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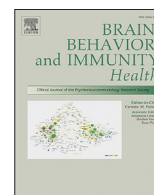
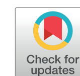


Full Length Article

Morphological and biochemical repercussions of *Toxoplasma gondii* infection in a 3D human brain neurospheres model



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A R T I C L E I N F O

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A B S T R A C T

Background: Toxoplasmosis is caused by the parasite *Toxoplasma gondii* that can infect the central nervous system (CNS), promoting neuroinflammation, neuronal loss, neurotransmitter imbalance and behavioral alterations. *T. gondii* infection is also related to neuropsychiatric disorders such as schizophrenia. The pathogenicity and inflammatory response in rodents are different to the case of humans, compromising the correlation between the behavioral alterations and physiological modifications observed in the disease. In the present work we used BrainSpheres, a 3D CNS model derived from human pluripotent stem cells (iPSC), to investigate the morphological and biochemical repercussions of *T. gondii* infection in human neural cells.
Methods: We evaluated *T. gondii* ME49 strain proliferation and cyst formation in both 2D cultured human neural cells and BrainSpheres. Aspects of cell morphology, ultrastructure, viability, gene expression of neural phenotype markers, as well as secretion of inflammatory mediators were evaluated for 2 and 4 weeks post infection in BrainSpheres.
Results: *T. gondii* can infect BrainSpheres, proliferating and inducing cysts formation, neural cell death, alteration in neural gene expression and triggering the release of several inflammatory mediators.
Conclusions: BrainSpheres reproduce many aspects of *T. gondii* infection in human CNS, constituting a useful model to study the neurotoxicity and neuroinflammation mediated by the parasite. In addition, these data could be important for future studies aiming at better understanding possible correlations between psychiatric disorders and human CNS infection with *T. gondii*.

1. Introduction

Toxoplasmosis is caused by an obligatory intracellular parasite, the protozoan *Toxoplasma gondii*, one of the world's most common zoonoses that affects around one third of population (Montoya and Liesenfeld, 2004). The majority of exposed individuals are asymptomatic (Nissen

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et al., 2017), and the acute form of the disease evolves into a latent, chronic condition, with *T. gondii* inside tissue cysts. *T. gondii* can cross the blood-brain barrier, infecting the central nervous system (CNS), mainly the amygdala, hippocampus, olfactory bulb and cortex (Berenreiterova et al., 2011; Hermes et al., 2008; Schluter and Barragan, 2019). The infection disturbs neural circuits, inducing neuroinflammation, neuronal loss and CNS deficits, indicating a possible correlation between *T. gondii* infection and neurodegenerative disorders, like Alzheimer and Huntington diseases (Donley et al., 2016; Kusbeci et al., 2011). Moreover, *T. gondii* prenatal exposure increases congenital syndromes probability, including CNS disturbances like intellectual disability and seizures in the affected newborns (Jones et al., 2001).

The latent infection is also related to psychomotor deficits, lower intelligence quotient and personality changes (Flegr et al., 2003; Havlicek et al., 2001), generating strong interest in elucidating how *T. gondii* infection affects human behavior (Tyebji et al., 2019). Toxoplasmosis has been associated with suicidal tendencies, traffic accidents, cognitive deficits, psychiatric disorders, anxiety, aggressiveness and violence (Dickerson et al., 2017, 2018; Flegr et al., 2002; Flegr and Horacek, 2018; Hamdani et al., 2017; Markovitz et al., 2015; Pedersen et al., 2012; Sutterland et al., 2015; Tyebji et al., 2019). For suicidal individuals, higher *T. gondii* IgG levels were reported, suggesting that infection could enhance human behavioral alterations (Arling et al., 2009), also observed in infected rodents that develop predation risk changes as observed through their reduced fear of cats (Berdoy et al., 2000; Ingram et al., 2013), also reported for other predators (Boillat et al., 2020; Mitra et al., 2013; Vyas et al., 2007). Additionally, infected rodents display impaired motor performance, as well as learning and memory deficits (Hay et al., 1983; Witting, 1979).

T. gondii infection has also been related to neuropsychiatric disorders like schizophrenia spectrum disorders, bipolar disorder and anxiety (Del Grande et al., 2017; Elsheikha et al., 2016; Mahmoudvand et al., 2015); these reports are reinforced by the higher *T. gondii* IgG levels in schizophrenic patients than in control individuals (Dickerson et al., 2014; Torrey et al., 2007). These neuropsychiatric disorders may be related to neurotransmitter imbalance promoted by the CNS parasite invasion. The infection affects several neurotransmitter levels like dopamine, glutamate, GABA and others (Brooks et al., 2015; David et al., 2016; Martin et al., 2015; Tonin et al., 2014; Wang et al., 2019), and their deregulation is already known to be related to mental disorders (Berk et al., 2007; Brisch et al., 2014). Also, infected rodents show an increase in dopamine release (Martin et al., 2015) and its secretion by the cysts in infected rodents and humans, contributing to its local increased levels that may be associated with behavioral changes, as observed in schizophrenic patients (Eyles et al., 2012; Martin et al., 2015; McConkey et al., 2013). However, recent studies have shown the neuroinflammation mediators importance in the behavioral alterations observed in the infection, a process also observed in neuropsychiatric disorders (Boillat et al., 2020; Martynowicz et al., 2019). Behavioral alterations were observed in mice infected with a strain incapable of inducing cyst formation, suggesting that neuroinflammation could be responsible (Ingram et al., 2013; Martynowicz et al., 2019).

Neurotransmitter and neuroinflammation imbalance may play an important role in the behavioral alterations in infected animal models and humans. However, the precise nature of the correlation remains to be investigated. The pathogenicity and inflammatory characteristics of disease response differ in humans compared to rodents. Animal models have provided a useful tool to study *T. gondii* infection, in particular to address the initial phase of host-parasite interaction at the cellular and biochemical levels. Despite the usefulness of animal data, these models fall short of addressing specific aspects of human cell physiology and kinetics. In this work, we used a human 3D CNS model derived from human pluripotent stem cells (iPSC), BrainSpheres, to investigate morphological and biochemical repercussions of *T. gondii* infection.

2. Methods and materials

2.1. iPSC generation

iPSCs were generated from Human Exfoliated Deciduous Teeth Stem Cells (SHED), from control donors recruited through the Tooth Fairy Project initiative (USP, Ethics Committee Protocol 1001). Reprogramming using the Sendai virus (Life Technologies) carried Oct4, Sox2, Kfl4 and c-Myc (Takahashi and Yamanaka, 2006). SHED were transduced and transferred two days later to a feeder layer condition (murine embryonic fibroblasts, MEFs (Millipore)) and maintained in DMEM/F12 (Life Technologies), 20% KSR (Invitrogen), 1% NEAA, and 100 μM beta-mercaptoethanol. The iPSCs colonies were identified after 3 weeks and transferred to Matrigel (BD Biosciences) coated plates and maintained in mTeSR medium (Stem Cell Technologies), changed daily.

