METHOD ARTICLE

REVISED Modelling host-*Trypanosoma brucei gambiense* interactions *in vitro* using human induced pluripotent stem cell-derived cortical brain organoids [version 2; peer review: 2 approved]

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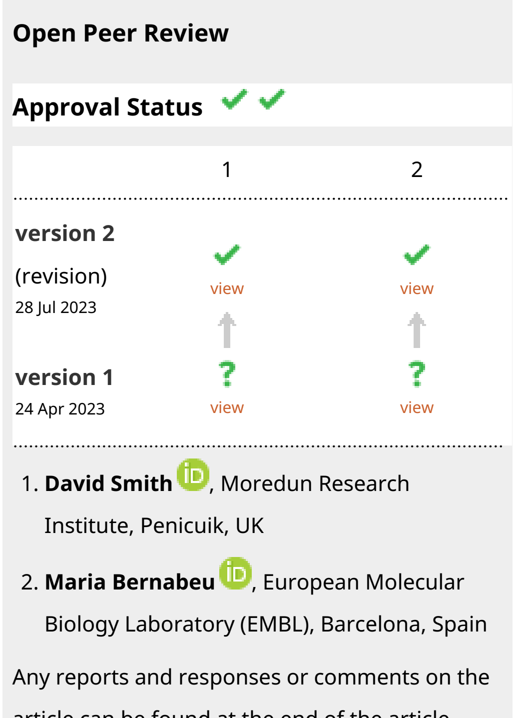
Abstract

Background: Sleeping sickness is caused by the extracellular parasite *Trypanosoma brucei* and is associated with neuroinflammation and neuropsychiatric disorders, including disruption of sleep/wake patterns, and is now recognised as a circadian disorder. Sleeping sickness is traditionally studied using murine models of infection due to the lack of alternative *in vitro* systems that fully recapitulate the cellular diversity and functionality of the human brain. The aim of this study is to develop a much-needed *in vitro* system that reduces and replaces live animals for the study of infections in the central nervous system, using sleeping sickness as a model infection.
Methods: We developed a co-culture system using induced pluripotent stem cell (iPSC)-derived cortical human brain organoids and the human pathogen *T. b. gambiense* to model host-pathogen interactions *in vitro*. Upon co-culture, we analysed the transcriptional responses of the brain organoids to *T. b. gambiense* over two time points.
Results: We detected broad transcriptional changes in brain organoids exposed to *T. b. gambiense*, mainly associated with innate immune responses, chemotaxis, and blood vessel differentiation compared to untreated organoids.
Conclusions: Our co-culture system provides novel, more ethical avenues to study host-pathogen interactions in the brain as alternative models to experimental infections in mice. Although our data support the use of brain organoids to model host-pathogen



interactions during *T. brucei* infection as an alternative to *in vivo* models, future work is required to increase the complexity of the organoids (*e.g.*, addition of microglia and vasculature). We envision that the adoption of organoid systems is beneficial to researchers studying mechanisms of brain infection by protozoan parasites. Furthermore, organoid systems have the potential to be used to study other parasites that affect the brain significantly reducing the number of animals undergoing moderate and/or severe protocols associated with the study of neuroinflammation and brain infections.

Keywords
Brain organoids, sleeping sickness African trypanosomes, brain infection, in vitro culture

This article is included in the Pathogens gateway.

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REVISED **Amendments from Version 1**

In addition to minor text edits, the updated manuscript version contains the following changes:

1. Abstract
- Under the "Conclusion" subheading, we have updated the abstract to strengthen the suitability and impact of the model developed in our study.

2. Methods
- Under the subheading "Cerebral organoid generation and in vitro culture", we have clarified that the cortical brain organoids were matured for ~ 2 months prior to co-culture experiments with *Trypanosoma brucei*.
- Under the "*Trypanosoma brucei gambiense* - human brain organoids co-culture system", we have explained in the text that the organoids are not actively colonised by the parasites, but rather that they were co-cultured with the organoids.
- Under the subheading "Bulk RNA sequencing and data analysis", we have clarified the number of replicates used for the bulk transcriptomics analysis presented in this study.

3. Results, Discussion and outlook
- We have removed *PECAM1* from the text and table as a marker for endothelial cells.
- We have clarified that our morphological assessment was based on two staining approaches (H&E and IHC using anti-MAP2 antibodies).

Any further responses from the reviewers can be found at the end of the article

Research highlights

Scientific benefit(s)
- Human-derived brain organoids can be used to study neuropathogenesis during *Trypanosoma* infection. This has been challenging to study in human tissues due to ethical implications and lack of complex *in vitro* culture systems.
- Evaluation of putative human brain cell populations associated with innate responses to protozoan pathogens.

3Rs benefit(s)
- Adoption of stem cell-derived 3D organoids can reduce ~47% of the mice used to study trypanosome infection, which would typically undergo protocols considered moderate or severe.
- Further ~20% reduction of donor mice required to generate infectious parasites, which is a moderate procedures.

Practical benefit(s)
- Possible to effectively introduce mutations of interest into the organoids without the need to established complex and expensive breeding schemes.
- Reductions in the number of animals required for *in vivo* work reduces breeding and husbandry costs.

Current applications
- Evaluation of global responses to a human pathogen.

Potential applications
- Screening for drugs acting on the CNS for treatment of infectious diseases.
- Can be combined with additional organoids, cell type/s of interest ("building blocks"), and/or organic matrices or scaffolds to generate more complex tissues/organs.
- Potential to manipulate genes/pathways (*e.g.*, CRISPR-Cas9 gene editing) to assess their function in pathogenesis to infection.

Introduction

Neurotropic pathogens encompass a wide range of parasitic organisms, from viruses to protozoan parasites, and are the causative agents of debilitating conditions affecting the central nervous system (CNS), often resulting in life-long impairments and death if left untreated. To date, most of these infections are studied using murine models of infection. Although these infection models often recapitulate the clinical outcomes observed in humans, there are serious ethical and biological implications associated with *in vivo* host-pathogen interaction studies. More recently, the generation

of organoids developed *in vitro* from human stem cells have provided novel insights into developmental biology, and their potential application as alternative models to study host-pathogen interactions is starting to be recognised. These models offer an opportunity to interrogate human tissues that are difficult to access, such as CNS tissue. Indeed, human brain organoids comprising the diversity of cell types representative of the complex neuroepithelium are an increasingly attractive model system to interrogate how human nervous cells respond to infection. Currently, *in vitro* brain organoid systems are being used to study infections from ZIKA and SARS-CoV-2,[1–4] and have proven insightful for understanding other parasitic infections, including toxoplasmosis and malaria.[5,6] However, these *in vitro* systems have not been used to explore the pathogenesis of human African trypanosomiasis, a parasitic infection traditionally known for its devastating neurological effects.[7–10]

Here, we explored whether human brain organoids can be used to model host-trypanosome interactions *in vitro.* Using bulk RNA sequencing, we observed that the human cortical brain organoids transcriptionally respond to the human pathogen *T. brucei gambiense* by upregulating gene pathways associated with innate immune functions, amongst others. Some of the upregulated genes are proposed to have antimicrobial properties, suggesting that human brain organoids are able to sense and respond to pathogens in the absence of innate immune cells (*e.g.*, microglia). Using this novel *in vitro* system, we estimate a direct reduction of ~47% of animals required to achieve similar conclusions, and ~20% of animals used as donors to generate infectious parasites. The methods and results presented here have the potential to open new research avenues for the adoption of human brain organoids to model host-pathogen interactions with important implications for the 3Rs principles—replacement, refinement, and reduction.

