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ALTERAÇÕES COMPORTAMENTAIS E NEUROCOGNITIVAS EM MODELOS DE INFECÇÕES PARASITÁRIAS CRÔNICAS: RELAÇÃO COM NEUROINFLAMAÇÃO E PROPOSTAS DE TERAPIA

LEDA MARGARITA CASTAÑO BARRIOS

Rio de Janeiro
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Orientadora: Profa. Dra. Joseli Lannes-Vieira

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Ata da defesa de tese de doutorado acadêmico em Biologia Parasitária de **Leda Margarita Castaño Barrios**, sob orientação da Drª. Joseli Lannes Vieira. Ao sétimo dia do mês de janeiro de dois mil vinte e dois, realizou-se às nove horas e trinta minutos, de forma síncrona remota, o exame da tese de doutorado acadêmico intitulada: "**Alterações comportamentais e neurocognitivas em modelos de infecções parasitárias crônicas: relação com neuroinflamação e propostas de terapia**", no programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutora em Ciências - área de concentração: Imunologia e Patogenia, na linha de pesquisa: Imunologia e Patogênese de Doenças Infecciosas e Parasitárias. A banca examinadora foi constituída pelos Professores: Dr. Daniel Pedra Adesse – IOC/FIOCRUZ (Presidente), Dr. Rudimar Luiz Frozza– IOC/FIOCRUZ, Drª. Paula Campello Costa Lopes – UFF/RJ, e como suplentes: Drª. Maria Regina Reis Amendoeira- IOC/FIOCRUZ e Dr. Roney Santos Coimbra - CPQRR/FIOCRUZ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado acadêmico. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Presidente da Banca atesta a decisão e a participação da aluna e de todos o membros da banca de forma síncrona remota. O Coordenador do Programa Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 7 de janeiro de 2022.

Dr. Daniel Pedra Adesse (Presidente da Banca):

Dr. Rafael Maciel de Freitas (Coordenador do Programa):

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“Vivir sin ataduras, caminar bajo la luna, andar por el mundo a dos. Ser un ave de alas largas”.

Leda Castaño Barrios

RESUMO

O aumento da frequência de doenças neurológicas, nas últimas décadas, tem estimulado estudos sobre a patogênese destas e estratégias terapêuticas. Trabalhos apontam associação entre processos infecciosos e o estabelecimento ou progressão destas doenças, sejam associados à neuroinflamação, ou infecções sistêmicas, com ativação do sistema imune e produção de mediadores inflamatórios, como citocinas. Portadores crônicos da doença de Chagas e da toxoplasmose, causadas, respectivamente, pela infecção por *Trypanosoma cruzi* e *Toxoplasma gondii,* apresentam alterações comportamentais (depressão, mudanças de personalidade) e alterações neurocognitivas como deficit de memória de diversos tipos. Neste estudo, abordarmos a hipótese que infecções parasitárias crônicas, na presença ou ausência de neuroinflamação, podem levar a alterações comportamentais e neurocognitivas. Dessa forma, a presença do parasito no sistema nervoso central (SNC) seria relevante e, portanto, a redução do parasitismo por meio da resposta imune intrínseca e, sobretudo, por terapias etiológicas, teria impacto benéfico nestas alterações. Nossos resultados foram compilados em três artigos científicos. Em todos os estudos foram usados camundongos C57BL/6 (H-2^b) fêmeas. Em modelo de infecção crônica pela cepa Colombiana do *T. cruzi*, mostramos a instalação progressiva de alterações neurocognitivas (memória de longo prazo) com a evolução da fase crônica, associadas à atrofia cerebral, persistência do parasito e estresse oxidativo no SNC, mas na ausência de neuroinflamação. A intervenção terapêutica com benznidazol impediu a instalação ou mesmo reverteu alterações neurocognitivas, associado à redução da carga parasitária e do estresse oxidativo no SNC (**artigo 1**). Também desafiamos a participação do parasito no SNC, em presença de neuroinflamação, na indução de alterações comportamentais e neurocognitivas com a infecção de camundongos com a cepa ME-49 do *T. gondii*. Alterações comportamentais em fase crônica precoce e tardia foram relacionadas à presença de cistos no SNC e ruptura da barreira hematoencefálica (BHE), com presença de citocinas e CC-quimiocinas intracerebrais, que precedem a instalação da neuroinflamação, e perfil inflamatório sistêmico (**Artigo 2**). Desafiamos o efeito da resposta imune intrínseca, que leva à redução do número de cistos no SNC, e da associação do tratamento etiológico, sulfadiazina e pirimetamina (S+P), nas alterações comportamentais e neurocognitivas. A terapia S+P impactou, a depender da alteração, de forma transitória, parcial ou permanente, impedindo progressão ou, mesmo, as revertendo. A terapia melhorou o controle intrínseco da carga de cistos no SNC, alterações histopatológicas, ruptura da BHE e níveis séricos de citocinas Th1. Finalmente, a análise de componentes principais evidenciou três *clusters* distintos (não infectados; infectados tratados com veículo e com S+P) e a associação entre os níveis séricos de citocinas pro-inflamatórias, carga de cistos no SNC e alterações comportamentais e neurocognitivas (**Artigo 3**). Em conjunto, nossos dados indicam que a presença dos parasitos estudados no SNC exerce papel fundamental no estabelecimento de alterações comportamentais e neurocognitivas, associada ou não a neuroinflamação, com impacto benéfico do tratamento etiológico. Apesar das limitações para desvendar mecanismos neurobiológicos e moleculares envolvidos na persistência destes parasitos no SNC, que contribuiriam para estas alterações, nossos dados sugerem que terapias etiológicas na fase crônica destas infecções poderiam impactar a saúde mental e melhorar a qualidade de vida dos portadores.

