at 6 h p.i., correlating with the onset of parasite replication, impacts on several processes, including signal transduction, cell death and biosynthetic processes, increased. Most processes were up-regulated but anatomical structure development, cell differentiation, cell adhesion, cell motility, cell proliferation and signal transduction showed evidence of both up and down regulation throughout 24 h of infection, indicating a more complex regulatory effect on these processes (Figure 3).

In contrast, infection with the type II strain, induced only a modest effect on host transcription within the first 2 h p.i., with the only significantly impacted biological pathways immune system processes and response to stress (Figure 2B). Similar to type I infection, response to stress and immune system processes, remained highly affected throughout the 24 h course of infection. Beginning at 6 h p.i. type II infection induced strong effects on cell division related processes, including significant impact on DNA metabolic processes, cell cycle, mitosis, and chromosomal segregation. Similar to type I infection, strong effects on biosynthetic processes were also found by 6 h p.i. correlating with the beginning of parasite replication. By 24 h p.i., a broader range of host cell processes were affected by type II infection, although the dominant effects were seen on immune system processes and processes related to cell proliferation including the cell cycle, DNA metabolic processes, cell division, chromosomal segregation, chromosomal organization and mitosis.

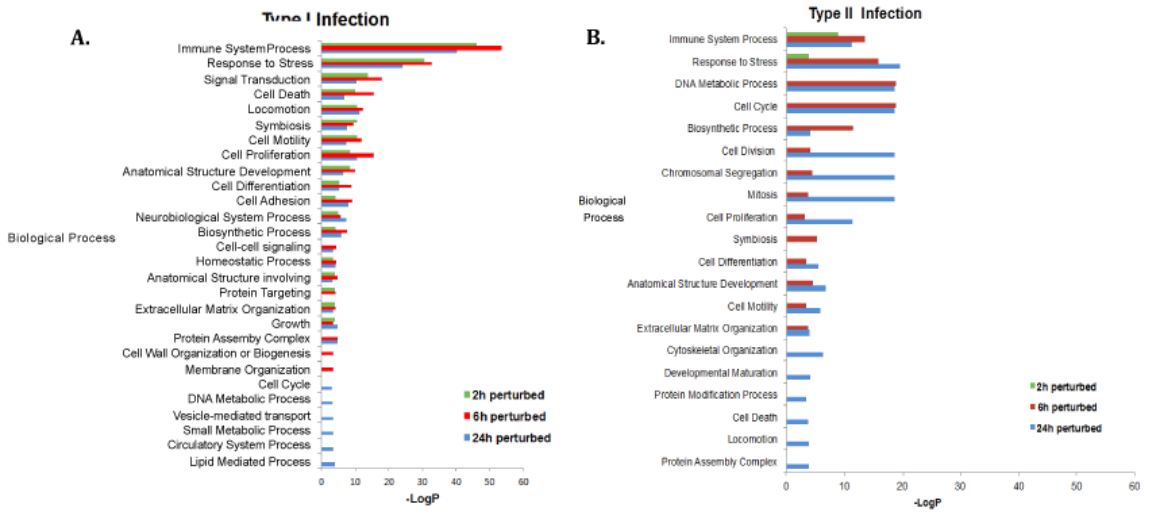

Figure 2: GSEA Analysis of biological processes differentially regulated by infection with type I vs. type II *Toxoplasma* strains in astrocytes. Comparison of biological processes perturbed (up or down-regulated) by type I strain (A) vs. type II strain (B) infection in astrocytes, as compared to mock-infected astrocytes using GO-SLIMS categories (n=72). P values of <0.05 and adjusted FDR of p<0.01 were used as a threshold to select significance.

Despite these differences induced by type I vs. type II infection, 258 many host cell processes were commonly affected by both strains by 24 h p.i. (Figure 4). Immune system processes were the most significantly perturbed pathway by infection with both type I and type II strains of *T. gondii*.

Other common host cell processes affected by both strains included effects on cell locomotion, cell proliferation, cell adhesion, neurobiological system processes and biosynthetic processes amongst others (Figure 4). In general, however, the effect of these host cell processes was more significantly impacted by type I strain than the type II strain. The most dramatic differences between the type I and II strains were on immunological processes, cell locomotion, cell death, cell signaling and neurobiological system processes. A notable difference to this trend were the effects on the cell cycle and related processes, such as mitosis, chromosomal segregation, and DNA metabolic processes, which was much more significantly impacted by type II strain than the type I strain.