Methods

Cerebral organoid generation and *in vitro* culture

This work was conducted jointly at the Heinrich-Heine-Universität and the University of Glasgow.

1. IMR90 human induced pluripotent embryonic stem cells (IPS(IMR90)-2 (RRID:CVCL_C435)), maintained at 80% confluency, were seeded at ~10,000 cells per well in mTeSR1 (Stem Cell Technologies, Vancouver, Canada) in 24-well, Matrigel-precoated plates (Corning, NY, USA). Cells were incubated at 37°C and 5% CO_2 with medium changed daily.

2. Colonies were observed to form after 7-10 days.

3. To detach colonies, mTeSR1 media was removed and the wells were washed once with 1 ml of 1X D-PBS without Calcium and Magnesium (Stem Cell Technologies) at 37°C.

4. PBS was removed and discarded. A total of 1 ml ReLeSR™ at 37°C temp (Stem Cell Technologies) was added per well.

5. Plates were incubated for 5-7 minutes at 37°C, after which 1ml TeSR™ was added to each well and the plates were vortexed for 2-3 minutes at room temperature (17-22°C) until the cultures were fully detached. Note that the mean aggregate size should be approximately 50-200 µm.

6. Cell pellets were resuspended in 500 µl AggreWell medium and 10% Clone R (Stem Cell Technologies) and seeded at 9,000 cells per well in round-bottom 96-well plates (in ~200 ml) and incubated at 37°C and 5% CO_2.

7. After two days, the medium was replaced with fresh AggreWell without Clone R, and the cells were incubated for an additional three days at 37°C and 5% CO_2. The medium was changed by gently placing the plate in a 45° angle to medium change.

8. On day six, the medium was replaced with neural induction medium (NIM) containing DMEM/F12, N2 supplement (Thermo Fisher Scientific, Waltham, MA, USA), minimum essential medium-nonessential amino acids (MEM-NEAAs), GlutaMAX (Thermo Fisher Scientific) and 1 µg/ml heparin (Sigma, MO, USA), and the cells incubated for five days 37°C and 5% CO_2.

9. After the NIM medium was changed, embryoid bodies (EBs) were transferred by pipette onto Matrigel droplets (Corning) that were 3 mm in diameter on the inner part of a 75 mm petri dish. The EBs should appear as located at the centre of the droplet. These droplets were incubated for 1 hour at 37°C, transferred to 24-well plates and maintained in cerebral organoid differentiation medium (CORD) containing DMEM/F12 and Neural Basal Medium (in 1:1 ratio), supplemented with 1:200 N2 (Thermo Scientific), 1:100 l-glutamine (Stem Cell Technologies), 1:100 B27 without vitamin A (Thermo Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin,

23 μM insulin (Sigma-Aldrich), 0.05 mM MEM non-essential amino acids (NAA), and 0.05 mM β-mercaptoethanol (Life Technologies) was used to differentiate the Matrigel embedded droplets. The medium was replaced every three days until usage (∼2 month in culture to ensure organoid maturation).

Trypanosoma brucei gambiense in vitro culture

1. Culture-adapted bloodstream slender forms of *Trypanosoma brucei gambiense* Eliane strain (MHOM/CI/52/ITMAP 2188)[11] were used in all experiments. This strain, originally isolated from an infected patient in Côte d'Ivoire (Ivory Coast)[11] was previously adapted in the laboratory to grow in HMI-9 culture medium supplemented with 20% foetal calf serum (FCS).

2. Cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2. Pleomorphic parasites were typically maintained at a density of 10^5 and 10^6 parasites/ml at 37°C and 5% CO_2.

Trypanosoma brucei gambiense - human brain organoids co-culture system

1. A total of 10^5 parasites at log-phase of growth were co-cultured with the brain organoids on 12 well plates (Final ratio of 1 organoid:10^5 parasites per well) for a period of 24 or 72 hours in HMI-9 media diluted 50:50 with CORD media at 37°C and 5% CO_2 in round-bottomed 96-well plates. These two time points were chosen to mimic acute (24 hours) and chronic (72 hours) responses and we determined that parasites grew well under these conditions, at least during the first 72 hours in culture (Figure 1B).

2. In parallel, organoids cultured in HMI-9 media diluted 50:50 with CORD media at 37°C and 5% CO_2 but without parasites were also seeded in round-bottomed 96-well plates and were included as controls to assess the effect of diluted media on the organoids transcriptome. As controls, we included organoids kept in 50:50 HMI-9:CORD organoid media alone.

3. After 24 or 72 hours, the organoids were gently washed 5 times with 1X PBS (Gibco) and wide bore pipette tips to remove parasites. Then, some organoids were fixed in 4% PFA for 24 hours at room temperature and preserved as paraffin-embedded blocks for immunohistochemistry analysis. The rest of the organoids were processed for bulk transcriptomics.

Immunohistochemistry

1. Paraformaldehyde-fixed organoid were processed into paraffin blocks for long-term storage.

2. We prepared 5 μm thick paraffin sections, which were placed on Superfrost Plus™ slides (Fisher Scientific) and stained with Mayer's haematoxylin Solution (Sigma-Aldrich), Bluing Buffer (Dako) for 5 minutes and 1:10 dilution of Eosin Y solution (Sigma-Aldrich) in 0.45 M of Tris-acetic acid buffer, pH 6.0, for 5 minutes in distilled water, with 3-4 washing steps in ultrapure water between each step. All solutions were kept at room temperature. The H&E staining was conducted using a Dako Autostainer Link 48 (Dako) with all the incubation steps at room temperature (17-22°C).