ABSTRAC

In the recent decades, the increase in the frequency of neurological diseases has stimulated studies on their pathogenesis and therapeutic strategies. Studies point to the association between infectious processes and the establishment or progression of these diseases, either associated with neuroinflammation or systemic infections, with activation of the immune system and production of inflammatory mediators, such as cytokines. Chronic patients of Chagas disease and toxoplasmosis, caused, respectively, by *Trypanosoma cruzi* and *Toxoplasma gondii* infection, present behavioral changes (depression, personality changes) and neurocognitive alterations such as different types of memory deficit. In this study, we address the hypothesis that chronic parasitic infections, in the presence or absence of neuroinflammation, can lead to behavioral and neurocognitive changes. Thus, the presence of the parasite in the central nervous system (CNS) would be relevant and, therefore, the reduction of parasitism through the intrinsic immune response and, mainly, through etiological therapies, would have a beneficial impact on these changes. Our results were compiled in three scientific articles. In all studies, female C57BL/6 (H-2^b) mice were used. In a model of chronic infection by the Colombian strain of *T. cruzi*, we show the progressive onset of neurocognitive changes (long-term memory) with the evolution of the chronic phase, associated with brain atrophy, persistence of the parasite and oxidative stress in the CNS, but in the absence of neuroinflammation. Therapeutic intervention with benznidazole prevented the onset or even reversed neurocognitive changes, associated with a reduction in the parasite load and oxidative stress in the CNS (Article 1). We also challenged the participation of the parasite in the CNS, in the presence of neuroinflammation, in the induction of behavioral and neurocognitive alterations with the infection of mice with the ME-49 strain of *T. gondii*. Behavioral changes in early and late chronic phases were related to the presence of CNS cysts and rupture of the blood-brain barrier (BBB), with the presence of intracerebral cytokines and CC-chemokines, which precede the onset of neuroinflammation, and systemic inflammatory profile (Article 2). We challenged the effect of the intrinsic immune response, which leads to a reduction in the number of cysts in the CNS, and the association of etiological treatment, sulfadiazine and pyrimethamine (S+P), on behavioral and neurocognitive alterations. Depending on the alteration, the S+P therapy had a transient, partial, or permanent impact, preventing progression or even reversing them. The therapy improved the intrinsic control of CNS cyst burden, histopathological changes, BBB disruption, and serum Th1 cytokine levels. Finally, principal component analysis revealed three distinct clusters (uninfected; infected treated with vehicle and with S+P) and the association between serum levels of pro-inflammatory cytokines, CNS cyst load, and behavioral and neurocognitive changes (Article 3). Together, our data indicate that the presence of the studied parasites in the CNS plays a fundamental role in the establishment of behavioral and neurocognitive alterations, associated or not with neuroinflammation, with a beneficial impact of the etiological treatment. Despite the limitations to unravel neurobiological and molecular mechanisms involved in the persistence of these parasites in the CNS, which would contribute to these changes, our data suggest that etiological therapies in the chronic phase of these infections could impact mental health and improve the quality of life of patients.

ÍNDICE

ÍNDICE DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

AVC	Acidente vascular cerebral
ADHD/TDAH	Attention-deficit/ hyperactivity disorder/ Transtorno de deficit de atenção e hiperatividade
AIDS	Acquired immunodeficiency syndrome/ Sindrome de imunodeficiencia adquirido
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance/ Análise de variância
BBB/BHE	Brain blood barrier/ Barreira hematoencefálica
Bz	Benznidazole/ Benznidazol
CB	Cerebellum
CBA	Cytometric Bead Array/ Imunoensaios baseados em esferas
CCC	Cardiomiopatia chagásica crônica
CCL2/MCP-1	C-C Motif Chemokine Ligand 2/ monocyte chemotactic protein-1
CCL3/ MIP-1α	C-C Motif Chemokine Ligand 3/ Macrophage inflammatory protein-1 alpha
CCL4/MIP-1β	C-C Motif Chemokine Ligand 4/Macrophage inflammatory protein-1β
CCL5/RANTES	C-C Motif Chemokine Ligand 5/ Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
CD/ DC	Chagas disease/ Doença de Chagas
CD1	Cluster of differentiation 1
CD8+	Cluster of differentiation 8
CEA-CF	Housed in the Experimental Animal Facility- Cardoso Fontes/ Centro de experimentação animal- Cardoso Fontes
Cm	Centrimeter
CNS/ SNC	Central nervous sistem / Sistema nervoso central
CNU	Cerebral Nuclei
Covid-19	Coronavirus disease 2019
DA	Doença de Alzheimer
Db	Decibel
DHFR	Dihidrofolato redutase
DHPS	Dihidropteroato sintetase
DI	Discrimination index
Diam	Diameter
DPI	Days postinfection/ Dias pós infecção
DT	Talidomida, 3,6'-ditiothalidomida
DTU	Discreet Typing Units/ Unidade de tipagem discretas
EB	Evans blue
EPMT	Elevated plus-maze test
FACS	Fluorescence-activated single cell sorting
Fiocruz	Oswaldo Cruz Foundation/ Fundação Oswaldo Cruz
FST	Forced-swimming test
G	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Gf	gram-force
GSMT	Grip strength meter test
HIV	Human immunodeficiency virus/ Vírus da imunodeficiência humana
HPF	Hippocampal formation
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HY	Hypothalamus
ICBIM	Instituto de Ciências Biomédicas
ICTB	Institute of Science and Technology in Biomodels/ Instituto de Ciência e Tecnologia em Biomodelos
ICTX	Isocortex
IFNα	Interferon-alpha
IFNγ	Interferon gamma
IL	Interleukin/ Interleucina
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-6	Interleukin 6
Kg	Kilograms
LBI-IOC	Laboratory of Biology of the Interactions
LPS	Lipopolysaccharides
mA	Milliampere
MAPK	mitogen-activated protein kinase
MB	Midbrain
MDA	Malondialdehyde
Mg	Milligram
MMSE	Mini-Mental State Exam
mRNA	Messenger ribonucleic acid,
MY	Medulla
NaOH	Hidróxido de sódio
NF-kB	Nuclear factor kappa B
NI	Non-infection/ Não infectado
NIH	National Institutes of Health
Nm	Nanômetro
NMDA	N-methyl-D-aspartate
NO	Óxido Nítrico
OFT	Open field test
OLF	Olfactory areas
P	Pons
P	Pyrimethamine
PBS	Phosphate-buffered saline
PCA/ ACP	Principal-component analysis / Analise de componente principal
PCR	Polymerase chain reaction
qPCR	Real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
S	Second / segundo
S	Sulfadiazine
Sec	Second

S+P	Sulfadiazineplus-pyrimethamin/ Sulfadiazina e pirimetamina
SatDNA	Satellite Deoxyribonucleic acid
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error of the mean
TAR	Terapia anteiretroviral
T. cruzi	*Trypanosoma cruzi*
T. gondii	*Toxoplasma gondii*
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive species
TH	Thalamus
Th1	T helper cells
TNF	Tumor necrosis factor / Fator de necrose tumoral
TNFR	Tumor necrosis factor receptor/ Receptor do fator de necrose tumoral
TNFR1	Tumor necrosis factor receptor 1 / Receptor 1 do fator de necrose tumoral
TST	Tail suspension test
UFU	Universidade Federal de Uberlândia
Veh	Vehicle
w/v	Weight per-volumen
µg	Micrograma
µL	Microlitro

1. INTRODUÇÃO

Melhoras em saúde pública, condições ambientais, desenvolvimento de vacinas, descoberta de agentes antimicrobianos e na capacidade de identificar novos organismos patogênicos e propor medicamentos e/ou vacinas, entre outros avanços e descobertas em biomedicina que tiveram destaque durante o século XX, permitiram a melhoria de qualidade e aumento da expectativa de vida da população mundial (1). Assim, chegamos ao século XXI com a expectativa da redução da frequência de mortes por doenças infecciosas, e aumento de mortes por doenças crônico-degenerativas (2), ainda que esta expectativa tenha sido afetada pela pandemia de Covid-19 (3). Este conjunto de alterações e avanços, com aumento da expectativa de vida de 25-35 anos para além de 65-75 anos em cerca de 100 anos, permitiu um inquietante aumento na frequência de doenças neurodegenerativas, alterações comportamentais e neurocognitivas na população, sobretudo em pessoas acima de 60 anos de idade (4, 5).