Immune Response Genes

The immune system processes affected by infection with both type I and type II strains of *T. gondii* in astrocytes were analyzed further. Immune system processes were primarily up-regulated by both type I and type II strains (Supplemental Table 2). Immune response genes up-regulated included chemokines, cytokines, innate immune system receptors including toll-like receptors (TLRs) and members of the C-type lectin domain family (Clec), MHC class I and II genes and some members of the immune response gene (IRG) and guanylate binding protein (GBP) families (Table 2). Chemokines were a dominant category of immune responses genes, induced by both type I and type II strains inducing up-regulation of both CC and CXC-type chemokines by 24h p.i. although the type I strain induced a greater range of CC type chemokines than did the type II strain infection (Supplemental Table S1 and S2).

Neurobiological Genes

The differential impact on neurobiological processes by infection with type I vs. type II strain was further investigated by probing the differentially regulated genes induced by type I strain vs. the type II strain, against the gene ontology (GO) categories, Nervous System Development (GO:007399) and Transmission of Nerve Impulse (GO: 0019266) and against several Schizophrenia genes datasets assembled by Schizophrenia Gene Resource (http://bioinfo.mc.vanderbilt.edu/SZGR/index.jsp), using murine gene homologues of human genes. The following datasets maintained on the Schizophrenia Gene Resource website were used. Schizophrenia Core genes, a manually collected gene set (n=33), Schizophrenia Gene Association dataset, a gene set created from schizophrenia association studies and analyzed for significance using a combined odds ratios method (n=263), a Schizophrenia Literature dataset, a gene set collected based upon co-occurrence of genes and schizophrenia-related keywords (n=1468) and Schizophrenia Gene Network dataset, a dataset based upon the shortest path distance of schizophrenia core genes in the human protein-protein interaction network (n=1010). Using these gene lists, it was found that type I strain significantly impacts Nervous system Development and Transmission of Nerve Impulse pathways, whereas infection with the type II strain did not (Supplemental Table 3). Infection with neither the type I nor type II strain significantly impacted the Schizophrenia Core genes. However type I strain had significant effects on Schizophrenia Gene Association, Schizophrenia Literature and Schizophrenia Network datasets. Significant effects of type I strain infection on these neurobiological datasets were seen in both up and down regulation of genes, although there was a stronger down-regulatory effect seen in transmission of nerve impulse, neurodevelopmental-related genes and Schizophrenia literature datasets, while conversely the Schizophrenia Association dataset was more significantly up-regulated.