2.2. NPC generation

The iPSC medium was changed to N2 medium: DMEM/F12 with 1× N2 (Invitrogen) and dual SMAD inhibitors: 1 μM dorsomorphin (Tocris) and 10 μM SB431542 (Stemgent). Colonies in suspension as Embryoid Bodies (EBs) after 5 day at 90 rpm in N2 medium were plated on matrigel-coated plates with NGF medium: DMEM/F12 with 0.5× N2, 0.5× Gem21 (Gemini Bioproducts), 20 ηg/mL FGF2 and 1% Pen/Strep. Rosettes containing neural progenitor cells (NPCs) were dissociated and plated in poly-ornithine (10 μg/mL, Sigma) and Laminin (2.5 μg/mL, ThermoFisher) coated plate. NPCs expansion used NGF medium.

2.3. ME49 Toxoplasma gondii strain maintenance

The *T. gondii* strain ME49-Luc, a type II strain expressing luciferase and GFP (Boyle et al., 2007) was provided by Dr. John C. Boothroyd, Stanford University. Tachyzoites were maintained in Normal Human Dermal Fibroblasts-Neonatal (NHDF, Lonza; provided by Dr. Sheila Nardelli, ICC/FIOCRUZ-BR) monolayers. The parasites released from infected cells were quantified using a Neubauer chamber.

2.4. NPC maintenance, 2D neuronal culture and infection

NPCs differentiated from iPSC were maintained up to 12 passages in proliferation medium: KO DMEM/F12 medium with 1× StemPro, 20 ng/mL human bFGF andEGF, 4 mM L-glutamine and 500/500 Units/μg Pen/Strep (ThermoFisher).

NPC were resuspended in the differentiation medium: Neurobasal Electro medium with B-27-electro, 10 ng/mL BDNF and GDNF, 4 mM L-glutamine, 500/500 Units/μg Pen/Strep (ThermoFisher). The cells were seeded on glass coverslips coated with 10 μg/mL poly-L-ornithine and 2.5 μg/mL laminin (Sigma) at 5×10^4 cells per well in 24-well plates and maintained at 37 °C and 5% CO_2. Cells were used after 4 weeks of differentiation.

After parasites dilution in differentiation medium at 1:100 (parasite/cell), cells were infected after 4 weeks of differentiation (day 28). Bright field images were acquired in a Leica DMI3000 microscope (Wetzlar, Germany).

2.5. BrainSpheres culture and infection

For BrainSpheres differentiation (described in (Pamies et al., 2017)), cells were seeded at 4×10^3 cells per hydrogel hollow mold with 400 μm diameter made in the laboratory using the differentiation medium and maintained at 37 °C and 5% CO_2. The ME49-Luc strain was diluted in the differentiation medium at 1:25 (parasite/cell). BrainSpheres were infected after 4 weeks of differentiation (day 28). The spheroid size was quantified in bright field micrograph images using a Leica DMI3000 microscope and ImageJ software (https://imagej.nih.gov/ij/index.html, NIH).

2.6. Immunofluorescence

Samples were fixed, blocked with 2% BSA (Sigma) and incubated overnight with primary antibodies: MAP2 (chicken, Abcam) at 1:1000, Synapsin-1 (rabbit, Millipore) at 1:400 and SOX2 (goat, Cell Signaling) at 1:200. For primary antibodies detection, samples were incubated with Alexa Fluor 647 for MAP2 and SOX2 and Alexa Fluor 555 for Synapsin-1 secondary antibodies (Life Technologies) at 1:500 and mounted using Prolong Gold Antifade (Invitrogen). Images were obtained using 40X objectives on a Zeiss microscope (Carl Zeiss Microscopy GmbH, Germany).

2.7. Histological staining and confocal microscopy

BrainSpheres were fixed with 4% formaldehyde, incubated with blocking/permeabilization buffer (1% BSA, 5% goat serum, 0.15% saponin (Sigma)), followed by overnight incubation with *Dolichos biflorus* lectin conjugated to Tetramethylrhodamine-5-(-6)-isothiocyanate (Vector Lab) at 10 µg/mL in blocking buffer. Samples were mounted on glass slides with Prolong Gold Antifade with Dapi. Z-stacks were acquired with ~100–200 nm optical sections. Images were obtained using a Structured Illumination Microscopy (SIM) mode in a Zeiss ELYRA PS.1 (Zeiss) microscope with identical time exposure and settings. Quantification of DBA-TRITC stained vacuoles were performed analyzing 10 different fields from two independent experiments.

2.8. Scanning electron microscopy (SEM)

NPCs and BrainSpheres were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Samples were post-fixed with 1% osmium tetroxide in sodium cacodylate buffer, followed by cacodylate buffer and distilled water washing steps. After gradual dehydratation in ethanol series, drying with CO_2 critical-point method in a CPD300 (Leica), mounting on aluminum stubs and coating in Balzers Apparatus, micrographs were obtained with 25 kV in FEI QUANTA 250 SEM (ThermoFisher).

2.9. Transmission electron microscopy (TEM)

BrainSpheres were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer, followed by sodium cacodylate buffer washing steps. Samples were post-fixed with 1% osmium tetroxide, 5 mM calcium chloride and 1.25% potassium ferrocyanide in sodium cacodylate buffer, followed by cacodylate buffer and distilled water washing steps. After gradual dehydratation with acetone and embedding in Epoxy resin (PolyBed812, Polysciences), ultrathin sections were obtained with Leica C7 ultramicrotome, collected with copper grids and stained with uranyl acetate and lead citrate. Grids were observed with 120 kV in FEI Tecnai Spirit TEM.

2.10. qRT-PCR

Total RNA was extracted from NPC cultures and BrainSpheres using RNeasy mini kit (Qiagen), according to manufacturer's instructions. The amount and purity were evaluated in UV spectrophotometer (NanoDrop, 2000; Thermo Fisher), by A260/280 and 260/230 ratios. RNA samples were treated with RNase-free DNase I and cDNA synthesis was performed using First-Strand cDNA synthesis kit (GE Healthcare), according to manufacturer's instructions. The DNA amplification was performed using GoTaq qPCR Master Mix (Promega) and carried out on a Real-Time PCR System (StepOne, Applied Biosystems) with conditions previously described by our team (Brito et al., 2012). Amplification was performed using specific primers for GAPDH, MAP2, GFAP, OLIG1, Synaptophysin, vGLUT1, GAD65, TH and TOXO. Data were analyzed using the StepOne software (Applied Biosystems) and the relative quantity was calculated according to $2^{-\Delta\Delta Ct}$.

2.11. Lactate dehydrogenase (LDH) release

LDH release in BrainSpheres supernatants was determined with CytoTox 96 Cytotoxicity Assay (Promega) according to manufacturer's instructions. Briefly, the supernatants were incubated with substrate solution, followed by stop solution. Absorbance was measured at 490 nm.

2.12. Multiple secreted mediators analysis

Cytokines, chemokines and growth factors secreted by infected BrainSpheres were determined in Bio-Plex Magpix apparatus (Bio-Rad) through Luminex (Austin TX, USA) xMAP magnetic technology using a human cytokine 27-plex assay kit (Biorad). Analysis was performed as previously described by our team (Leite et al., 2019).