O termo doença mental se refere a qualquer transtorno mental ou alteração comportamental diagnosticável. Estas são condições de saúde caracterizadas por alterações de pensamento, humor ou comportamento, associadas a fatores como sofrimento e/ou detrimento do bem estar que podem levar à deficiência, dor ou morte (6). Mundialmente, os transtornos mentais compõem uma parte importante da carga de doença, especialmente a depressão e o transtorno de ansiedade que custam US$ 1 trilhão por ano à economia global (7).

Estima-se que até 2017 um bilhão de pessoas no mundo, ou seja 1 de cada 7 pessoas, sofreu um ou mais transtornos mentais ou decorrentes de uso de substâncias, incluindo depressão, ansiedade, transtorno bipolar, transtornos alimentares, transtornos por uso de álcool ou drogas, esquizofrenia (8), e doenças neurológicas como Doença de Alzheimer (DA), Parkinson, acidente cérebro vascular (ACV), esclerose múltipla, epilepsia, lesões cerebrais e infecção do sistema nervoso central (SNC) (9). Independentemente da idade, sexo, educação ou renda, pessoas em todos os países são afetadas (9), sendo ao redor de 20% das crianças e adolescentes e mais de 20% dos idosos (4, 5). Ainda, em 2019, DA e outras demências foram consideradas como a sétima causa de morte mundialmente e a segunda causa em países de alta renda (10). No Brasil, estima-se que há cerca de 31,06 milhões de indivíduos afetados por alterações neuropsiquiátricas (ansiedade,

depressão) e 1,5 milhões com doenças neurodegenerativas, como DA e demências senis (8, 11).

As causas de transtornos mentais podem ser diversas e multifatoriais: fatores sociais, culturais, psicológicos, ambientais, genéticos, dieta, estresse e infecções podem desencadear doenças mentais (5). De fato, infecções têm sido relacionadas com o estabelecimento e manutenção de inflamação crônica sistêmica (12), que pode favorecer a instauração de transtornos mentais como depressão e ansiedade (13, 14). Além disto, muitas infecções estão associadas ao desenvolvimento de doenças neurodegenerativas como DA, por mecanismos envolvidos no desenvolvimento de neuroinflamação (15). Nesta condição, células inflamatórias, sobretudo, células mononucleares, como linfócitos T e macrófagos, infiltram o SNC (16). Também, infecções sistêmicas e níveis séricos elevados de citocinas pro-inflamatórias, como interleucina (IL)-1 β, em pacientes com DA, se relacionam com maior declínio cognitivo, que pode persistir por até dois meses após a resolução da infecção (17). Assim, a patogênese de alterações mentais e neurodegenerativas pode ter aspectos complexos e múltiplos, envolvendo alterações intrínsecas ao tecido do SNC, permissividade do endotélio à adesão de células inflamatórias e invasão do SNC, com geração de neuroinflamação, infecções *in situ* no SNC, infecções sistêmicas e geração de perfil inflamatório sistêmico (18). Nas últimas décadas, alterações comportamentais associadas a aumento de níveis sistêmicos de citocinas inflamatórias foram descritas, como por exemplo, o uso de IFNα para tratar hepatite C induziu depressão e ansiedade nos pacientes tratados (13). Assim, processos que atingem diretamente o SNC, com neuroinflamação, ou infecções sistêmicas, como ativação do sistema imune, poderiam contribuir para alterações comportamentais e neurocognitivas.

Diversos estudos vêm sugerindo que portadores crônicos da doença de Chagas e da toxoplasmose, causadas, respectivamente, pela infecção pelo *Trypanosoma cruzi* e pelo *Toxoplasma gondii*, apresentam alterações comportamentais (19-25) e neurocognitivas (20, 22, 26-30). Estas são doenças parasitárias crônicas de relevância epidemiológica, sendo um terço da população mundial infectada pelo *T. gondii* (31) e 7-8 milhões pelo *T. cruzi* (32).

Diante do exposto acima, nos artigos que aqui trazemos como resultados de nosso estudo, objetivamos avaliar a presença de alterações comportamentais e neurocognitivas induzidas pelos parasitos protozoários (i) *Trypanosoma cruzi* e (ii) *Toxoplasma gondii*, em condições de infecção crônica de camundongos, buscando

replicar aspectos semelhantes às alterações descritas em portadores destas infecções. Ambos os parasitos persistem no SNC de camundongos C57BL/6 (H-2^b) cronicamente infectados. Camundongos desta linhagem cronicamente infetados pela cepa Colombiana do *T. cruzi*, em condição de imunossupressão, apresentam reativação da infecção ou parasitos no SNC, com ausência de parasitemia (33). Já no modelo de infecção crônica pelo *T. gondii* com a cepa ME-49, cistos cerebrais são observados ainda após tratamento antiparasitário com redução da carga parasitaria (34). Se por um lado, a infecção crônica em portadores da doença de Chagas (DC), assim como na infecção experimental pelo *T. cruzi* cursa com ausência de neuroinflamação (33, 35, 36), por outro lado a infecção crônica pelo *T. gondii* pode transcorrer na ausência de neuroinflamação em pessoas imunocompetentes (37) ou com neuroinflamação sustentada em modelos experimentais (38-40). Os modelos foram avaliados durante a fase crônica das infecções e, adicionalmente, desafiamos o papel da presença do parasito, por meio da terapia etiológica de primeira escolha estabelecida para cada patógeno (41, 42). Estes modelos permitiram avaliar a relação das alterações comportamentais e neurocognitivas com a presença de neuroinflamação e do parasito.

1.1. *Trypanosoma cruzi* E DOENÇA DE CHAGAS

O protozoário *Trypanosoma cruzi* é o agente etiológico da doença de Chagas, uma doença tropical negligenciada (43), descoberta pelo brasileiro Carlos Chagas, em 1909 (44). A DC é endêmica em 21 países da América Latina, e estima-se que 65 milhões de pessoas vivam em áreas de exposição e estejam sob risco de infecção (45). Como consequência da dinâmica migratória da população latino-americana durante o final do século XX e início do século XXI, a DC está presente em países não-endêmicos da América do norte, Europa, Japão e Austrália (32, 45). Atualmente, acredita-se que a doença de Chagas afete entre 7 e 8 milhões de pessoas no mundo, principalmente na América Latina (32). No Brasil 1,9 a 4,6 milhões de pessoas estão cronicamente infectadas pelo *T. cruzi* (46) e ainda, nas Américas mais de 10 mil pessoas morrem a cada ano em decorrência de complicações clínicas da doença. Também, estima-se cerca de 8 mil novos casos de doença de Chagas congênita anualmente (43).