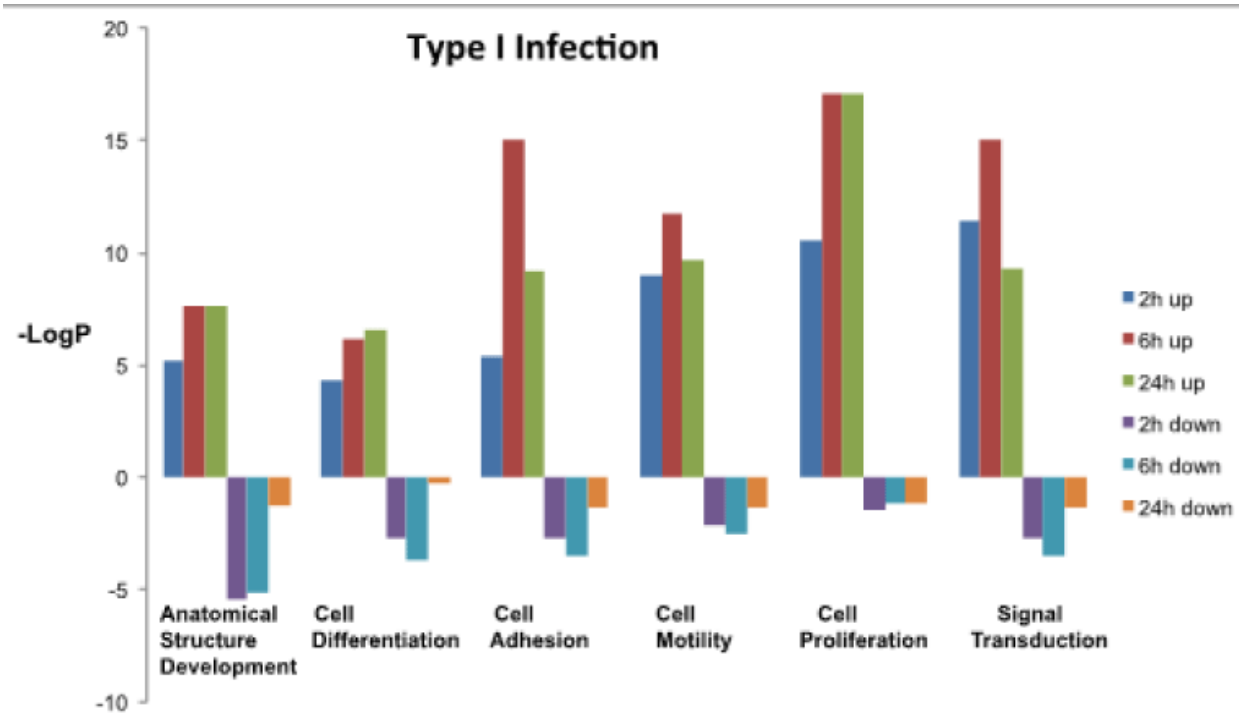

Figure 3: Biological Processes significantly up and down regulated by Type I infection in Astrocytes. Processes up or down-regulated by type I strain infection in astrocytes, as compared to mock-infected astrocytes, using GO-SLIMS categories (n=72). P values of <0.05 and adjusted FDR of p<0.01 were used as a threshold to select significance.

Network datasets was only significantly up-regulated. Of these schizophrenia datasets, type II infection only had a significant impact on the Schizophrenia Network dataset, inducing an up regulation of these genes.

Immune Response Gene Functional Category	Type I strain	Type II strain
Chemokines (CC-type)	Ccl2, Ccl3, Ccl4, Ccl7, Ccl9, Ccl12, Ccl20, Ccl22, Ccl24	Ccl2, Ccl3, Ccl7, Ccl9, Ccl22
Chemokines (CXC-type)	Cxcl1, Cxcl3, Cxcl10, Cxcl11, Cxcl12, Cxcl16	Cxcl1, Cxcl10, Cxcl11, Cxcl12, Cxcl16, Xcl1
Chemokine receptors	Ccr1, Ccr1	
Cytokines	IL-1α, IL-1β, IL-6, IL-10, IL-12β IL-21, IL-23α	IL-6, IL-12β, IL-15
Cytokine Receptors	IL-2r gamma subunit, IL-13 alpha-1, IL-4 alpha, IL-12r- beta	IL-2r gamma subunit, IL-13r alpha 1 subunit
TLR	TLR1, TLR2	TLR1, TLR2
Clec family	Clec4a2, Clec4d, Clec4n, Clec7a	Clec4d, Clec4n, Clec5a, Clec7a
MHC Class I	H2-K1	H2-K1
MHC Class II	H2-D1, H2-Q7, H2-Q8, H28	H2-Aa, H2-Ab1, H2-D1, H2-Q8
IRG family	Iigp1	Iigp1
GBP family	Gbp1, Gbp2, Gbp3, Gbp6, Gbp8	Gbp1

Table 2: Immune Response Genes Up-regulated by type I and II Toxoplasma strains infection in astrocytes1.

	P valuesa				
	N genes	Type II	Type II	Type I	Type I
Neurobiological Category	In category	Up	Down	Up	Down
Nervous System Development (GO:007399)	1303	NS	NS	0.03503	0.01047
Transmission of Nerve Impulse (GO:0019266)	519	NS	NS	0.03503	1.1E-05
Schizophrenia Core Genes	33	NS	NS	NS	NS
Schizophrenia Gene Association	263	NS	NS	0.00005	0.01713
Schizophrenia Literature	1468	NS	NS	0.00304	0.00014
Schizophrenia Network	1010	0.0009	NS	0.00007	NS

Table 3: Impact of type I vs. type II-infection in astrocytes on Nervous System Processes and Schizophrenia Associated Genesa ; aAll comparisons against uninfected astrocytes at 24 h p.i. using murine homologues of genes (**http://www.informatics.jax.org/function.shtml**); FDR adjusted P values, NS–Non-significant.