2.13. Statistical analysis

Mean and standard errors was calculated with GraphPad Prism 7 (GraphPad Software). One- or two-way ANOVA and unpaired t tests were applied to obtain statistical significance. Secreted inflammatory mediator concentrations were quantified with xPONENT software version 4.2 (Biorad). Differences were considered significant at the 0.05 level of confidence.

3. Results

Cyst formation and proliferation of T. gondii ME49 strain in neural cell monolayer cultures.

BrainSpheres express mature neurons (MAP2) after 6 and 8 weeks of differentiation, while NPC (SOX2) exhibit a slight reduction (Supplemental Fig. 1). BrainSpheres were differentiated for 4 weeks, infected with the *T. gondii* ME49 strain and observed until 4 days post-infection (dpi). After 1 dpi, it was observed neural projections in both control and infected cultures, but at 4 dpi, we observed differences in cellular morphology (Fig. 1A).

Intact and disrupted cysts were observed at 4 dpi. It was also observed the parasites emerging from infected cells and spreading to the neighbor NPC in differentiation (Fig. 1B). In addition, *T. gondii* was able to proliferate due to increase of B1 gene transcripts (~60× after 3 dpi, $p < 0.001$) (Fig. 1C).

3.1. T. gondii ME49 strain induces neural death in BrainSpheres

BrainSpheres are reproducible in size (200–260 µm) from batch to batch and experiment to experiment. Their small size prevents a necrotic core development as many larger sized organotypic models do (Pamies et al., 2017), making our model very suitable for neural infection studies. BrainSpheres were infected with 4 weeks (28 days) of differentiation and observed for a further 2 and 4 weeks (6 and 8 weeks of differentiation, respectively). Control BrainSpheres showed spherical shape and ~250 µm in diameter from day 28–42 (6-weeks of differentiation) (Fig. 1D and E). However, infected BrainSpheres showed irregular shape and a reduced diameter (Fig. 1D and E). Infected BrainSpheres showed an average diameter of 150 ± 12 µm at day 42, corresponding to 42% reduction compared to control (±7%, $p < 0.001$) (Fig. 1E).

At day 32 of differentiation (4 dpi), *T. gondii* infection induced a large increase in LDH leakage ($p < 0.001$), reaching maximum levels at day 42 (6 weeks of differentiation) ($p < 0.001$) (Fig. 1F).

3.2. Morphological alterations and cystogenesis of T. gondii ME49 strain in BrainSpheres

After 4 weeks of differentiation, uninfected BrainSpheres showed typical morphology with viable appearance (Fig. 2A, C, E). However, infected BrainSpheres showed altered morphology and degenerated cells