T. cruzi é dividido em seis unidades de tipagem discretas (Discreet Typing Units, DTU): TcI, TcII, TcIII, TcIV, TcV e TcVI, que variam segundo a distribuição

geográfica, especificidade do hospedeiro e patogenicidade. (47, 48). Além do genótipo Tc Bat, associado com morcegos no Brasil, Panamá e Colômbia (49-52).

O ciclo biológico do *T. cruzi,* envolve dois tipos de hospedeiros, (i) o hospedeiro invertebrado, insetos estritamente hematófagos, triatomíneos da ordem hemíptera, popularmente conhecidos no Brasil como barbeiro, bicudo, chupão e procotó, entre outros, e agem como vetor da DC, e (ii) hospedeiros vertebrados(44). Até 2010 foram reportados um total de 180 espécies de mamíferos selvagens e domésticos das ordens Didelphidomorphia, Lagomorpha, Chiroptera, Rodentia, Pilosa, Cingulata, Carnivora, Primata, Perisodactyla, infectados naturalmente pelo *T. cruzi*, incluindo ao humano, que também agem como reservatórios. Outros vertebrados como aves, anfíbios, repteis e peixes são refratários à infecção, mas agem como fonte de alimentação para os vetores (53).

As formas evolutivas do *T. cruzi* são constituídas por (i) amastigota, forma intracelular obrigatória, proliferativa e infectante, exclusiva de hospedeiros vertebrados, (ii) tripomastigota sanguínea, forma infectante circulante no sangue de hospedeiros vertebrados, (iii) epimastigota, forma proliferativa, presente no hospedeiro invertebrado e gambás e (iv) tripomastigota metacíclica, forma infectante presente no reto do vetor e eliminado nas fezes do mesmo (44, 54). A forma de transmissão do parasito classicamente conhecida é a transmissão vetorial, se dá através do contato com fezes e urina de triatomíneos infectados. Que eliminam formas tripomastigotas metacíclicas nas fezes durante o repasto sanguíneo, estas entram no organismo através de fissuras, a lesão ocasionada pelo inseto e/ou através da mucosa conjuntiva e oral. A transmissão também pode ocorrer quando há consumo de alimentos ou bebidas contaminados pelo *T. cruzi* (contaminadas com fezes ou urina de triatomíneos), pela transfusão de sangue ou hemoderivados e transplante de órgãos de doadores infectados, transmissão vertical, e acidentes laborais (objetos perfurocortantes contaminados) (32).

A DC apresenta duas fases: (i) a fase aguda, que geralmente é assintomática, ou se apresenta com sintomas inespecíficos (febre, cansaço, edema de gânglios), caracterizada pela presença de tripomastigotas circulantes no sangue periférico e em células do tecido subcutâneo, e (ii) a fase crônica, se caracteriza pela ausência ou presença intermitente de raros parasitos circulantes e pode apresentar as seguintes formas clinicas: (a) Forma indeterminada, caracterizada pela ausência de sinais e sintomas clínicos, mesmo na presença de positividade para anticorpos anti-*T. cruzi*. (b) Forma cardíaca, esta forma se desenvolve em 20 a 30% dos portadores

crônicos, e resulta em alterações no sistema condutor elétrico cardíaco, bradiarritmias, taquiarritmias, aneurismas apicais, insuficiência cardíaca, tromboembolismo e morte súbita. Acumulação de infiltrado inflamatório, lesão nas fibras cardíacas e remodelamento do tecido e fibrose, também podem estar presentes no tecido cardíaco, contribuindo à instauração da cardiomiopatia chagásica crônica (CCC). (c) Forma digestiva, nesta forma geralmente há alterações na secreção, motilidade, e na absorção. A presença do parasito e da inflamação produzida pela resposta imune pode ocasionar disperistalsia do esôfago e do cólon pela destruição do plexo mioentérico, culminando na instauração de megaesôfago e megacólon. (d) Forma cardio/digestiva ou mista, padecida por uma pequena porcentagem dos portadores crônicos, que apresentam as duas formas concomitantemente (54).

Uma quinta forma clínica desta doença, tem sido proposta e ainda é matéria de debate, a forma nervosa. O comprometimento do SNC na DC tem sido reportado desde o descobrimento da doença por Carlos Chagas, quem descreveu alterações neurocognitivas em crianças com perturbações funcionais com condições intelectuais precárias, e infantilismo em adultos (44). Contudo, seus dados não tinham controles não infectados submetidos às mesmas condições. Além disso, Chagas reportou crises convulsivas, especialmente em crianças, e a presença de meningoencefalite com lesões de múltiplos focos disseminados em diversas áreas do SNC, observadas durante a autópsia de uma criança de 3 meses de vida, vítima da DC aguda (55). A presença de formas amastigotas do *T. cruzi* no cérebro de tal criança, junto com focos inflamatórios irregularmente distribuídos de infiltração leucocitária, além da presença do parasito e a divisão intracelular de formas amastigotas em células neuronais e gliais, foram descritas posteriormente por avaliação histológica, trazendo aportes importantes sobre a patogênese da DC aguda nervosa (56).

O estabelecimento de modelos de infecção experimental permitiu corroborar dados observados em humanos por Carlos Chagas. Infecção experimental em gatos resultou em alterações locomotoras, presença de inúmeros focos inflamatórios múltiplos disseminados no cerebelo, núcleos da base, medula e protuberância tanto na substância branca, quanto na cinzenta, assim como a presença do parasito nestas regiões cerebrais (56). Por outro lado, um estudo anatomopatológico, em modelo experimental agudo de infecção pelo *T. cruzi* em cães descreveu a presença de um quadro de encefalomielite com presença de diversos focos constituídos por

células da glia hipertrofiadas e amastigotas, majoritariamente localizados na substância branca e na medula. De modo importante, foi descrita a ausência de processos inflamatórios nas meninges, assim como a conservação da estrutura das células do córtex e da medula (57). Modelos murinos de infecção experimental crônica também têm sido importantes no entendimento do comprometimento do SNC durante a fase crônica da doença de Chagas. Na fase aguda da infecção experimental em camundongos, pode ocorrer ou não presença de meningoencefalite, que depende da suscetibilidade ou resistência do hospedeiro e está relacionada à ativação de leucócitos circulantes e a ativação do endotélio, mas ausente na fase crônica em ambos os modelos (33). De fato, na fase aguda, mas não na fase crônica, o endotélio ativado é permissivo à adesão de leucócitos (33, 58). Por outro lado, camundongos C57BL/6 cronicamente infectados pelo *T. cruzi*, submetidos à imunossupressão, apresentam reativação da infecção com presença de parasitos no SNC e ausência de formas circulantes (33). Além disso, foi demonstrado que camundongos Swiss Webster jovens são mais suscetíveis ao desenvolvimento do quadro encefálico durante a fase aguda da infecção, devido, hipoteticamente, à imaturidade do sistema imune e maior permeabilidade dos capilares nessa fase de vida (59), apoiando a existência de maior suscetibilidade à encefalite chagásica em crianças abaixo de 4 anos de idade (59, 60).