RT-PCR Validation

Several genes were validated by RT-PCR for type I and type II infection at 24 h p.i. For type I infection, Hif1α, Ccl17, Cxcl12 and IL-6 were validated by RT-PCR. For type II infection, Ccl7, Ccl9, Cxcl1 and Cxcl10 were validated. Fold changes as determined by RT-PCR were in general agreement with microarray values.

	Fold Change1			
Gene	**Type I-24 h**	**Type I-24 h**	**Type II-24 h**	**Type II-24 h**
	RT-PCR	**Microarray**	**RT-PCR**	**Microarray**
Hif1α	2.1	1.8	---	---
Ccl7	---	---	15	12
Ccl9	---	---	15	7
Ccl17	157	51	---	---
Cxcl1	---	---	7	5
Cxcl5	131	80	---	---
Cxcl10	---	---	4	18
Cxcl12	19	143	---	---
IL-6	72	218	---	---

Table 4: RT-PCR Validation of Selected Genes; 1- fold change determined from infected vs. uninfected for type I (RH) and type II (Me49) strain infected astrocytes respectively at 24h p.i.; absence of values indicates no transcripts were detected.

Discussion

Astrocytes are an important host cell for the parasite in the brain, capable of harboring the cyst stage and supporting the tachyzoite stage. In this study we examined gene expression in murine astrocytes infected with a type I strain vs. type II strain of *T. gondii*. The results showed several important findings. First, *T. gondii* infection with both type I and type II strains, induced a strong transcriptional response in astrocyte host cells with immune response genes the dominant pathway affected. Secondly, the host cell response to type I strain was more robust than the type II strain inducing an earlier response to infection and larger changes in gene transcription. Thirdly, the host response to type I strain infection was qualitatively distinct, with only approximately 20% of the differentially regulated host cell genes in common between the type I and type II strains and with the dominant host cell processes affected distinct. Finally, while both type I and type II infection significantly affected host cell genes involved in neurobiological processes, the effect of type I strain on these processes was greater than the type II strain, inducing significant effects on neurodevelopmental processes and nerve impulse transmission, including many gene homologues associated with Schizophrenia. Transcriptional studies investigating the effects of infection of *Toxoplasma* virulent vs. avirulent strains have been done in human neuro epithelial cells, human fibroblasts and murine macrophages [32,35,36]. In all these cell types, the same trend of greater differential regulation of host cell genes by type I strain vs. avirulent type II and III strains were found. In this study in astrocytes and in neuroepithelial cells, in addition to the differential quantitative transcriptional response to infection with *Toxoplasma* strains, significant qualitative differences in the transcriptional response to type I vs. a virulent type II and III strains were also found, supporting the idea that virulent strains use different adaptive strategies than the a virulent strains to exploit the host cell. The robust and early transcriptional response induced by virulent type I strain observed in this study in astrocytes, affecting multiple host cell pathways early after infection, may represent a reprogramming of host cell processes, serving to subvert host cell functions to promote parasite growth and replication. This could in part, provide a mechanistic basis to explain rapid parasite growth and replication that is characteristic of type I strains. Conversely, the strategy taken by avirulent type II strains, which induce a mild host cell transcriptional response, may help maintain normal host cell functions resulting in slower parasite growth but allowing for long-term persistence in the host cell. This strategy may be of particular importance in the brain, where loss of host cells and host cell function has more costly effects than in tissues where cells can replace themselves and which have more robust repair mechanisms such as in the gut or in the skin.