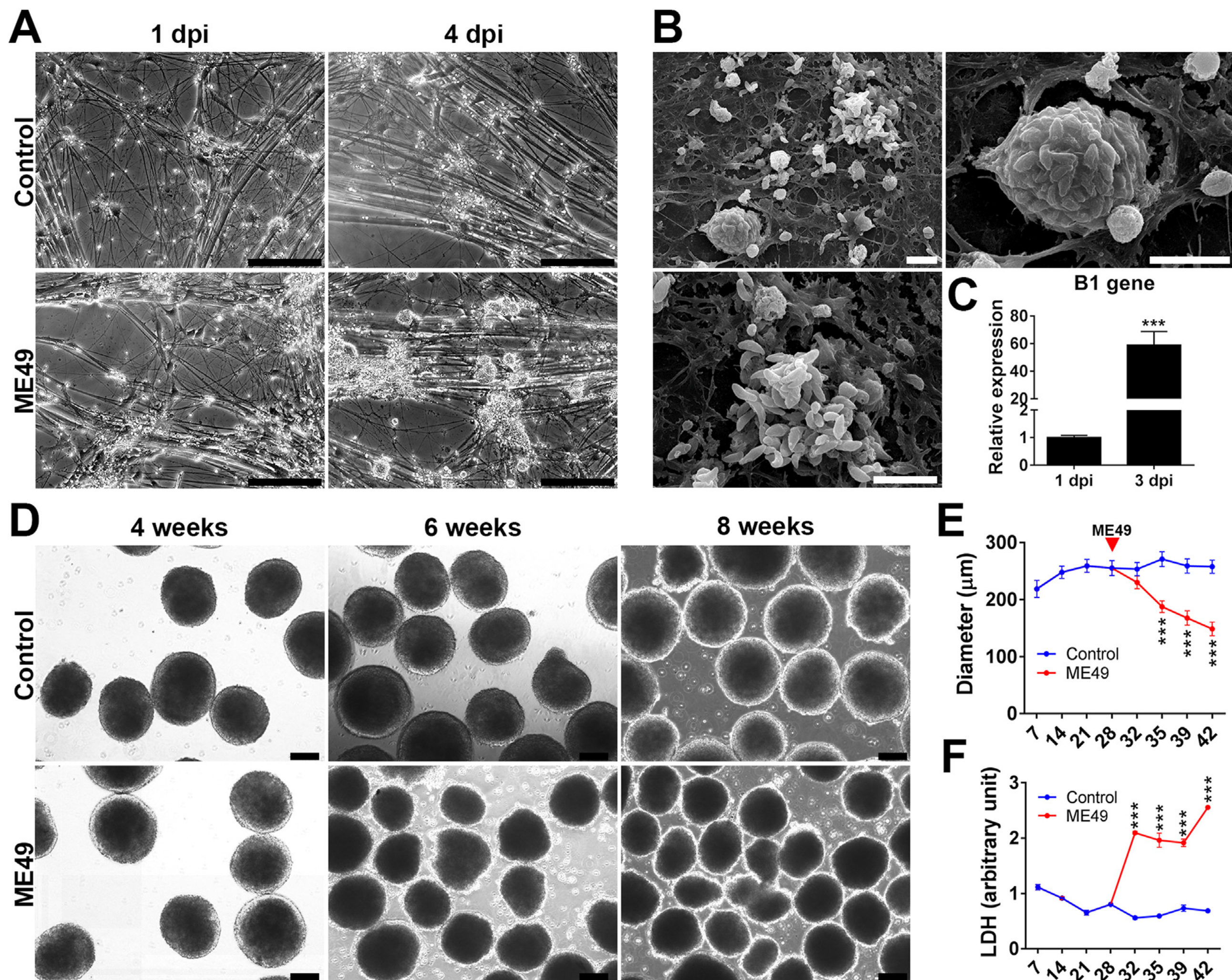

Fig. 1. Cyst formation and proliferation of ME49 strain in monolayers of neural cell cultures and analysis of cell death in BrainSpheres. (A) Phase contrast images of monolayer neural cells differentiated during 4 weeks without infection (Control) and after 1 and 4 dpi with ME49 strain of *T. gondii*. (B) SEM micrographs showing an intact cyst and parasites from a disrupted cyst spreading to neighbor cells of monolayers of neural cell cultures. (C) qRT-PCR of *B1* gene of *T. gondii* after 1 dpi and 3 dpi of NPC cultures. (D) Bright field images of BrainSpheres differentiated for 4, 6 and 8 weeks without infection (Control) and after infection when BrainSpheres reach 4 weeks (day 28) of differentiation (ME49). (E) Quantification of BrainSpheres diameter during differentiation from day 7–42 (1–6 weeks of differentiation). Blue line corresponds to control non-infected and red line corresponds to ME49-infected. Red arrowhead indicates the moment of infection with ME49 strain of *T. gondii* at day 28. (F) Quantification of LDH in the BrainSpheres supernatant during differentiation from day 7–42. Blue line corresponds to control non-infected and red line to ME49-infected. Scale bars: (A) 100 μm; (B) 10 μm; (D) 100 μm. All data were collected from three independent experiments, with three technical replicates representing fold changes (±SD) for (C) and mean (±SEM) for (E) and (F). Student *t*-test was used to analyze the statistical significance for (C) and two-way ANOVA with Bonferroni's post-test for (E) and (F) (***$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on their surface (Fig. 2B). Moreover, many parasites were observed inside the core of infected BrainSpheres (Fig. 2D and F).

After 2 weeks of infection (6 weeks of differentiation), BrainSpheres showed a dense material deposition under the parasitophorous vacuole membrane, leading to a gradual membrane thickening, indicative of cystic wall formation (Fig. 2G–I) and cystogenesis. DBA lectin labeled samples confirmed the cyst wall formation (Zhang et al., 2001). BrainSpheres showed a large number of parasitophorous vacuoles labeled with DBA, indicating cystogenesis due to tachyzoites differentiation to bradyzoite (Fig. 2J and K). Analysis of DBA staining showed the presence of vacuoles with complete (arrowheads, Supplemental Fig. 2A) and partial staining for DBA (arrows, Supplemental Fig. 2A), which is an indicative of the beginning of cyst conversion. Quantification of cyst conversion rate showed that 30.6% ($p < 0.01$) of vacuoles were positive for DBA (Supplemental Fig. 2B).

3.3. *T. gondii* ME49 strain into the BrainSpheres

BrainSpheres showed their features and viability maintenance during differentiation (Fig. 3A and B). BrainSpheres showed *T. gondii* infection progression for at least 4 weeks. After 1 week of infection, the BrainSpheres contained several parasitophorous vacuoles with parasites showing numerous cytoplasmic amylopectin-like granules (Fig. 3C–E), a bradyzoite stage characteristic. The bradyzoites development was observed in the whole organoid (Fig. 3C–E).

3.4. *T. gondii* ME49 strain alters the gene expression of neural cells in BrainSpheres

Specific mRNA levels related to cell types, synapses and neuronal phenotypes were analyzed at different stages of BrainSpheres

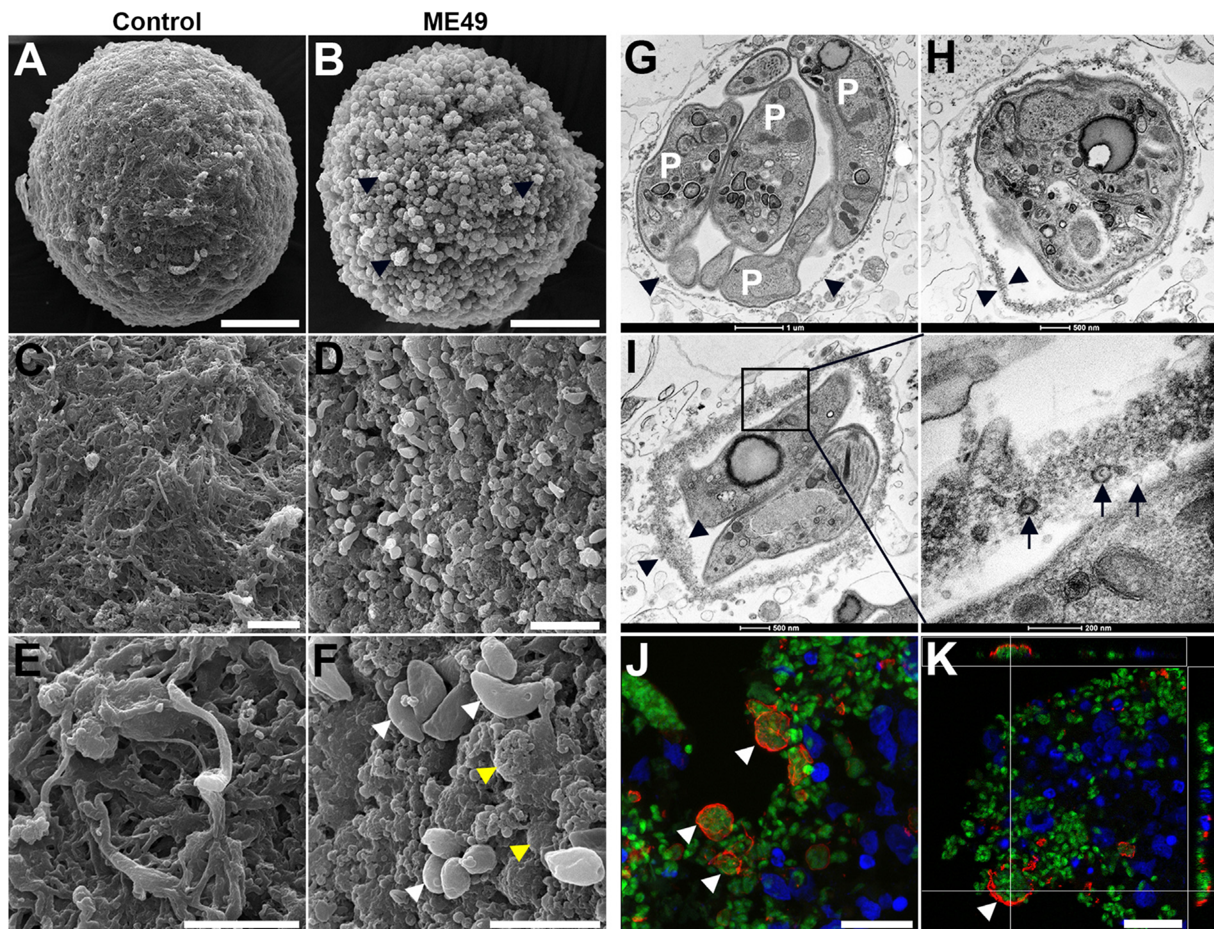

Fig. 2. SEM, TEM and confocal microscopy images showing cellular damage and cystogenesis after infection of 4 weeks differentiated BrainSpheres with *T. gondii* from ME49-Luc (expressing luciferase and GFP) for 2 weeks (6 weeks total). (A, C, E) BrainSpheres maintained without infection (Control), showing (C, E) structures similar to neural projections. (B, D, F) SEM images of BrainSphere infected with *T. gondii*, it is possible to observe a pattern of generalized degeneration of the cells on the sphere surface (black arrowheads in B). (D, F) A BrainSphere was ruptured allowing the detection of several parasites (white arrowheads in F) and degenerated cells (yellow arrowheads in F) inside the 3D sphere. (G-I) It is possible to observe by TEM observations the deposition of a dense material under the membrane of the parasitophorous vacuole, which leads to a gradual thickening (black arrowheads). This indicates the formation of the cystic wall of bradyzoites. (inset in I) The cystic wall shows a particular composition with secreted materials by the parasites (black arrows). P = parasites. (J, K) Confocal laser-scanning microscopy analysis after labeling infected BrainSpheres with *Dolichos biflorus* lectin (DBA-TRITC) (red) confirmed the presence of *T. gondii* cysts (white arrowheads). The parasites from ME49-Luc show green fluorescence, DAPI labeled cell nuclei represented by blue fluorescence. Scale bars: (A, B) 50 μm; (C, D) 10 μm; (E, F) 5 μm; (G) 1 μm; (H, I) 500 nm; (inset in I) 200 nm; (J, K) 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