O comprometimento do SNC durante a fase aguda da DC, foi bem caraterizado após a descoberta da doença em humanos e modelos experimentais, sendo aceita a existência da forma nervosa aguda caracterizada pela presença de meningoencefalite com infiltrados inflamatórios dispersos por todo o SNC (55-57). Encefalite chagásica na fase aguda afeta principalmente crianças menores de dois anos de idade. O desenvolvimento de meningoencefalite pode levar à morte, especialmente quando há comorbidade, como miocardite e insuficiência cardíaca (60). Em humanos, casos de reativação da DC em pacientes com síndrome de imunodeficiência adquirida (AIDS) têm sido reportados. Ainda, alterações neurológicas em portadores crônicos da doença de Chagas, co-infectados com HIV, foram relacionadas à presença do *T. cruzi* no SNC, corroborado pela melhora significativa do quadro de meningoencefalite após a administração do tratamento etiológico tripanossomicida (61). Acometimento cerebral por reativação da infecção pelo *T. cruzi* em pacientes HIV positivos ou imunossuprimidos também pode ser fatal (61-64).

Durante a fase crônica, alterações neurológicas como nódulos gliais residuais e pequenos focos de infarto no tecido cerebral estão presentes, porém considerados como sequelas da fase aguda. Sendo que se destaca a ausência de neuroinflamação crônica persistente ou infiltrados perivasculares e conservação da estrutura celular (60, 65, 66), ainda que atrofia cerebral tenha sido descrita por exame de tomografia computadorizada, independente de cardiopatia (66). Distúrbio cortical funcional discreto e lesões na substância branca também podem ser achados no SNC em portadores crônicos (67). Embora alterações inflamatórias focais muito discretas ou insignificantes tenham sido descritas em pacientes com DC crônica, não há uma base histopatológica que permita demostrar a existência desta forma (60). Assim, o comprometimento do SNC na DC crônica tem sido amplamente relacionado à disfunção cardíaca, que resulta em isquemia cerebral por hipoperfusão e eventos embólicos com consequente comprometimento neurocognitivo e comportamental (68). Contudo, atrofia cerebral e cerebelar com ausência de neuroinflamação foi demonstrada em portadores crônicos da DC (36) e em infecção em modelo experimental (69). Ainda corroborado em pacientes com CCC avaliados por tomografia computadorizada de crânio, quando comparados com pacientes com outras cardiomiopatias, os pacientes com CCC apresentaram menor volume cerebral total, indicando a associação de atrofia cerebral à DC, como o possível principal substrato anatômico do comprometimento cognitivo na doença de Chagas (66). Assim, foi postulada a hipótese de que a inflamação crônica na DC, como mecanismo de defesa pela persistência do parasito, pode constituir, de forma progressiva, um mecanismo para a instauração do dano endotelial, ativação plaquetária, aceleração de aterosclerose e apoptose de células endoteliais, resultando em acidente vascular cerebral e atrofia cerebral, como a base histopatológica que demostra o comprometimento do SNC na DC crônica (70), embora a forma nervosa ainda continue sem um consenso.

Adicionalmente, infecções por *T. cruzi* têm sido apontadas como indutoras de alterações neurocognitivas. Pacientes com CCC apresentam recordação atrasada do teste da Figura Complexa de Rey, que avalia a capacidade de construção visual-espacial e memória não verbal, quando comparados com pacientes com outras cardiomiopatias (71). Déficit de atenção, prejuízos no desempenho da leitura, à escrita e da capacidade de compreensão (20), transtornos da linguagem (72), alterações de memória não-verbal e de aprendizagem (28), perda de memória (27), baixo rendimento no mini exame do estado mental (MMSE) (26), prejuízo da função

cognitiva, detectada por encefalograma, na ausência de comprometimento cardíaco (73). Ademais, alterações comportamentais como depressão (19-21), em pacientes com CCC ou com função cardíaca preservada (74), mudanças de humor (72), têm sido descritas em portadores crônicos da DC, fornecendo evidências do comprometimento do SNC na fase crônica infecção pelo *T. cruzi.*

Alterações comportamentais e neurocognitivas também têm sido descritas em modelos de infecção experimental pelo *T. cruzi*. Aumento do período de vigília e redução do sono foi reportado em ratos Wistar infectados com a cepa H4, durante fase aguda (60 dias pós-infecção, dpi) da infecção e alteração de memória de trabalho durante a fase aguda e crônica (40 e 90 dpi) (75). Na fase crônica de infecção de camundongos C57BL/6 ($H2^b$) com a cepa Colombiana do *T. cruzi*, anteriormente demonstramos a presença de comportamento semelhante à ansiedade, de forma independente de comportamento semelhante a doença ("sickness behavior"), como perda de peso e alteração de temperatura (76). Adicionalmente, mostramos que animais infectados apresentam depressão na fase crônica da infecção (35), sem a previa presença de neuroinflamação e meningoencefalite durante a fase aguda e na ausência de destas na fase crônica (33). Estes dados reforçam que a instauração e desenvolvimento de alterações comportamentais e neurocognitivas durante a fase crônica não seria uma consequência ou sequela do parasitismo e neuroinflamação descritos durante a fase aguda da infecção nestes modelos experimentais. Portanto, muito há para desvendar sobre fatores da relação parasito/hospedeiro que levam a alterações comportamentais e neurocognitivas na doença de Chagas, podendo modelos experimentais contribuir para tal. No nosso **artigo 1**, usamos modelo de infecção crônica de camundongos C57BL/6 pela cepa Colombiana do *T. cruzi* e exploramos a presença de alterações neurocognitivas e a relação com alterações neurológicas, presença do parasito e alterações intrínsecas ao SNC.