Signal transduction was one of the dominant processes significantly impacted by type I strain infection in astrocytes, suggesting some of the differential host gene expression between strains may be due to parasite effects on host signaling pathways. The parasite kinase, ROP38, has been identified as a potent down regulator of host cell transcription, and lower levels of this parasite kinase in RH type I strain, has been shown to account for the greater induction of host cell genes by the RH strain vs. the type III strain, which expresses very high levels of ROP38 [32]. However ROP38 levels in Me49 and many other type II strains are also relatively low, indicating other factors are involved that account for differences in host cell transcriptional response between type I and type II strains [32,36]. Transcription factors up-regulated by type I but not type II infection in astrocytes in this study, included Hif1α, which has been reported in other transcriptional studies to be up-regulated by parasite infection and found to promote parasite growth, possibly via acquisition of host glucose and iron uptake [27, 29]. In astrocytes the early growth response genes 2 and 3 (EGR2 and EGR3) showed strong differential regulation by type I strain vs. type II strain infection with EGR2 induced 50-fold and EGR3 induced 168-fold by type I infection at 24 h p.i. vs. only 1.5-2-fold increases in EGR2 and EGR3 respectively by type II at 24 h p.i. (see Supplemental Tables S1 and S2). EGR2 and EGR3 regulate genes involved in cell growth, survival and differentiation and are immediate early response genes that are activated by growth factors, cytokines and other stimuli. In neuronal cells they are involved in cognitive processes including learning and memory [43]. Additionally, EGR3 has been identified as a susceptibility gene for Schizophrenia associated with neurodevelopment dysfunctions [44-46]. Differential effects on EGR2 and EGR3 could be involved in some of the *Toxoplasma* strain-specific regulation of host cell genes observed in this study in astrocytes and EGR3 may be of relevance to the parasites effects on neurodevelopmental pathways and development of Schizophrenia. A recent transcriptional study in murine macrophages has similarly identified strain-specific effects on host cell signaling pathways, although in macrophages the predominant strain-specific responses were in the type I interferon signaling pathway [36].

The strongest host cell response to both type I and type II infection was induction of immune response genes. Immune response genes have also been found to be the major class of genes affected by type I, II and III strains of *Toxoplasma* from transcriptional studies analyzing peripheral lymphocyte and brain tissues *in vivo* [37,47]. Collectively data from our study and the *in vivo* studies, indicate the importance of modulation of the host immunological response regardless of the parasite genotype. Also, while the strong induction of the host immune responses by parasite infection in immune cells is not surprising, data from our study in astrocytes and the in vivo study analyzing brain tissue, indicate non-immune cells and tissues also respond strongly to parasite infection, stimulating the host immune response. In astrocytes, the immune response to infection by type I strains was more significantly affected than type II infection indicating there are strain-specific effects on the immune responses in the brain, as discussed more fully below. A broad range of immune functions was induced by infection with both type I and II parasite strains in astrocytes, supporting results from many previous studies indicating the importance of astrocytes as resident immune effector cells in the brain [38,41,48].