3. For staining with the monoclonal neuron-specific microtubule associated protein 2 (MAP 2, Clone M13, Thermo Fisher Scientific Cat. No. 13-1500. RRID: AB_2533001), 5 μm thick paraffin sections were treated in a pressure cooker (~140°C) for 5 minutes in citrate buffer pH 6.0, followed by staining with the monoclonal NSE antibody (Cell Signalling Technologies, clone E2H9X, Cat. No. 24330. RRID: AB_2868543) diluted in 1:1,000 in 1× blocking buffer (Dako) and incubated overnight at 4°C. Staining was conducted using a goat anti-Rabbit antibody coupled to Horseradish peroxidase (1:1,000, Thermo Fisher Scientific Cat. No. A16104. RRID: 2534776) for 1 hour at room temperature.

4. The samples were mounted in VECTASHIELD Antifade Mounting Media with DAPI (Vectorlabs, Cat. No. H-1200. RRID: AB_2336790) and visualised on a Axio Imager 2 instrument (Zeiss. RRID: SCR_018876).

Bulk RNA sequencing and data analysis

1. Before proceeding with the RNA extraction step, all the pipettes and surfaces were thoroughly cleaned with RNAZap (Thermo) to remove RNAses. For this protocol, we used sterile filtered tips.

2. At the selected time points, brain organoids (n = 3 replicates / experimental condition) were harvested and washed in 500 μl of 1X PBS at 4°C twice. The washes were conducted by letting the organoids settle at the bottom of a 1.5 ml Eppendorf tube before removing the supernatant.

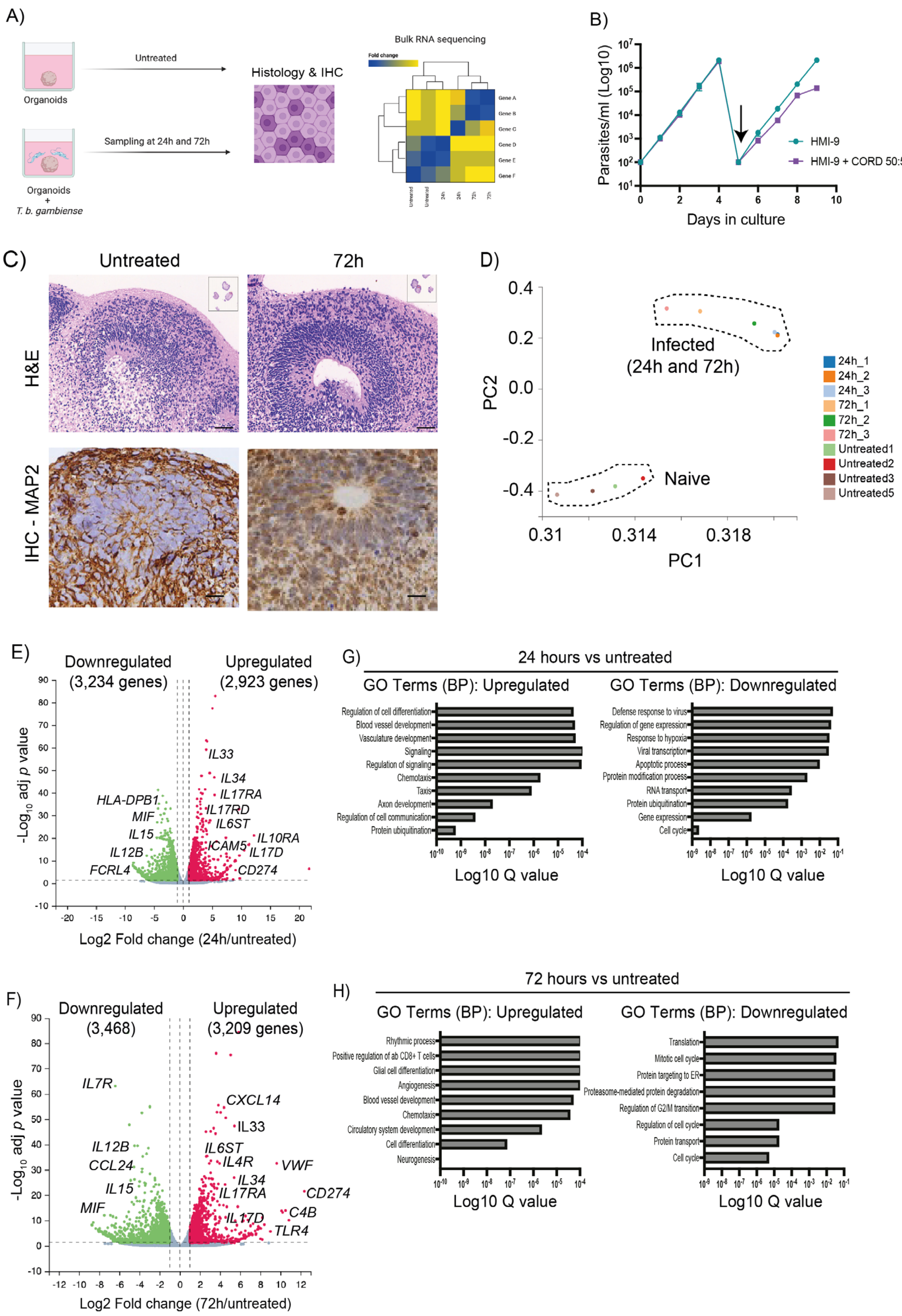

Figure 1. Bulk transcriptomics analysis of human cortical brain organoids co-cultured with *T. brucei gambiense.* A) Schematic representation of the experimental design developed for this study. B) Growth curve assay for *T. b. gambiense* in HMI-9 alone (teal) or diluted 50:50 with CORD media (magenta). The arrow indicates a dilution step to bring the parasite cultures down to 10^2 parasites/ml. Data shown as mean ± standard deviation from three independent experiments. C) Representative H&E staining and MAP 2 Immunohistochemistry from naïve (left) and *T. brucei gambiense*-infected (right) organoid after 72 hours of *in vitro* co-culture. Scale bar = 50 µm. D) Principal component analysis of the samples including the bulk RNA sequencing analysis. Volcano plot of differentially expressed genes between untreated organoids and after (E) 24 hours and (F) 72 hours in culture with T. *brucei gambiense.* Dotted line represents the significance ($-0.5 < Log_2FC > 0.5$ and p adjusted value < 0.05). Pathway analysis of the genes dysregulated at (G) 24 hours and (H) 72 hours in culture with *T. brucei gambiense.* The adjusted p value (Q value) for each of the enriched pathways is included. MAP 2, microtubule associated protein 2.

3. Once washed, the organoids were resuspended in 500 µl of Qiazol (Qiagen) and dissociated firmly by pipetting up and down using a wide bore p1000 pipette tip.