1.1.1. TRATAMENTO DA DOENÇA DE CHAGAS

Atualmente há dois medicamentos disponíveis para o tratamento etiológico da DC, benznidazol (Bz), que constitui o tratamento de primeira escolha e o nifurtimox (Nfx), eleição alternativa em casos de reações adversas severas ao tratamento com Bz (42, 46). O Bz e Nfx contêm um grupo nitro ligado a um anel imidazol (nitroimidazol) ou furano (nitrofurano), respectivamente. Ambos funcionam como pró-droga, ou seja, precisam ser ativados por redução para exercer efeitos citotóxicos no

parasito (77). Durante o processo de bioativação, radicais livres são formados, sendo que a nitroredução do Bz gera o radical livre hidronitroxido (77), porém a detecção deste radical ocorre apenas em concentrações muito superiores às necessárias para atingir o *T. cruzi*. Enquanto a nitroredução do Nfx gera o radical livre de nitroânion, que, na presença de oxigênio, leva à geração de intermediários reativos de oxigênio, gerando uma maior toxicidade que o Bz, pelo que sua comercialização foi descontinuada em alguns países (42, 77). Acredita-se que a toxicidade do Bz se deve à interação de seus metabólitos reativos com o DNA. Estes mecanismos podem estar envolvidos não apenas no efeito tripanossomicida nas formas intracelular e extracelular do parasito, mas também no efeito de toxicidade em mamíferos, de modo que a atividade antiparasitária está intimamente ligada à toxicidade no hospedeiro (77). Esta pode gerar diversas reações adversas, destacando-se a hipersensibilidade, dermatite com erupções cutâneas, edema febril generalizado, linfadenopatia, dor articular e muscular, depressão da medula óssea, agranulocitose, púrpura trombocitopenica, polineuropatia, parestesia e polineurite dos nervos periféricos (42). Como consequência, taxas variadas de reações adversas, entre 13,4% (78, 79) e 85,9% (80), têm sido reportadas, sendo a maioria leves. Além de taxas de 10-20% de interrupção do tratamento, como consequência das reações adversas em pacientes crônicos tratados com Bz (81). Atualmente, estudos clínicos avaliam estratégias terapêuticas que permitam a diminuição da dose com a consequente diminuição de reações adversas e aumento de tolerância e adesão ao tratamento (82-84).

O uso de BZ na fase crônica ainda é tema de debate, pois os benefícios clínicos são questionados. Ademais, BZ oferece eficácia elevada na fase aguda e crônica inicial da DC, com uma taxa de 60 a 80% de cura parasitológica estéril, enquanto que na fase crônica as probabilidades de cura parasitológica diminuem drasticamente (42, 46, 54). Embora o Bz consiga reduzir a carga parasitária sem agravar a doença, há resultados discordantes sobre o efeito benéfico em portadores de CCC (79, 85-88). O estudo clínico BENEFIT, com portadores crônicos da DC com a forma cardíaca, mostrou um efeito benéfico do Bz, com redução da carga parasitária em sangue durante a fase crônica e redução do número de hospitalizações por causas cardiovasculares. No entanto, este estudo não determinou um efeito benéfico na interrupção da progressão da doença em pacientes com CCC (79). Ainda, uma análise crítica da população estudada avaliou os portadores brasileiros que participaram do estudo BENEFIT. Esta análise mostrou

que Bz reduziu a proporção de pacientes com PCR positivo ao final do tratamento dos pacientes tratados no Brasil, que tiveram 86,3% de conversão negativa de PCR, versus 24,3% no grupo placebo (85). Portadores crônicos, sem alterações no eletrocardiograma e fração de ejeção normal, tratados com Bz tiveram um efeito protetor na progressão da doença (86). Adicionalmente, efeitos benéficos de Bz têm sido descritos em portadores crônicos da DC: (i) aumento dos níveis plasmáticos de IL-17 (89), uma citocina pro-inflamatória com papel protetor em modelos murinos experimentais da DC (90); (ii) preservação da função miocárdica (89) e (iii) diminuição da incidência de progressão da DC de portadores com a forma indeterminada para a forma cardíaca e também a uma diminuição do risco de eventos cardiovasculares (78).

Em estudos do nosso grupo, mostramos que a dose sub-ótima de Bz (25 mg/kg, ¼ da dose total), em camundongos cronicamente infectados, tem efeito na redução de alterações da forma cardíaca na fase crônica da infecção, como alterações elétricas e fibrose, de modo associado à redução da miocardite e do perfil inflamatório no tecido cardíaco, com redução de expressão de quimiocinas e citocinas pró-inflamatórias como IFN-γ (91, 92). De modo importante, na infeção crônica pelo *T. cruzi*, mostramos presença de alterações comportamentais semelhantes à depressão, com persistência do parasito e aumento de expressão de IDO. A terapia com Bz na fase aguda levou à eliminação dos parasitos circulantes e mostrou efeito benéfico no comportamento semelhante à depressão na fase crônica (35). No **artigo 1** da presente tese, utilizamos o modelo de infecção crônica de camundongos C57BL/6 ($H2^b$) pela cepa Colombiana do *T. cruzi* e exploramos o efeito do tratamento com Bz na carga parasitária e alterações intrínsecas ao SNC, relacionando com o impacto nas alterações neurocognitivas.

1.2. *Toxoplasma gondii* E TOXOPLASMOSE

O *Toxoplasma gondii* é o agente etiológico da toxoplasmose. Atualmente, o *T. gondii* atinge um terço da população mundial, com soroprevalência que varia de 0,8 a 94%, dependendo das condições ambientais e socioeconômicas, incluindo hábitos alimentares, higiene, suscetibilidade do hospedeiro e localização geográfica (31, 93, 94). Em países sul americanos como o Brasil e Colômbia a soroprevalência é alta, com taxas de até 92% e 93,75% da população brasileira e colombiana, respectivamente (93-95). Nos EUA, embora sejam reportadas taxas baixas de

soroprevalência de até 22,5% (96), o protozoário é considerado uma das principais causas de hospitalização (8%) e de morte (24%) atribuídas a doenças transmitidas por alimentos (97). Estes dados mostram a relevância de se estudar a relação parasito/hospedeiro na infecção pelo *T. gondii*.

O *T. gondii* foi descrito em 1908 por Splendore em São Paulo, Brasil, durante a avaliação de coelhos mortos a consequência de um quadro infeccioso agudo causado por um organismo desconhecido (98), e simultaneamente por Nicolle e Manceaux, na Tunísia. África, por um acaso, enquanto pesquisavam *Leishmania* em fígados do roedor *Ctenodactylus gundi*, que gerou o nome da espécie do protozoário (99). Desde a descoberta até os dias de hoje, diversos pesquisadores se destacaram por sua contribuição na descrição da patologia em humanos e animais que permitiram reconhecer a forma congênita da toxoplasmose, transmissão congênita, hospedeiros intermediários e definitivos e o ciclo de vida do parasito (100). Especialmente, foi demonstrada a colonização do SNC pelo parasito, associados a lesões necróticas e granulomatosas, no cérebro de uma criança recém nascida, que apresentava convulsões, dificuldade respiratória e sinais de comprometimento do coluna espinhal, que veio a óbito por causa de um quadro grave de encefalomielite, além de evidenciar que parasitos provenientes da criança, eram capazes de infectar animais inoculados experimentalmente (101).