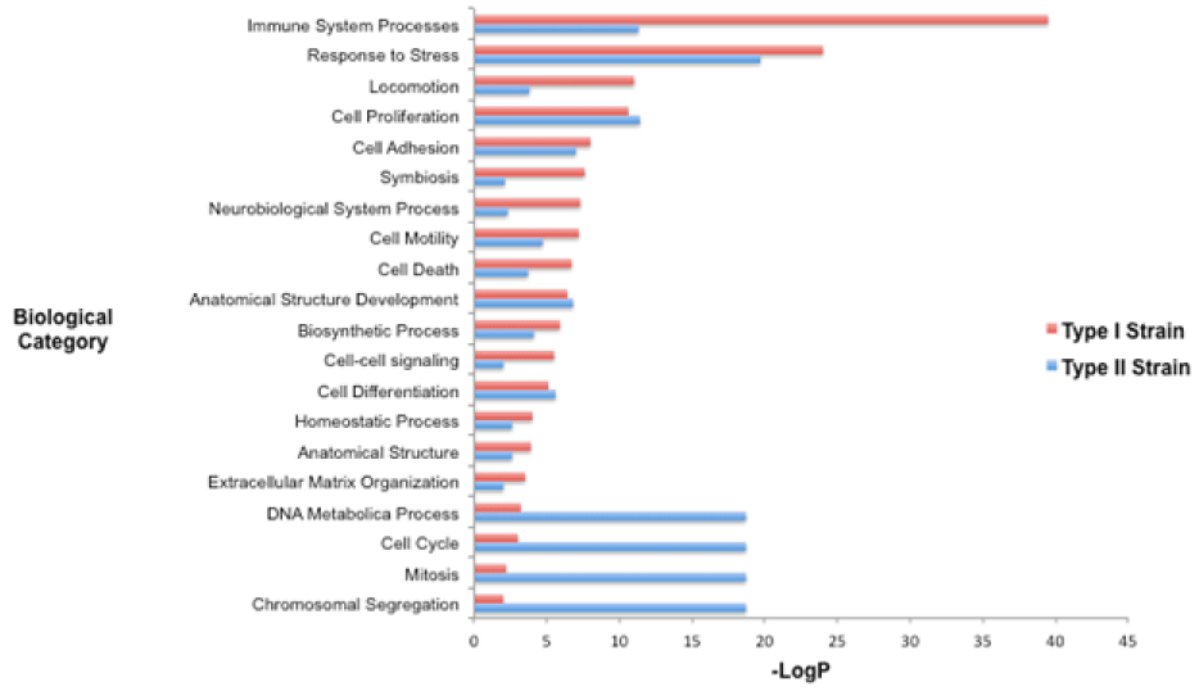

Figure 4: Common Pathways affected by type I vs. type II strains at 24h p.i. in astrocytes. Comparison of pathways perturbed (up or down-regulated) by type I vs. type II strain infection in astrocytes, as compared to mock-infected astrocytes using GO-SLIMS categories (n=72). P values of <0.05 and adjusted FDR of p<0.05 were used as a threshold to select significance.

Amongst the immune responses induced by infection were innate immune receptors including toll-like receptors and C-type lectin receptors, MHC class I and II genes, chemokine's and cytokines and members of both IRG/GBP protein families, which are endogenous immune effectors. Infection of astrocytes by both type I and type II strains induced a wide variety of CC and CXC type chemokine's, indicating infected astrocytes can induce monocytes, T lymphocytes, and neutrophils to traffic into the brain. As astrocytes are the dominant glial cell in the brain and support tachyzoite growth at least in the early phases of the infection in the brain, chemokine secretion by infected astrocytes is likely important both to immune cell recruitment into the brain, and specifically to direct these infiltrating leukocytes to the site of infection and parasite replication in the brain. Finally, while type I and type II strains induced CC and CXC chemokines, in g 403 eneral type I infection induced a more diverse and rapid chemokine response than type II infection, with over a dozen chemokine's induced by 2 h p.i., and higher levels of chemokine's expressed by 24 h (data not shown). Stronger chemokine responses to infection with type I vs. type II strains have also been reported in macrophages [49]. The quantitative difference of host chemokine transcripts elicited by *T. gondii* type I vs. type II strains likely has significant contributions to different levels of immunopathology and severity of toxoplasmosis in the brain. Additionally, in the brain, where neurons express chemokine receptors, chemokine's in addition to acting as inflammatory mediators in the brain, may also affect neuronal functions. For example, under non-pathological conditions, where chemokine's levels are low, chemokine's play a neuro modulatory role regulating neurotransmission, but in the presence of prolonged neuro inflammation, higher chemokine levels can lead to neuronal death [50-52]. Thus the strong chemokine response induced by infection with virulent strains, may also lead to more severe consequences in the brain such as increased neuronal cell death.

In addition to immune response, other universal or 'core' host responses to type I vs. type II infection included effects on cell-cell signaling pathways, cell locomotion, and cell proliferation. The effects of the parasite on these host cell processes is well known and collectively thought to ensure establishment of infection in the host with effects on cell-signaling pathways subverting the immune response, modulation of migratory properties of infected cells facilitating dissemination in the host, and induction of a 'proliferation response' serving to fulfill the growth requirements of the parasite during the intracellular phase of the infection [28,53,54]. In this study we found strain-specific effects on these common host responses. For example infection with type I strain induced a greater effect on cell locomotion than did the type II infection. Enhanced migration by infection with type I strains has been found in macrophages and dendritic cells facilitating a faster dissemination in the host and trafficking into the brain [54,55]. Analogously, a hypermotility phenotype of type I infected astrocytes, a resident brain cell, could lead to greater parasite dissemination within the brain and resulting in greater pathology. Cell proliferation and cell death were other core responses that were differentially affected by strain types in our study in astrocytes with type II infection inducing a stronger effect on cell proliferation and related processes such as the cell cycle and mitosis, while conversely type I strains more significantly impacted cell death. A similar differential response on cell division and apoptosis between type I and type II strains has been reported in trophoblast cells and HeLa cells where S phase indices were higher in Me49 vs. RH-infected cells, while the apoptotic indices were higher in RH-infected cells than Me49-infected cells [56]. These results indicate type I vs. II strains use different strategies to exploit the host cell. In the brain, the type II strategy of maintaining host cell proliferation while minimizing cell death could be especially important as loss of host cells may have more severe consequences. These strain-specific effects on cell proliferation, cell death, and cell motility, could have important impacts on the host-parasite relationship in the brain and remain to be investigated further.