differentiation and infection. The parasite tightly reduced MAP2 and GFAP mRNA levels. OLIG1 mRNA levels were drastically increased soon after the infection, mainly in the sixth week of differentiation, corresponding to the second week of infection. The infection induced a sharp increase in vGLUT1 and GAD65 mRNA levels, while tyrosine hydroxylase (TH) mRNA levels sharply reduced, mainly in the sixth week of differentiation. Indeed, synaptophysin mRNA levels were also lower than in non-infected BrainSpheres, suggesting synapse loss. The synaptophysin mRNA levels were stable from the fourth week (without infection) until the eighth week of differentiation (with infection) (Fig. 4A).

Ultrastructural observations showed differences between the pattern of myelin-like structures in uninfected and infected BrainSpheres (Fig. 4B and C). While non-infected cells showed a normal morphology with concentric arrangement (Fig. 4B), the infection caused a remarkable alteration similar to myelin decompaction (Fig. 4C). Indeed, infected BrainSpheres (Fig. 4E) showed a reduction of synapsin-1 immunodetection, a pre-synaptic protein marker, compared to control group (Fig. 4D). These results indicate that *T. gondii* infection differentially affects the neuronal phenotypes and synapses in BrainSpheres.

3.5. T. gondii ME49 strain induces the release of cytokines, chemokines and growth factors in BrainSpheres

Multiple secreted proteins analysis from non-infected BrainSpheres showed that most analytes were secreted in the initial phases of differentiation (7–21 days), decreasing over time. Despite TNFα and IL-1β reduction, these cytokines showed an increase (~28–42 days) (Fig. 5).

Infected BrainSpheres evidenced a sharp increase in all shown analytes. The pro-inflammatory cytokines levels like TNFα, IFNγ, IL-1β and IL-6 increased with a plateau up to 42 days. IL-15 also seems to be maintained during the period of culture. The pro-inflammatory IL-7, IL-8, IL-12 and IL-13 showed a quick response soon after infection, persisting for 7 days (up to 35 days) and then started to drop (Fig. 5A). The anti-inflammatory mediators IL-1ra and IL-10 showed a sharp increase; however, while IL-1ra was maintained, IL-10 showed a strong but gradual decrease (Fig. 5B). Chemokines displayed a pattern similar to pro-inflammatory cytokines, but with a delayed response: IP-10 and RANTES showed no measurable levels until at least 4 days after infection (32 days). In contrast, MCP-1 showed increased levels soon after infection (***$p < 0.001$) (Fig. 5C). Growth factors like GM-CSF and PDGF-BB

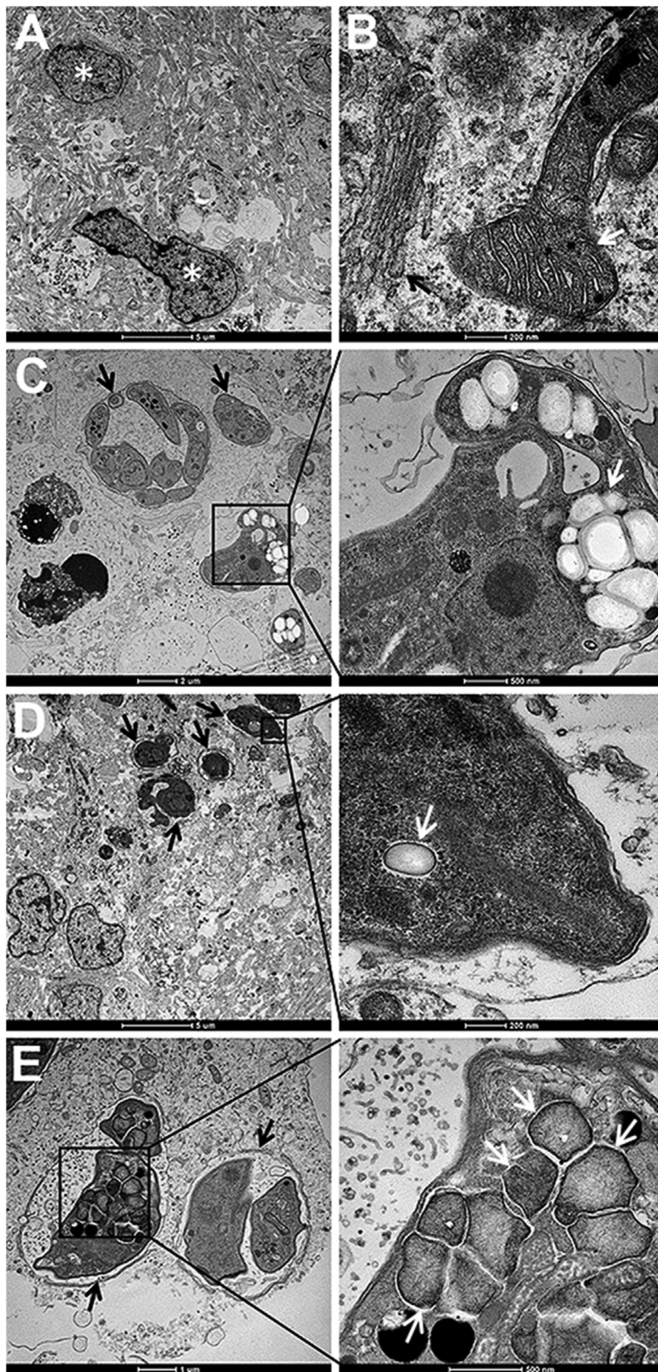

Fig. 3. TEM images showing BrainSpheres infected with ME49 strain of *T. gondii*. (A, B) BrainSpheres differentiated for 4 weeks and maintained without infection (Control) for another week. The BrainSpheres were accessed and show neural cells with typical cytoplasmic organization and cellular nuclei (asterisks in A). Indeed, organelles such as mitochondria (white arrow in B) and Golgi (black arrow in B) were observed. (C-E) After 4 weeks of differentiation, BrainSpheres were infected with *T. gondii* and then cultured for another week. Infected cells show parasitophorous vacuoles containing several parasites (black arrows in C, D and E) with preserved structures. The presence of parasites harboring granules similar to amylopectin confirms the differentiation process of tachyzoites to bradyzoites (white arrows inset in C, D and E). Scale bars: (A) 2 μm; (B) 200 nm; (C) 2 μm, inset 500 nm; (D) 5 μm, inset 200 nm; (E) 1 μm, inset 500 nm.

showed a delayed response, increasing their levels only after 7 days of infection (day 35). bFGF and VEGF showed a sharp increase immediately after infection (day 32), but bFGF was almost undetectable at day 35, whereas VEGF showed a strong but gradual decrease (Fig. 5D).