T. gondii é classificado em três linhagens clonais predominantes, denominadas como tipo I, II e III, sendo que as cepas do tipo II são maiormente associadas à toxoplasmose humana, e cepa do tipo III apresenta uma alta frequência em animais. As cepas do tipo I são altamente virulentas, e as do tipo II e III são relativamente não virulentas. Além destas três linhagens clonais, genótipos que não se encaixam nesta descrição, têm sido reportados como genótipos exóticos ou atípicos. A linhagem tipo II é predominante na Europa e na América do Norte, e o tipo I e linhagens atípicas, mais virulentas, na América do Sul (102, 103) .

T. gondii apresenta três formas evolutivas infectantes. (i) oocistos, contendo esporozoítas, forma altamente infecciosa, produzidos exclusivamente no epitélio intestinal dos felídeos, (ii) taquizoítas, forma infecciosa de reprodução rápida, que se multiplica em diversos tipos celulares no hospedeiro intermediário e nas células epiteliais não intestinais do hospedeiro definitivo, responsáveis pela fase aguda da infecção (iii) cistos teciduais, contendo bradizoítas, forma infectante de reprodução lenta, que determinam a instauração da fase crônica da toxoplasmose (37). Este protozoário infecta praticamente todos os animais. Aves, mamíferos, répteis,

anfíbios, peixes, moluscos e humanos, agem como hospedeiros intermediários e reservatórios. Nestes hospedeiros ocorre apenas a reprodução assexuada do parasito. Por sua vez, os insetos e vermes podem agir como veículos de disseminação do parasita (37, 104). A reprodução sexuada ocorre exclusivamente nos felídeos, hospedeiros definitivos. Os gatos podem eliminar milhões de oocistos nas fezes, porém, os oocistos são eliminadospor curtos períodos, menos de três semanas, e menos de 1% da população de gatos pode ser encontrada liberando oocistos (37, 105).

Assim, a transmissão da toxoplasmose pode ocorrer por duas vias principais: (i) pela ingestão de oocistos eliminados nas fezes de felídeos, presentes em alimentos, água, solo, ou pela ingestão de cistos teciduais alojados em carne ou vísceras cruas ou malcozidas dos hospedeiros intermediários infectados. (ii) pela via vertical, por transmissão transplacentária dos taquizoítas. Taquizoítas podem ser encontrados em diversos órgãos e fluidos corporais. O parasito também pode ser transmitido por transplante de sangue ou órgãos de uma pessoa infectada (37).

A toxoplasmose apresenta duas fases: (i) uma rápida fase aguda, caracterizada pela pre sença de formas taquizoíta e (ii) uma fase crônica, definida pela presença de formas bradizoíta contidos em cistos teciduais, descrita como uma infecção latente, onde não há manifestação de sinais clínicos da infecção (37). O conceito de uma infecção latente, silenciosa, com cistos cerebrais que evadem o sistema imune é amplamente aceito (37). No entanto, este conceito vem sendo questionado e deve ficar obsoleto, pela constante demonstração de alterações comportamentais e neurocognitivas em pessoas imunocompetentes infectadas cronicamente (22-25, 29, 30, 106). Além da recente descrição do estabelecimento de uma resposta imune inata e adaptativa ativa, mediada por macrófagos e células T $CD8^+$, dependentes de perforina, capaz de eliminar cistos maduros, expondo assim, a inviabilidade do conceito de infecção crônica silenciosa (107).

Em pacientes imunocompetentes, a toxoplasmose geralmente cursa de forma assintomática ou com leves sinais clínicos, porém pode causar um quadro grave em crianças com infecção congênita. *T. gondii* é considerado um parasito oportunista em pacientes imunocomprometidos e pode causar altas taxas de morbidade e mortalidade nesta população. A infecção pode ser reativada em condições como HIV-1 podendo levar a severas encefalites que apresentam alta letalidade (108) Calcula-se que mundialmente existam 36,7 milhões de pessoas infectadas pelo HIV-1, e mais de 13 milhões de pacientes co-infectados com *T. gondii,* a maioria em

países de baixa renda (109). Embora o acesso universal à terapia antirretroviral (TAR), tenha contribuído na redução significativa da incidência de toxoplasmose cerebral em pacientes com HIV/AIDS, de países de alta e média renda, ainda há uma grande proporção de indivíduos infectados pelo HIV que se apresentam tarde ao atendimento clínico. Pacientes com doença avançada ao iniciar a TAR têm menos probabilidade de atingir supressão virológica, mais probabilidade sofrer eventos adversos e taxa de mortalidade mais alta, pelo que ao iniciar a TAR com contagens de CD4 baixas ou com um evento definidor de AIDS, existe uma maior probabilidade de progredir para estágios avançados de AIDS clínica, resposta imunológica fraca e morte (108). A reativação da doença, com encefalite toxoplásmica, é frequentemente caracterizada por déficits neurológicos subagudos, lesões focais cerebrais, convulsões generalizadas, com ou sem encefalite e pleocitose mononuclear do líquido cefalorraquidiano. Além disso, múltiplas lesões de baixa densidade na junção cortico-medular ou nos gânglios da base que aumentam após a administração de contraste intravenoso podem ser observadas por diagnóstico de imagens (108). Sequelas neurológicas, neurocognitivas e deficiência visual são frequentes em crianças com toxoplasmose congênita (110). Pacientes transplantados, portadores de neoplasias e usuários de terapia imunossupressora com corticosteroides ou drogas citotóxicas, também estão em alto risco de desenvolver encefalite, pneumonite, miocardite, e infecção disseminada pelo *T. gondii* devido ao estado de imunossupressão resultante da doença ou do comprometimento do sistema imunológico celular e humoral (108).

Mais recentemente, vem-se tentando estabelecer em pacientes com infecções latentes pelo *T. gondii* uma associação entre a soropositividade para toxoplasmose e alterações neurocognitivas e comportamentais como esquizofrenia, transtornos bipolar (22, 29), depressivo, obsessivo-compulsivo (22), mudanças de personalidade (106), tentativas de suicídio (23, 24), traços de agressividade, transtorno do sono, impulsividade (24), prejuízo cognitivo da capacidade de aprendizado e recordação (30) e maior taxa de envolvimento em acidentes de trânsito (25). Inclusive, IgG positiva na sorologia para *T. gondii* foi relacionada a casos mais graves de transtorno de déficit de atenção e hiperatividade (TDAH) em crianças (111). A infecção crônica pelo *T. gondii* em camundongos pode induzir alterações comportamentais e neurocognitivas. A atração à urina do gato, ou perda de medo especifica ao predador (felinos), é um paradigma clássico e a alteração mais amplamente conhecida, causada pelo *T. gondii*. Considerada como uma forma

de manipulação do parasitoa ao hospedeiro, como estratégia para uma reprodução e transmissão bem sucedida no hospedeiro definitivo e tem sido descrita por vários autores (112-114). Embora recentemente tenha sido documentado que esta alteração não é específica ao predador, podendo estar presente ante qualquer estímulo ao que o camundongo infectados seja exposto (39). Camundongos BALB/c, infectados com 10 cistos da cepa ME-49 mostraram deficiência no aprendizagem e na memória de curto prazo (115), prejuízo da memória espacial, foi observada em camundongos BALB/c infectados com 400 taquizoítas da cepa ME-49. Hiperatividade também foi observada nesta linhagem quando infectada com 400 taquizoítas da cepa PRU (114). Em camundongos C57BL/6 infectados com 10 cistos da mesma cepa, também foi observada alteração de memória especial, além de alteração do reconhecimento de novidade social e sensibilidade olfativa reduzida (116). Comportamento semelhante à ansiedade foi descrito em diversos modelos de infecção, usando linhagens de camundongos e cepas diferentes do parasito (39, 116-120). A perda de medo, como, por exemplo, a aversão ao odor de urina de felinos sendo convertida em atração, também foi reportada em camundongos BALB/c infectados com $5X10^5$ parasitos da cepa RHΔrop5 ou CEP (112).