4. The dissociated tissue was then subjected to total RNA extraction using the mRNeasy kit (Qiagen), following the recommended volume of chloroform. All the solutions were kept at room temperature (17-22°C) unless indicated otherwise by the manufacturer. All the centrifugation steps were conducted at 4°C. We eluted the total RNA from brain organoids in 50 ml of EB buffer (Qiagen). On average, we detected a recovery of ~100 µg/ml of total RNA, as quantified by Qubit.

5. The quality of the RNA was assessed on a Bioanalyzer RNA Pico chip (Agilent). We considered an RNA Integrity Number (RIN) value >8.0 to be ideal for bulk RNA sequencing. All the samples analysed here consistently had a RIN value >8.0.

6. Once assessed, 1 µg of total RNA per sample was submitted to the Beijing Genomic Institute (BGI; RRID: SCR_011114) for RNA sequencing and processed for 150 bp paired-end sequencing on the DNBSeq platform (RRID: SCR_017981).

7. Once sequenced, raw reads were filtered using SOAPnuke (RRID:SCR_015025) software (v1.5.2) developed by BGI, allowing for the removal of reads containing adapters, reads with N content >5%, or with a base quality score <15.

8. Clean reads were then aligned the to the human genome using the package Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) (RRID:SCR_015530) (v2.0.4) and Bowtie 2 (RRID:SCR_016368) (v2.2.0), with default parameters using the Genome reference consortium Human Build 38 patch release 12 (GRCh38.p12).

9. Subsequent downstream analysis, including differential gene expression and pathway analysis using Gene Ontology (RRID: SCR_002811), were conducted on the Dr. Tom analysis suite built by BGI. For gene expression analysis, differentially expressed genes were considered significant if the adjusted p value < 0.05, and Log_2 fold change of -2< or >2.

Results

African trypanosomes cause extensive neurological changes resulting in neuropsychiatric disorders and culminating in death if not treated adequately. Although this disease is frequently modelled using experimental infections in mice, for ethical reasons around the use of animals in research, we were motivated to explore the utility of induced pluripotent stem cell (iPSC)-derived human cortical brain organoids to model brain-trypanosome interactions *in vitro* as alternatives to *in vivo* infections. Thus, using an *in vitro* co-culture system, we set out to characterise the transcriptional responses of the iPSC-derived human brain organoids to the human pathogen *T. brucei gambiense* (Figure 1A),[22,23] compared to organoids that were not incubated with the parasites. These time points were selected to evaluate early (24 hours) and late (72 hours) responses, in an attempt to gain as much insight as possible into the temporal dynamics associated with tissue responses to infection. Importantly, we did not detect significant morphological or histological changes in the organoids exposed to the parasites based on the morphological aspects observed upon H&E staining and MAP2 staining (Figure 1C), suggesting that *T. brucei gambiense* does not seem to elicit tissue damage over a 72 hour culture period, at least not with the markers used in this study. Principal component analysis demonstrates that at a transcriptional level, the samples segregate mainly based on infection status and experimental time point, but with limited transcriptional variation between samples harvested at 24 and 72 hours (Figure 1D). In these brain cortical organoids, we identified a total of 6,157 dysregulated genes at 24 hours (3,234 and 2,923 downregulated and upregulated genes, respectively) and 6,677 dysregulated genes at 72 hours (3,468 and 3,209 downregulated and upregulated genes, respectively) (Figure 1E and 1F, and Table S1 in *Underlying data*),[22,23] defined as genes with an adjusted p value < 0.05 and a Log_2 Fold change of -2< or >2. To obtain a broad overview of immune related pathways, we examined cytokine, chemokine, and immune receptors that were significantly dysregulated in brain organoids exposed to *T. b. gambiense.* We detected several genes with canonical immune functions such as *CD274*, that encodes for Programme death ligand 1(PD-L1), the complement factor *C4B*, and the glial fibrillary acidic protein (*GFAP)*, typically associated with gliosis during CNS inflammation[12] (Table 1 and Table S2 in *Underlying data*).[22,23] Additionally, we detected the expression of several interleukins and chemokines such as interleukin-34 (*IL34)* that promotes monocyte and macrophage survival and differentiation,[13] the chemokine *CXCL14* involved in immune cell recruitment,[14] transforming growth factor beta 1 (*TGFB1*), and the alarmin *IL33*, which is a critical mediator of innate immune responses and inflammation[15] (Table 1 and Table S2 in *Underlying data*).[22,23] We also detected significant expression of the interleukin-17 receptor subunit A and D (*IL17RA* and *IL17RD*, respectively), interleukin-10 receptor subunit a (*IL10RA*), and the Interferon gamma receptor 1 (*IFNGR1*) (Table 1 and

Table 1. Upregulated immune-related genes in iPSC-derived human brain organoids in response to *Trypanosoma brucei gambiense*.

Gene of interest	24 h co-culture		72 h co-culture	
	Log2FC	Adjusted *p* value	Log2FC	Adjusted *p* value
CD274	6.316	1.33^{-10}	5.578	1.26^{-07}
C4B	5.734	2.29^{-03}	8.151	1.86^{-08}
IL34	3.909	1.73^{-10}	3.522	4.41^{-08}
IL33	1.241	8.75^{-04}	1.389	5.55^{-04}
IL17D	0.8711	0.0351	ND	ND
IL6ST	0.694	0.0270	1.070	9.66^{-04}
CXCL14	ND	ND	2.123	2.74^{-04}
VWF	ND	ND	9.765	6.60^{-03}
VEGFC	1.480	0.01404	1.886	3.55^{-04}
CCL25	2.723	0.0453	ND	ND
IFITM1	2.277	7.10^{-03}	3.168	9.28^{-16}
IL10RA	1.585	7.93^{-03}	ND	ND
IL17RD	1.512	1.29^{-07}	1.582	1.24^{-08}
IL17RA	1.191	3.27^{-03}	1.384	6.34^{-04}
IL1RL1	ND	ND	5.857	0.016
IL4R	ND	ND	3.109	0.0452
IL13RA1	1.887	3.90^{-03}	1.986	9.32^{-04}
TLR4	ND	ND	5.05	0.0315
GFAP	3.642	1.7^{-09}	3.363	7.96^{-09}
TGFB1	0.9907	8.82^{-03}	1.22	6.99^{-03}
IFNGR1	ND	ND	0.605	0.043
CD47	1.225	2.41^{-06}	1.151	2.80^{-05}
CDH5	ND	ND	7.178	0.015

iPSC = induced pluripotent stem cell; ND = Not detected; h = hours.