4. Discussion

Though rodent *in vivo* models are important tools for the analysis of various aspects of *T. gondii* infection, some of the results are not reproducible in humans due to differences between the species, highlighting the need for new models able to more closely represent human infection. Recently, our group established BrainSpheres as a useful tool to study the toxicity and biocompatibility of nanomaterials for brain drug delivery (Leite et al., 2019). Compared to 2D cell culture, the 3D model displays more cell interactions and can better reproduce the *in vivo* physiology (Pamies et al., 2014; Zafeiriou et al., 2020). Thus, in this work, we used BrainSpheres as a 3D human CNS model to study the *T. gondii* infection repercussions in the human brain.

T. gondii from avirulent strain ME49 was able to infect, proliferate and convert to cyst forms in both 2D human neural cell cultures and in BrainSpheres surface and deeper layers. Cysts and tachyzoites were found in BrainSpheres core, indicating *T. gondii* invasiveness in the 3D model. Cyst formation was observed and the bradyzoites cystic wall was confirmed by DBA lectin labelling (Zhang et al., 2001). Structural markers like amylopectin granules (Coppin et al., 2003) and dense granular material deposition under PV membrane were also observed. Cystogenesis is an important event related to the *T. gondii* presence and permanence in the CNS, two processes mostly studied in rodents and 2D models (Barbosa et al., 2020; Boillat et al., 2020; Evans et al., 2014).

In BrainSpheres, we observed that *T. gondii* infection reduces cellular viability. A significant infection effect was the spheroid size reduction (~42%), which correlated with the augmented neural cell death. BrainSpheres extensive damage was also evidenced by LDH leakage. Since the CNS is a high infection load, an important clinical manifestation of toxoplasmosis in reactivated patients is encephalitis (Bertoli et al., 1995; Luft and Remington, 1992). In addition, focal brain necrosis is frequently observed in congenitally infected newborns. Thus, the cellular damage in infected BrainSpheres could result from the natural parasite replication cycle, causing cell lysis (Blader et al., 2015).

BrainSpheres expressed neuronal and glial markers with distinct neuronal phenotypes similar to other studies (Pamies et al., 2017; Zafeiriou et al., 2020). We observed decreased mRNA levels of neuronal and astrocytic markers, consistent with the cell death findings. In contrast, we also observed increased OLIG1 mRNA levels and alterations resembling myelin decompaction. We have not observed *T. gondii* infection predilection for any particular cell type, concurring with *in vitro* studies using rodent and human cells that show cyst formation in neurons and astrocytes (Fischer et al., 1997a; Halonen et al., 1996). Cysts in neuronal processes usually result in neurite and synapse loss (Barbosa et al., 2020; Cabral and Koshy, 2014). This is in agreement with our data showing infection reducing synaptophysin mRNA levels and synapsin-1 immunodetection compared to non-infected BrainSpheres, which can be interpreted as a reduction in synapse density. Neurite and synapse loss are observed in psychiatric disorders like schizophrenia spectrum disorders (Penzes et al., 2011) and may be another manifestation of the correlation between *T. gondii* infection and mental disorders.

Neuronal communication changes due to alterations in neurotransmitter release are an important consequence of the infection. Our data discloses increased vGLUT1 and GAD65 mRNA levels in the infected BrainSpheres, suggesting that the glutamate and GABA levels may increase as a consequence of *T. gondii* infection. Extracellular glutamate levels were increased with *in vivo* infection, and the glutamate reuptake system mediated by astrocytes was affected as evidenced by a decrease in GLT-1 (glutamate transporter) levels (David et al., 2016). However, some glutamatergic synapse proteins like AMPA and NMDA receptor subunits were downregulated after infection, suggesting reduced glutamatergic