A significant finding of this study was the differential effects of the type I vs. type II strain on neurobiological processes. Similar differential effects of infection with type I vs. avirulent type II and III strains on neurobiological processes have been found in neuroepithelial cells and infected brain tissue [35,47]. But significantly, in our study we also identified that infection with the type I strain, but not type II strain, significantly affects neurodevelopmental processes, transmission of nerve impulse and several gene sets associated with Schizophrenia. *T. gondii* in immune competent individuals has been implicated, via wide variety of epidemiological studies and via studies of human personality profiles, to be associated with behavioral, affective and cognitive abnormalities in humans and strongly associated with Schizophrenia [9,12,57]. Results presented in this study are consistent with the idea that *Toxoplasma* infection plays a role in these neurological disorders and supports the suggestion that type I strains have a more significant impact on brain functions relevant to neurological disorders such as Schizophrenia. A high prevalence of infection with type I strains and Ocular Toxoplasmosis and Cerebral Toxoplasmosis in some immune compromised patients, has been found indicating type I infection can result in more severe neurological outcomes [18,19,22]. Furthermore, offspring of mothers with a serological pattern consistent with type I infection, have been found to be at a greater risk of development of psychosis, supporting the idea that type I infection can play a role in the etiology of neuropsychiatric disorders [58]. Schizophrenia is a complex disorder is widely regarded as a dysregulation of dopamine and/or glutamate transmission [59]. The neurodevelopmental hypothesis of schizophrenia postulates schizophrenia pathogenesis begins early in life, and environmental influences, such as infectious agents, interact with causative genes leading to abnormalities in neurite outgrowth and neuronal differentiation that may result in dysregulation of dopamine cellular signaling pathways [59-61]. The finding presented in this study that host cells infected with type I strain of *Toxoplasma* affect neurotransmission and neurodevelopmental pathways, are consistent with this neuro developmental hypothesis of schizophrenia and dysregulation of neurotransmission in schizophrenia. While these studies were done in astrocytes, future studies in neurons, would be useful to further study the effect of type I infection on neurotransmission and developmental processes related to neurite growth and formation of the synapse. Astrocytes however interact with both the pre and post-synaptic terminals of neurons and are now believed to play a significant role in regulation of nerve transmission [62-64]. Additionally, astrocytes are also now recognized to be involved in synaptic plasticity and synaptogenesis during development and have been suggested to play a central role in neuropsychiatric disease [65,66]. Thus *Toxoplasma* type I strain infection of astrocytes may also contribute to dysregulation of neurotransmitter levels and/or affect synaptic transmission of neurons. Finally, while our transcriptional studies and transcriptional studies by others have been done with the tachyzoite stage, as the parasite exists in the cyst stage in immune competent individuals with a chronic infection, future studies

of the cyst stage would also be useful to further define the effects of *Toxoplasma* infection in the brain.

Transcriptional analyses have been used to gain deeper insight into the molecular basis of *Toxoplasma* interactions with its host cell in several different host cell types, including immune cells such as macrophages and non-immune host cells such as fibroblasts and neuro epithelial cells. This study contributes to the understanding of the molecular basis of host cell/parasite interactions and adds new insights into the differential effects of parasite virulent vs. avirulent strains in astrocytes, a clinically relevant host cell for the parasite in the brain. Additionally, this study found many strain-specific host cell responses in astrocytes (available in Supplemental Tables S1 and S2), which have not reported in other cell types that could be probed for future studies. Importantly results of this study reveal differential effects of virulent type I strains on neurobiological processes that may be relevant to our understanding of chronic Toxoplasmosis in the brain and the role of *Toxoplasma* in neuropsychiatric disorders such as Schizophrenia. A better understanding of the causative effects of *Toxoplasma* infection on neuronal host cells may lead to development of new methods of treatment and prevention of Schizophrenia and other neuropsychiatric disorders.

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