Table S2 in *Underlying data*),[22,23] indicating that these organoids are primed to sense and respond to *T. brucei gambiense* by activating IL-17, IL-10, and IFNγ signalling pathways. Furthermore, we also detected genes involved in angiogenesis and endothelial function, including the vascular endothelium growth factor subunit c (*VEGFC*), cadherin 5 (*CDH5*), the integrin associated protein *CD47*, and von Willebrand factor (*VWF*) (Table 1 and Table S2 in *Underlying data*),[22,23] suggesting that co-culture with *T. b. gambiense* also induces the expression of genes associated with vasculogenesis and vascular repair. Some of these genes showed a temporal expression dynamic, with some genes involved in immune sensing, recruitment and tissue repair (*e.g.*, *CXCL14*, *VWF*, *TLR4*, *IL4R*) being exclusively detected after 72 hours of exposure to *T. b. gambiense* compared to naïve controls.

To gain a better understanding of the broad transcriptional responses triggered in the human brain organoids to *T. b. gambiense infection*, we performed Gene Ontology analysis on genes significantly dysregulated. After 24 hours of exposure to *T. b. gambiense*, the iPSC-derived human brain organoids upregulate genes associated with blood vessel and vasculature development, signalling, and chemotaxis, with a concomitant reduction in genes associated with response to hypoxia, defence response against viruses, and protein ubiquitination (Figure 1G). At 72 hours, the pathways overrepresented in the organoids transcriptome were associated with glial cell differentiation, positive regulation of $CD8^+$ T cells, chemotaxis, and vascular and blood vessel differentiation, and a significant reduction of gene pathways associated with cell cycle progression, protein transport and proteasome-mediated protein degradation (Figure 1H). Taken together, these data suggest that *T. b. gambiense* trigger a broad innate-like immune response in the iPSC-derived human brain organoids accompanied by upregulation of genes involved in vascular development, immune chemotaxis, and cytokine-mediated immune signalling.

Use cases

Similar approaches have been recently implemented to study host-pathogen interactions in the context of viral infections and protozoan infections, including toxoplasmosis and malaria, *in vitro*. We anticipate that our detailed protocol can be used to explore further interactions between *T. brucei* and iPSC-derived human brain organoids in more detail, including novel effects of *T. brucei* on the function of human neurons, which remains unexplored. We additionally anticipate that the protocol provided here can be leveraged to study potential cytotoxic side effects of novel antiparasitic compounds.

Discussion and outlook

In this study, we tested the possibility of using stem cell-derived human brain organoids as an *in vitro* system as an alternative mode to live animals to study host-trypanosome interactions, in line with the 3Rs principles. We firstly set up an *in vitro* co-culture system whereby iPSC-derived human brain organoids were co-cultured with the human pathogen *T. b. gambiense* and assessed the response of these organoids to the pathogen using histology and transcriptomics as a proxy for global responses to the pathogen. The data presented here demonstrate that iPSC-derived human brain organoids trigger a transcriptional programme associated with an innate-like immune response when exposed to *T. brucei gambiense.* Bulk transcriptomics has enabled us to identify that the brain organoids specifically respond to *T. brucei gambiense in vitro* by upregulating several genes with putative immune functions such as *CXCL14*, the alarmin *IL33*, the complement component *C4B*, as well as vasculogenesis and vascular repair such as *VEGFC*. *CXCL14* is a potent antimicrobial cytokine secreted in response to inflammatory processes and is critical for human neutrophil recruitment,[14,16,17] which have been proposed as important players in controlling CNS infections.[18,19] Similarly, the upregulation of several genes critical for angiogenesis and development of vascular beds, including *VEGFA, VEGFB*, and *VWF*, suggests that they may potentially support vasculogenesis in the presence of this pathogen. All of these observations require further testing at the protein and functional level but provide an initial robust framework to dissect the relevance of these 3D culture systems to model brain-trypanosome interactions.

Our work provides an initial approach to explore the utility of complex 3D culture systems to study host-Trypanosoma interactions *in vitro,* adding African trypanosomes to the compendium of pathogens that have been tested to model host-pathogen interactions using brain organoids. However, there are many challenges and considerations that need to be addressed for the full implementation of these *in vitro* systems, with the aim of replacing animal models of infection. One of the critical hurdles is to generate fully mature organs *in vitro*, encompassing all the cell types typically identified *in vivo*, including microglia and vasculature cells,[20] which are likely to be the main drivers of and/or responders to infection. The incorporation of additional organoids (*e.g.*, vascular or choroid plexus organoids[1]) or inclusion of additional cell types (*e.g.*, endothelial cells, microglia), referred to as "building blocks",[21] will support the development of mature cortical brain organoids that could faithfully recapitulate the immunological responses observed *in vivo.* Given these technical and biological limitations, we are unable to examine the role of these cell types using our *in vitro* host-pathogen culture system. Future work addressing these key challenges will improve the quality of these organoids to model CNS infections *in vitro*, facilitating the reduction and/or replacement of animals in research. Our work provides a wealth of data that can be further mined to design, refine, or implement *in vitro* experiments (*e.g.*, using stem-cell derived astrocytes) as alternatives for *in vivo* work, and provides a foundation for future work in this area.

In summary, we delivered an initial proof-of-concept framework for future adoption of these *in vitro* systems for neuro-immunology research, motivated by the need to reduce and/or fully replace to use animals to study brain-pathogen interactions, in line with the 3Rs principles under the Animals (Scientific procedure) Act, 1986. Based on our estimations, with this model in place, animals used to study brain infections with African trypanosomes, typically considered to be moderate to severe procedures, would have been reduced by ~47%, with an additional ~27% reduction in the number of immunocompromised mice used as donors to generate infectious parasites.

Data availability

Underlying data

Gene Expression Omnibus: Modelling host-*Trypanosoma brucei gambiense* interactions in vitro using human induced pluripotent stem cell-derived cortical brain organoids. Accession number GSE220766; https://identifiers.org/geo:GSE220766.[22]

Figshare: Modelling host-*Trypanosoma brucei gambiense* interactions in vitro using human induced pluripotent stem cell-derived cortical brain organoids. https://doi.org/10.6084/m9.figshare.22491100.[23]

This project contains the following underlying data:

- Table S1 (Quality control and summary of the bulk transcriptomics analysis obtained from the iPSC-derived human brain organoids co-culture with *T. b. gambiense*)

- Table S2 (List of differentially dysregulated genes in iPSC-derived human brain organoids at 24 h and 72 h in co-culture with *T. b. gambiens*)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Reviewer Report 14 August 2023

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Maria Bernabeu
Tissue Biology and Disease Modelling Unit, European Molecular Biology Laboratory (EMBL), Barcelona, Spain

I endorse the indexing of the article but there is indeed a wrong statement on summary of changes: "We have removed *PECAM1* from the text and table as a marker for endothelial cells". PECAM1 is a marker for endothelial cells, EPCAM is not. EPCAM is a marker for epithelial cells. So I'm not sure why the authors deleted PECAM1 and kept EPCAM.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 3D brain bioengineered models, host-parasite interactions, malaria, vascular engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 August 2023

https://doi.org/10.5256/f1000research.153520.r191612

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David Smith
Moredun Research Institute, Penicuik, Scotland, UK

Thank you to the authors for addressing the comments raised in the initial review.