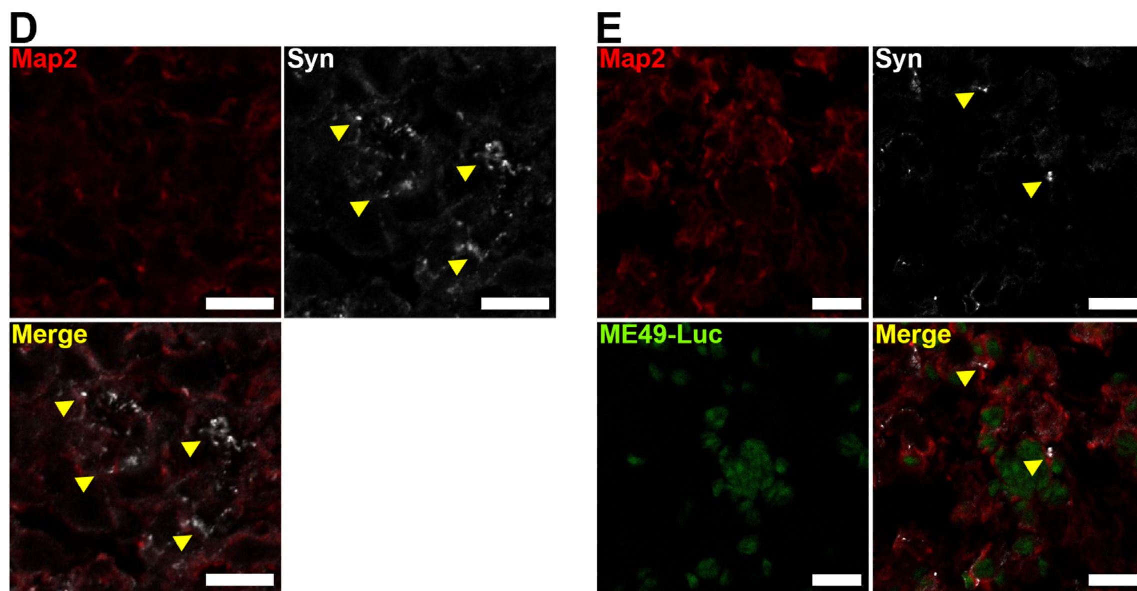

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Fig. 4. ME49 strain of *T. gondii* affects mRNA levels of cell types, synapses and neuronal phenotypes in BrainSpheres. (A) 3D human CNS model differentiated for 4, 6 and 8 weeks without infection (Control, blue line) and with infection (ME49, red line). The mRNA levels of different neural markers were analyzed: MAP2 (neuron), GFAP (astrocyte), OLIG1 (oligodendrocyte), synaptophysin (synapse), and vGLUT1, GAD65 and TH (glutamatergic, GABAergic and dopaminergic neurons, respectively). TEM images of (B) non-infected BrainSpheres differentiated for 4 weeks and cultured for 2 weeks, and (C) BrainSpheres differentiated for 4 weeks followed by *T. gondii* infection for 2 weeks. BrainSpheres without infection show a myelin-like pattern with normal concentric disposure (black arrowheads, B), whereas infected BrainSpheres show a myelin loss pattern (black arrowheads, C). Fluorescence images of (D) non-infected BrainSpheres differentiated for 4 weeks and cultured for 2 weeks, and (E) BrainSpheres differentiated for 4 weeks followed by *T. gondii* infection for 2 weeks. MAP2 (red), Synapsin-1 (Syn, white) and ME49-Luc (green). (E) Infected BrainSpheres showed reduced staining for Syn compared to (D) non-infected BrainSpheres (yellow arrowheads). Scale bars: (B) 100 nm; (C) 200 nm; (D) and (E) 10 μm. Data were collected from three independent experiments and represent mean (±SD). Student *t*-test and two-way ANOVA with Bonferroni's post-test were used to analyze the statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

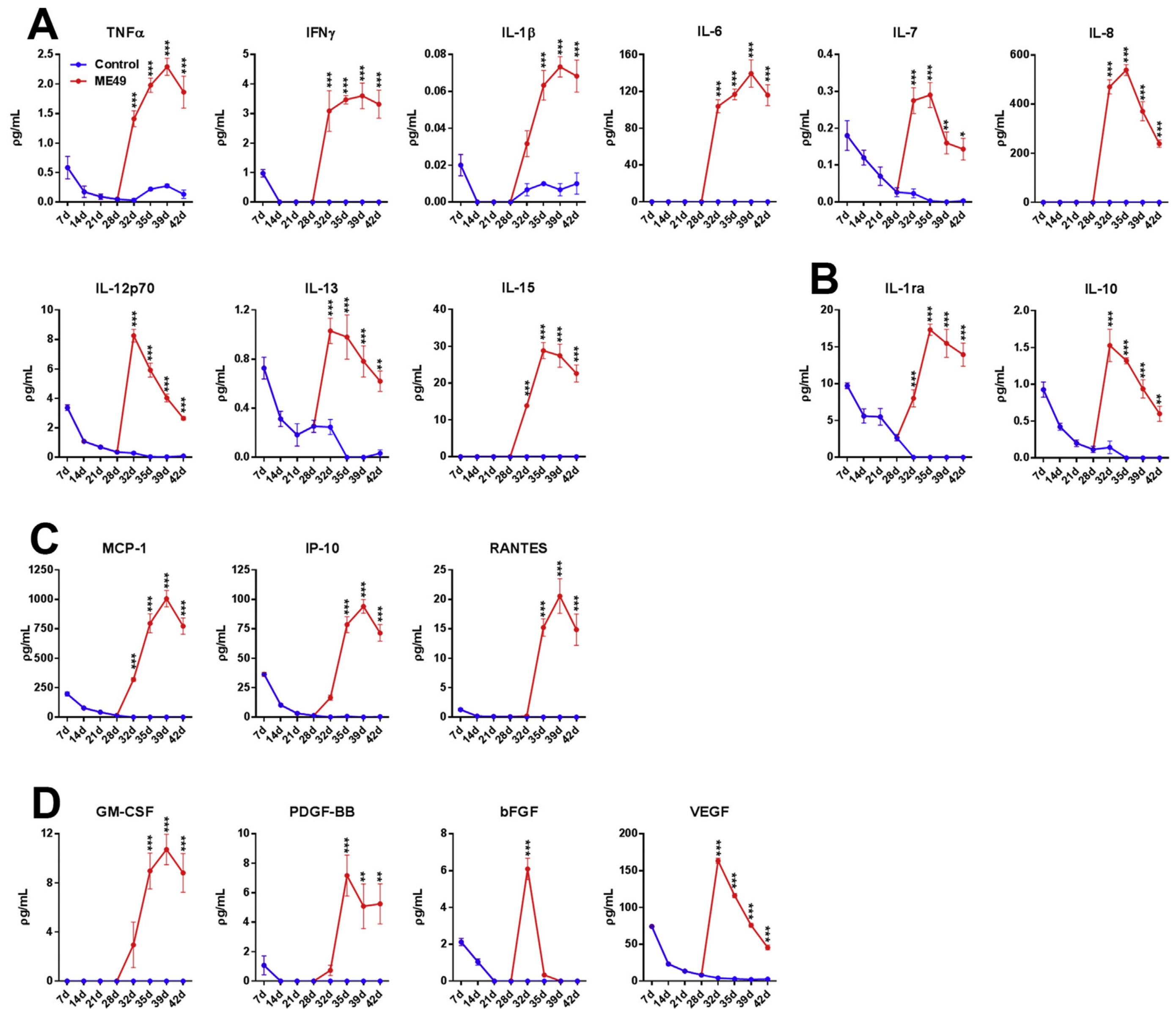

Fig. 5. ME49 strain of *T. gondii* induces the release of cytokines, chemokines and growth factors in BrainSpheres. Graphs showing the levels of different secreted mediators of BrainSpheres without infection (Control, blue line) and after infection (ME49, red line) in different periods of BrainSpheres culture. The infection with ME49 strain of *T. gondii* occurred at day 28. The protein concentration levels were grouped into (A) pro-inflammatory cytokines: TNFα, IFNγ, IL-1β, IL-6, IL-7, IL-8, IL-12p70, IL-13 and IL-15; (B) anti-inflammatory cytokines: IL-1ra and IL-10; (C) chemokines: MCP-1, IP-10 and RANTES; and (D) growth factors: GM-CSF, PDGF-BB, bFGF and VEGF. Data were collected from three independent experiments and represent mean (±SEM). Two-way ANOVA with Bonferroni's post-test was used to analyze the statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

neurotransmission (Lang et al., 2018). Another *in vivo* study showed that infection did not alter GAD67 protein levels but induced its mislocalization in neurons and the infected animals became more susceptible to seizures (Brooks et al., 2015). It is possible that the increased GAD65 mRNA levels in infected BrainSpheres constitute a compensatory mechanism for its mislocalization, reflecting the attempt to restore the excitatory/inhibitory homeostasis.

Our data also evidenced that the infection reduced TH mRNA levels, suggesting dopaminergic neuronal cell death. Several works investigated *T. gondii* infection effects on dopamine levels, with many describing an increase in its levels. An *in vitro* study reported the reduction of TH protein levels in the *T. gondii* presence without alteration of dopamine levels (Barbosa et al., 2020). Other authors did not observe TH mRNA and protein levels changes, but instead reported increases in dopamine synthesis in PC12 cells (Martin et al., 2015). Similarly, augmented dopamine levels were found in infected rodents (Xiao et al., 2014). Parasite cysts in rat cortical neuron cultures and mouse brains also display high dopamine levels. *T. gondii* expresses endogenous TH, contributing to the augmented neurotransmitter levels (Prandovszky et al., 2011). Dopamine neurotransmission is involved in motor control, cognition and emotion, and this neurotransmitter dysregulated levels could be related to the behavioral alterations observed in the infection. In addition, dopamine dysfunction is associated with neuropsychiatric disorders like schizophrenia spectrum disorders (Caton et al., 2020; Eyles et al., 2012).

Nicotinic cholinergic neurotransmission may also play a role in schizophrenia spectrum disorders and other psychiatric illnesses (Caton et al., 2020). The enteric system's infection produces the cholinergic neurons death in the submucosal plexus of the duodenum-ileum (Trevizan et al., 2019), indicating a possible parasite tropism for the cholinergic system. Another possible cholinergic system involvement in CNS pathology in toxoplasma infection is the astrocyte-derived tryptophan metabolite, kynurenic acid. Electrophysiological experiments indicate that kynurenic acid and nicotinic receptors regulate the GABAergic inputs to hippocampal CA1 pyramidal neurons (Banerjee et al., 2012), with kynurenic acid as an α7 nicotinic receptors antagonist (Albuquerque and Schwarcz, 2013). Although, this contention is still controversial. Instead, kynurenic acid is more likely to antagonize NMDA receptors (Stone, 2020). In addition, neuroinflammation increases kynurenic acid synthesis in CNS, maybe contributing to the glutamatergic, GABAergic and cholinergic system modulations observed in the infection. Animal and human *in vivo* studies suggest that kynurenic acid increased levels can be related to cognitive deficits observed in schizophrenia spectrum disorders (Schwarcz et al., 2012). Kynurenic acid increased production mediated by neuroinflammation may be another connection between *T. gondii* infection and neuropsychiatric disorders.

BrainSpheres exposed to *T. gondii* display increased release levels of several inflammatory mediators such as TNFα, IFNγ, IL-1β, IL-6, chemokines and growth factors. Astrocytes and microglia activated release inflammatory mediators during the CNS infection, including that mediated by *T. gondii* (Schluter and Barragan, 2019; Strack et al., 2002). As microglia is not present in our 3D human CNS model, these mediators are released by astrocytes and neurons; cells that are known to release inflammatory mediators in response to *T. gondii* (Schluter et al., 2001). Infected mice cerebellar granule neurons in culture produce IL-6, TGF-β1, MIP-1α and MIP-1β, while astrocytes release IL-1α, IL-6, GM-CSF, MCP-1 and IP-10 (Fischer et al., 1997a; Schluter et al., 2001; Strack et al., 2002). In addition, the CNS of infected animals displayed TNF-α, IFNγ and IL-6 increased levels (Mahmoudvand et al., 2016), in accordance with our data. IL-4 and IL-13 regulate the indoleamine 2,3-dioxygenase activity induction, affecting tryptophan availability, causing starvation and controlling *T. gondii* replication in human fibroblasts activated by IFNγ (Chaves et al., 2001). A recent study showed that infected mice have sustained inflammation and several inflammatory mediators are still upregulated after the parasite clearance (Boillat et al., 2020). In mice, some cytokines like IFNγ and TNFα are important to *T. gondii* infection control (Hunter et al., 1994) and their signaling deficiency may be related to severe and reactivated cerebral toxoplasmosis in humans. However, only two of 180 toxoplasmosis cases showed congenital immunodeficiency, in accordance with the low number of patients with severe toxoplasmosis and primary immunodeficiency (Robert-Gangneux et al., 2015).

Anti-inflammatory cytokines are also crucial to control the *T. gondii* inflammation. For instance, IL-10 from monocytes contribute to the cerebral toxoplasmosis regulation (Biswas et al., 2015). Besides, IL-10 decreases animal mortality promoted by *T. gondii* and downregulates the immune response in chronic cerebral toxoplasmosis (Deckert-Schluter et al., 1997; Gazzinelli et al., 1996). In BrainSpheres, IL-10 showed a sharp increase just after infection, but its levels gradually receded over time. Meanwhile, IL-1ra that binds to IL-1 receptor preventing IL-1 downstream signaling and inflammation activation (Akdis et al., 2016), was maintained over time. These data show that IL-10 and IL-1ra are responsive to *T. gondii* infection and may attempt to control the increased pro-inflammatory mediators.

Neuroinflammation mediated by *T. gondii* also promotes chemokine and growth factor release. Infected astrocytes in culture showed MCP-1 increased levels involved in CNS leukocyte infiltration (Brenier-Pinchart et al., 2004); its receptor (CCR2) also plays a relevant role in controlling CNS *T. gondii* infection (Benevides et al., 2008). Mice with toxoplasmic encephalitis display astrocytes producing IP-10, critical for effector T cell trafficking and host survival in *T. gondii* infection, and MCP-1 (Khan et al., 2000; Strack et al., 2002). Previously, it was shown that PDGF released from human platelets activated by *T. gondii* inhibited the parasite growth in human pulmonary fibroblasts (Chumpitazi et al., 1998), and that VEGF can control the *T. gondii* proliferation in human retinal pigment epithelium (Quan et al., 2020). Furthermore, GM-CSF levels were increased in cultured astrocytes and mouse brain infected with *T. gondii* (Fischer et al., 1997b; Gazzinelli et al., 1993). It is possible that the increased chemokine and growth factor levels in infected BrainSpheres are an attempt to maintain cell survival.

The behavioral alterations mediated by *T. gondii* are also impacted by neuroinflammation. Post-mortem human brain analyses showed that behavioral alterations correlate with high amounts of parasite DNA in the CNS (Samojlowicz et al., 2019). However, the correlation between behavioral alterations and presence of cysts in CNS specific regions is still controversial. While some studies suggest that there is a possible connection, other findings emphasize the importance of neuroinflammation more than cyst load or location for the altered behavior (Boillat et al., 2020; Evans et al., 2014; Martynowicz et al., 2019). The cyst load reduction in infected mice reversed the hyperactivity, but the regular locomotor activity was restored only through reduction of neuroinflammation. It appears that some behavioral alterations are more related to neuroinflammation than to a parasite effect (Martynowicz et al., 2019). In this sense, the authors of an *in vivo* study suggested that neuroinflammation is the major factor for behavioral alterations (Boillat et al., 2020). Neuroinflammation is also present in several neuropsychiatric disorders like schizophrenia, whose behavioral symptoms could be related to inflammation-mediated by *T. gondii*. Antipsychotic drugs with activity against *T. gondii* decreased the behavioral symptoms and inflammation in schizophrenic patients (Fond et al., 2018).

Our findings are in agreement with the literature reinforcing the possible connection between human CNS *T. gondii* infection, alterations of neurotransmitter systems, and neuroinflammation. In addition, BrainSpheres constitute a relevant tool to characterize the *T. gondii* infection in human neural tissue in a species-specific manner. We consider that these data could be important for future studies aimed at a better understanding possible correlations between psychiatric disorders and human CNS infection with *T. gondii*.

Declaration of competing interest

The authors report no financial interests or conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100190.

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