I consider all comments raised to have been addressed, either by manuscript edits or direct responses to the reviewer report. I therefore confirm support for the indexing of this publication.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Organoids, parasitology, host:parasite interactions, protozoa, helminths

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 May 2023

https://doi.org/10.5256/f1000research.144355.r170807

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Maria Bernabeu
[1] Tissue Biology and Disease Modelling Unit, European Molecular Biology Laboratory (EMBL), Barcelona, Spain
[2] Tissue Biology and Disease Modelling Unit, European Molecular Biology Laboratory (EMBL), Barcelona, Spain

In this manuscript, Chandrasegaran *et al.*, use for the first-time *in vitro* brain organoids to understand *Trypanosoma brucei gambiense* pathogenesis. This is an important topic, as brain organoids have already become as a valid strategy to study disease mechanisms. Although the team led by Juan Quintana is already a leader in animal models of *T. brucei,* I would like to praise their efforts on using alternative disease modelling tools that could reduce the number of research animals and overcome differences between mouse and humans. The authors show differential gene expression after brain organoid incubation with *T, brucei gambiense* after a 24h and 72h incubation. The manuscript is timely, and overall, scientifically sound and well written. I support the article indexing after the introduction of some modifications on the text.

Major

- Although the methods section is very clear, I couldn't find any reference on how long the brain organoids were grown. This is important because brain organoids are grown for long periods of time (up to a year) and depending on the maturation time, they acquire different brain developmental stages. I would suggest that the authors include this information in the methods section, as well as on the results or discussion section, as it is important for interpretation of the results.

- How does the 24h and 72h time point or the parasite concentration used compare with the

life cycle of *T. brucei gambiense* in the brain? This information could be useful for an audience not familiar with *T. brucei*.

- The authors couldn't find any brain damage markers on Fig 2C after staining with two different markers. However, other non-used markers could be affected. Although the sentence in the result section mildly implies that ("*Importantly, we did not detect significant morphological or histological changes in the organoids exposed to the parasites based on H&E staining and MAP 2 staining (Figure 1C), suggesting that T. brucei gambiense does not elicit tissue damage over a 72 hour culture period.*"), I would suggest to rephrase to make it more obvious. Furthermore, did the author quantify for differences in expression on MAP2, or is the analysis mostly qualitative?

- The increased expression of endothelial markers is intriguing, taking into account that brain organoids present minimal presence of endothelial cells. This is important because vascularization of brain organoids is one of the main challenges in the bioengineering field, and the presence of vasculature in these models could be used to study the molecular mechanisms of *T. brucei* crossing through the brain microvasculature. Did the authors checked for the presence of blood vessels by immunostaining. In a similar topic, I would like to clarify that EPCAM is not an endothelial marker, so I would recommend to delete any reference to EPCAM in the figures/tables, results and discussion section.

Minor

- Abstract *"Future work is required to increase the complexity of the organoids (e.g., addition of microglia and vasculature). We envision that the adoption of organoid systems will be beneficial to researchers studying mechanisms of brain infection by protozoan parasites."* Although it is important to showcase the limitations of the model, the second part of the sentence has somehow a negative connotation on the validity of the current results. In this paper, the authors validate the use of organoids systems to study *T. brucei* infection and the authors should highlight that they already represent an alternative to animal models.

- Research highlights (3Rs benefit(s)) and results page 3: Which methodology did you use to measure the reduction in animal models that could be used to study trypanosomiasis?

- There seems to be a reference missing in: *"For instance, there are intrinsic differences in the immune response between hosts (e.g., mouse vs. human) that pose limitations for translational science."*

- Results Page 7: "*We also detected significant expression of the interleukin-17 receptor subunit A and D (IL17RA and IL17RD, respectively), interleukin-10 receptor subunit a (IL10RA), and the Interferon gamma receptor 1 (IFNGR1) (Table 1 and Table S2 in Underlying data),22, indicating that these organoids are primed to sense and respond to IL-17, IL-10, and IFNγ signalling upon exposure to T. brucei gambiense*" - I would recommend to rephrase to something similar to "these organoids are prime to sense and response to *T. brucei gambiense* by activating IL-17, IL-10 and IFNg pathways."

Are a suitable application and appropriate end-users identified?

Yes

Are the 3Rs implications of the work described accurately?

Yes

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 3D brain bioengineered models, host-parasite interactions, malaria, vascular engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Jul 2023

Juan Quintana

In this manuscript, Chandrasegaran *et al.*, use for the first-time *in vitro* brain organoids to understand *Trypanosoma brucei gambiense* pathogenesis. This is an important topic, as brain organoids have already become as a valid strategy to study disease mechanisms. Although the team led by Juan Quintana is already a leader in animal models of *T. brucei,* I would like to praise their efforts on using alternative disease modelling tools that could reduce the number of research animals and overcome differences between mouse and humans. The authors show differential gene expression after brain organoid incubation with *T, brucei gambiense* after a 24h and 72h incubation. The manuscript is timely, and overall, scientifically sound and well written. I support the article indexing after the introduction of some modifications on the text.

We sincerely thank this reviewer for their positive assessment of our work. We are always motivated to explore novel and more ethical ways to conduct our research and are indeed excited by the possibility of implementing organoids and “organ-on-a-chip”

approaches to study brain responses to infection.

Major

- Although the methods section is very clear, I couldn't find any reference on how long the brain organoids were grown. This is important because brain organoids are grown for long periods of time (up to a year) and depending on the maturation time, they acquire different brain developmental stages. I would suggest that the authors include this information in the methods section, as well as on the results or discussion section, as it is important for interpretation of the results.

Authors: We apologise for this issue. The cortical brain organoids used in this study were ~2 months old, to ensure a minimum level of maturation, as reported in references 2 and 4. We have included this in the methods section of the revised manuscript.

- How does the 24h and 72h time point or the parasite concentration used compare with the life cycle of *T. brucei gambiense* in the brain? This information could be useful for an audience not familiar with *T. brucei*.

Authors: This is a great question, but a challenging one to address. Gambiense HAT is typically mild, lasting for years before the clinical symptoms associated with the second stage of the disease (e.g., sleep disturbances) become patent. Unfortunately, there is no clinical data indicating how many parasites there are in the brain at any given points during gambiense HAT, but CSF from second stage gambiense HAT patients can report anywhere tens to hundred parasites per ml of CSF (https://www.thelancet.com/journals/ebiom/article/PIIS2352-3964(22)00558-8/fulltext for reference). In our own experience infecting mice with *T. b. gambiense*, we typically observed around 10^2-10^4 parasites/gram of brain tissue (unpublished data), but we are unsure at this stage how comparable this is to the *in vitro* system we set out to develop here. We will focus on these aspects in future work.

- The authors couldn't find any brain damage markers on Fig 2C after staining with two different markers. However, other non-used markers could be affected. Although the sentence in the result section mildly implies that ("*Importantly, we did not detect significant morphological or histological changes in the organoids exposed to the parasites based on H&E staining and MAP 2 staining (Figure 1C), suggesting that T. brucei gambiense does not elicit tissue damage over a 72 hour culture period.*"), I would suggest to rephrase to make it more obvious. Furthermore, did the author quantify for differences in expression on MAP2, or is the analysis mostly qualitative?

Authors: We thank this reviewer for this useful suggestion. We have rephrased the text to highlight that we are basing our observations of a limited number of morphological markers (H&E and MAP2), as follow: *Importantly, we did not detect significant morphological or histological changes in the organoids exposed to the parasites based on the morphological aspects observed upon H&E staining and MAP2 staining (Figure 1C), suggesting that T. brucei gambiense does not seem to elicit tissue damage over a 72 hour culture period, at least not with the markers used in this study*.

It is important to note that we originally intended to keep some of these organoids to run western blotting for quantification of markers of interest (e.g., MAP2) but were unable to do so due to limited availability. We plan to dissect this aspect in more detail as part of future work.

- The increased expression of endothelial markers is intriguing, taking into account that brain organoids present minimal presence of endothelial cells. This is important because vascularization of brain organoids is one of the main challenges in the bioengineering field, and the presence of vasculature in these models could be used to study the molecular mechanisms of *T. brucei* crossing through the brain microvasculature. Did the authors checked for the presence of blood vessels by immunostaining. In a similar topic, I would like to clarify that EPCAM is not an endothelial marker, so I would recommend to delete any reference to EPCAM in the figures/tables, results and discussion section.

Authors: We agree with this reviewer. We were also surprised to see an upregulation of genes typically found in the endothelial compartment as these organoids do not typically contain vasculature, as this reviewer states. It is important to note that we failed to detect positive staining for VWF and CDH5 by immunohistochemistry, but there might be technical limitations to consider (e.g., antibody compatibility, detection level, etc) before we can confidently support or exclude the presence of a vasculature-like cell population in our dataset in response to *T. b. gambiense* exposure/co-culture. Although intriguing and exciting, we decided to err on the side of caution and limit our interpretations to present the transcriptional features, without concluding that these organoids in fact contain vasculature. Also, as suggested by this reviewer, we have removed references to *Epcam* from the text/table.

Minor

- Abstract *"Future work is required to increase the complexity of the organoids (e.g., addition of microglia and vasculature). We envision that the adoption of organoid systems will be beneficial to researchers studying mechanisms of brain infection by protozoan parasites."* Although it is important to showcase the limitations of the model, the second part of the sentence has somehow a negative connotation on the validity of the current results. In this paper, the authors validate the use of organoids systems to study *T. brucei* infection and the authors should highlight that they already represent an alternative to animal models.

Authors: We agree with this reviewer. As suggested, we have amended the abstract accordingly as follow: *Although our data support the use of brain organoids to explore and model host-pathogen interactions in the context of T. brucei infection as an alternative to in vivo models, future work is required to increase the complexity of the organoids (e.g., addition of microglia and vasculature). We envision that the adoption of organoid systems is beneficial to researchers studying mechanisms of brain infection by protozoan parasites*.

- Research highlights (3Rs benefit(s)) and results page 3: Which methodology did you use to measure the reduction in animal models that could be used to study trypanosomiasis?

Authors: The figures presented in the current version of the manuscript were based on a prediction assuming full adoption of this method, and the number of animals that undergo similar procedures (e.g., brain responses to infection) over the past 5 years of our current UK Home Office license. In this case, we determined that on a typical year of our license, 47/100 animals undergo moderate-to-severe procedures involving evaluating brain responses to infection that could be fully replaced with a suitable *in vitro* brain organoids system. To generate those infections, we also typically require donor mice from which we obtain infective trypanosomes, which we

estimated to be reduced by about 1/5 with the organoids system in place.

- There seems to be a reference missing in: *"For instance, there are intrinsic differences in the immune response between hosts (e.g., mouse vs. human) that pose limitations for translational science."*

Authors: We have removed this sentence from the manuscript.

- Results Page 7: "*We also detected significant expression of the interleukin-17 receptor subunit A and D (IL17RA and IL17RD, respectively), interleukin-10 receptor subunit α (IL10RA), and the Interferon gamma receptor 1 (IFNGR1) (Table 1 and Table S2 in Underlying data),22, indicating that these organoids are primed to sense and respond to IL-17, IL-10, and IFNγ signalling upon exposure to T. brucei gambiense*" - I would recommend to rephrase to something similar to "these organoids are prime to sense and response to *T. brucei gambiense* by activating IL-17, IL-10 and IFNg pathways."

Authors: We have rephrased the manuscript as suggested as follow: *indicating that these organoids are primed to sense and respond to T. brucei gambiense by activating IL-17, IL-10, and IFNγ signalling pathways*

Competing Interests: No competing interests were disclosed.

Reviewer Report 03 May 2023

https://doi.org/10.5256/f1000research.144355.r170892

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David Smith

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This manuscript from Chandrasegaran et al., reports the application of iPSC-derived cerebral organoids as a model for neurological *T. b. gambiense* infection. Organoids are increasingly powerful *in vitro* models that better represent *in vivo* tissues compared to simpler cell culture systems. This is allowing researchers to address research questions at a level of precision and detail that is difficult to achieve *in vivo*. It also greatly reduces reliance on animals to experimentally address specific research questions. Overall, the methods in this manuscript are very clear (some minor points to be addressed stated below) which, importantly, provides an accessible protocol for other researchers to follow for the cultivation of iPSC-derived cerebral organoids (for which the authors are commended). In the results, the authors demonstrate infection of cerebral organoids by *T. b. gambiense* parasites and the differential gene expression determined between uninfected organoids and organoids infected with *T. b. gambiense* for 24 or 72 hours. A large number of genes are differentially up and down regulated and the author's inclusion of GO analysis is helpful to provide broader context to these transcriptomics differences. This report is overall very clear (with some minor points to address below) and will provide a valuable addition to the literature. There is minimal modification necessary before this manuscript

is suitable for indexing.

1. How long were the cerebral organoids cultivated for before challenge with parasites? Were the consistently matured to the same started point? What is the timescale for “maturing” the cerebral organoids?

2. Point 1 under “*Immunohistochemistry”*(methods) should read “storage” instead of “maintenance”.

3. The results section indicates 3 experimental replicates were used in the RNA-seq analysis for each test group. Please make this clear in the associated methods section.

4. Perhaps there is not sufficient read depth, but if it is possible it would be interesting to map the parasite RNA-seq reads from the 24h and 72h group to determine any differences, potentially informing on how the parasite is adapting to co-culture and infection in the cerebral organoids and whether this is similar to what would be expected/observed in vivo. If this is possible, it would have been useful to have a test group of parasites in the RNA-seq analysis representative of parasites immediately prior to organoid challenge (to better assess how the parasite itself is responding to co-culture with the organoids) – this is just a point for future consideration for the authors. It would be useful in future experiments to see how long a chronic infection can persist in the organoids (i.e. how far beyond 72 hours post-challenge can the cultures be maintained?).

5. How did the authors determine the predicted reduction in animal usage by replacement with organoids.

6. Figure legend 1C should say (left) and (right) instead of (top) and (bottom)? Also use arrows in panel C to indicate parasites for non-specialists.

7. Did the authors quantify parasite burden in the cerebral organoids at 24 and 72 hours post-challenge, relative to the 10^5 parasites used to challenge organoids at the start of the experiment.

Are a suitable application and appropriate end-users identified?

Yes

Are the 3Rs implications of the work described accurately?

Yes

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Organoids, parasitology, host:parasite interactions, protozoa, helminths

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Jul 2023

Juan Quintana

This manuscript from Chandrasegaran et al., reports the application of iPSC-derived cerebral organoids as a model for neurological *T. b. gambiense* infection. Organoids are increasingly powerful *in vitro*models that better represent *in vivo* tissues compared to simpler cell culture systems. This is allowing researchers to address research questions at a level of precision and detail that is difficult to achieve *in vivo*. It also greatly reduces reliance on animals to experimentally address specific research questions. Overall, the methods in this manuscript are very clear (some minor points to be addressed stated below) which, importantly, provides an accessible protocol for other researchers to follow for the cultivation of iPSC-derived cerebral organoids (for which the authors are commended). In the results, the authors demonstrate infection of cerebral organoids by *T. b. gambiense* parasites and the differential gene expression determined between uninfected organoids and organoids infected with *T. b. gambiense* for 24 or 72 hours. A large number of genes are differentially up and down regulated and the author's inclusion of GO analysis is helpful to provide broader context to these transcriptomics differences. This report is overall very clear (with some minor points to address below) and will provide a valuable addition to the literature. There is minimal modification necessary before this manuscript is suitable for indexing.

Authors: We sincerely thank this reviewer for taking the time to provide feedback on our methods article. We hope the comments below clarify their questions.

1. How long were the cerebral organoids cultivated for before challenge with parasites? Were the consistently matured to the same started point? What is the timescale for "maturing" the cerebral organoids?

Authors: The cortical brain organoids used in this study were ~2 months old, to ensure a minimum level of maturation, as reported in references 2 and 4.

1. Point 1 under "*Immunohistochemistry"*(methods) should read "storage" instead of "maintenance".

Authors: We have amended the text accordingly.

1. The results section indicates 3 experimental replicates were used in the RNA-seq analysis for each test group. Please make this clear in the associated methods section.

Authors: We have clarified this in the methods section, in step 2 under “Bulk RNA sequencing and data analysis”.

1. Perhaps there is not sufficient read depth, but if it is possible it would be interesting to map the parasite RNA-seq reads from the 24h and 72h group to determine any differences, potentially informing on how the parasite is adapting to co-culture and infection in the cerebral organoids and whether this is similar to what would be expected/observed in vivo. If this is possible, it would have been useful to have a test group of parasites in the RNA-seq analysis representative of parasites immediately prior to organoid challenge (to better assess how the parasite itself is responding to co-culture with the organoids) – this is just a point for future consideration for the authors. It would be useful in future experiments to see how long a chronic infection can persist in the organoids (i.e. how far beyond 72 hours post-challenge can the cultures be maintained?).

Authors: Indeed, we agree with this reviewer, and this is something we plan to consider in future experiments. We had financial constraints that limited our ability to explore how the parasites responded to the co-culture system. It is important to note that in our hands, we did not see parasites penetrating the organoids *per se*, and so we decided to wash the organoids with 1X PBS to remove as many parasites as possible. This has now been clarified in the methods section, in step 3 under “Trypanosoma brucei gambiense – human brain organoids co-culture system”.

1. How did the authors determine the predicted reduction in animal usage by replacement with organoids.

Authors: The figures presented in the current version of the manuscript were based on a prediction assuming full adoption of this method, and the number of animals that undergo similar procedures (e.g., brain responses to infection) over the past 5 years of our current Home Office license. In this case, we determined that on a typical year of our license, 47/100 animals undergo moderate-to-severe procedures involving evaluating brain responses to infection that could be fully replaced with a suitable *in vitro* brain organoids system. To generate those infections, we also typically require donor mice from which we obtain infective trypanosomes, which we estimated to be reduced by about 1/5 with the organoids system in place.

1. Figure legend 1C should say (left) and (right) instead of (top) and (bottom)? Also use arrows in panel C to indicate parasites for non-specialists.

Authors: We have amended the figure legend as suggested. Unfortunately, we did not detect parasites inside the organoids by any means, and so the images in panel 1C are showing the integrity of the organoids post-culture compared to untreated organoids, and the staining for neurons using MAP2.

1. Did the authors quantify parasite burden in the cerebral organoids at 24 and 72 hours post-challenge, relative to the 10^5 parasites used to challenge organoids at the start of the experiment.

Authors: In short, we did not. We had originally intended to inoculate the parasites inside the organoids using microinjections, but this was not feasible for various reasons (e.g., access to adequate microinjection platforms, size of the organoids, etc).

Thus, we decided to co-culture the parasites with the organoids instead. Before doing so, we ensured that the parasite remained viable under the culture conditions (50:50 organoids:HMI9 media) using a growth curve approach, as shown in figure 1B.

Competing Interests: No competing interests were disclosed.

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