Os dados acima mostram estudos em diferentes modelos (linhagens de camundongo e cepas do parasito) que reproduzem alterações comportamentais e/ou neurocognitivas, com aspectos semelhantes a alterações descritas em pessoas infectadas pelo *T. gondii*. Contudo, os processos biológicos associados a estas alterações comportamentais e neurocognitivas são ainda pouco elucidados. Diversos mecanismos têm sido propostos para explicar a origem destas alterações. Ingram *et al*, propuseram que a infecção pelo *T. gondii* causa mudanças permanentes no cérebro durante a infecção aguda, o que manteria as alterações comportamentais na fase crônica, ainda na ausência do parasito e após a resolução da neuroinflamação. Eles estabeleceram dois modelos de infecção crônica com cepas tipo I (RHΔrop5, não cistogênica) e tipo III (CEP, cistogênica) em camundongos BALB/c, e verificaram que ambos os modelos exibiam alterações comportamentais relacionadas com o medo (como descrito acima). Porém, além de não detectar parasitos no SNC, o perfil dos leucócitos cerebrais dos animais infectados pela cepa tipo I foi similar ao dos animais não infectados, em quanto que a cepa tipo III induziu uma alta carga parasitária e perfil inflamatório com predominância de células T $CD4^+$ e $CD8^+$, indicando que a alteração do medo é independente da neuroinflamação e da persistência do parasito no SNC (112). Outro

mecanismo proposto refere-se à localização dos cistos em regiões cerebrais especificas (121). Afonso *et al.,* desafiaram esta hipótese em camundongos C57BL/6 cronicamente infectados pela via intraperitoneal com 10.000 a 100.000 taquizoítas da cepa ME-49. Após o estabelecimento de alterações exploratórias e de medo (risco), foi analisada a localização dos cistos através de análise de componente principal (ACP), que relacionou as alterações comportamentais à presença dos cistos, porém condicionados a combinações especificas da distribuição dos cistos em diversas regiões do SNC que não puderam ser agrupadas segundo a função exercida pela região (34).

Outro aspecto relevante para a modulação da função neural na toxoplasmose se dá pela alteração observada nos níveis de dopamina, dentre outras alterações neuroquímicas (39, 122) . Foi descrito que *T. gondii* possui uma enzima ortóloga à tirosina hidroxilase, que é responsável pela conversão de tirosina em DOPA (123, 124). A infecção experimental por *T. gondii* em camundongos induz aumento no metabolismo de dopamina nas células neurais. Ademais, células neuronais dopaminérgicas (PC12), infectadas com o parasito, aumentam a produção de dopamina, associada diretamente à taxa de infecção (123). Níveis elevados de dopamina também são relacionados com o desenvolvimento da esquizofrenia em humanos (125). Por último, o silenciamento funcional dos neurônios, também foi proposto como um mecanismo indutor de alterações comportamentais. Haroon *et al,* demonstraram que taquizoítas podem manipular a sinalização de cálcio dos neurônios, resultando em neurônios hiper ou hipo-responsivos à estimulação por glutamato, o que se traduz na desregulação da função desta célula. Além, neurônios infectados por cistos perdem a sua capacidade funcional. O soma, dendritos e axônios, podem ser colonizados por cistos persistentes, que, a medida em que aumentam de tamanho, alteram a função neuronal pela alteração morfológica da célula (126).

Assim, nos nossos **artigos 2 e 3** exploramos as alterações comportamentais e neurocognitivas induzidas pelo *T. gondii* durante a infecção crônica, precoce e tardia, e possíveis vias biológicas que podem participar no desenvolvimento, como a carga parasitária, localização e tamanho dos cistos, perfil inflamatório sistêmico e intracerebral e a integridade da barreira hematoencefálica (BHE).

1.2.1. TRATAMENTO DA TOXOPLASMOSE

A combinação de sulfadiazina (S) e pirimetamina (P) (S+P) é o tratamento de primeira escolha para toxoplasmose (41). Como acontece com o Bz na DC (42), este tratamento oferece cura parasitológica na fase aguda da doença, quando ainda não existem cistos tissulares. Mas a eficácia diminui na fase crônica, sendo pouco eficaz na eliminação total dos cistos (110). Os cistos teciduais evitam a resposta imune do hospedeiro por diversos mecanismos e são resistentes às drogas atualmente disponíveis, permitindo que permaneçam no SNC por toda a vida do hospedeiro, em um estado que se acreditava aparentemente silencioso (127). Sulfadímicos (como sulfametoxazol), diaminopiridinas (trimetoprima), lincosaminas (clindamicina) e macrolídeos (azitromicina, claritromicina e espiramicina) podem ser utilizados como alternativas em casos de reações adversas ao regime S+P ou em condições que impossibilitam o uso do tratamento de primeira escolha, como na gravidez (41). O uso de corticosteroides para minimizar o dano tecidual causado pela inflamação é comum na toxoplasmose encefálica (128) e ocular (129). No entanto, deve ser utilizado com cautela, devido à possibilidade da ocorrência de toxoplasmose ocular fulminante após a aplicação de corticosteroides em monoterapia ou simultaneamente ao tratamento antiparasitário (130).

O principal alvo dos medicamentos anti-*T. gondii* é a via do folato, envolvida na síntese de DNA com as enzimas dihidrofolato redutase (DHFR) e dihidropteroato sintetase (DHPS). P e a trimetoprima agem na DHFR do parasito, porém não consegue distingui-la da DHRF humana. A monoterapia destes inibidores da síntese de ácido folínico não é eficaz ou potente contra o parasito, e age em sinergismo com sulfonamidas, que bloqueiam a DHPS (41, 110). Ainda é desconhecido o mecanismo de ação da S, mas acredita-se que seja através de alterações do metabolismo do hospedeiro (131). S+P são inibidores da síntese de DNA em taquizoítas de *T. gondii,* mas podem inibir a síntese de DNA na medula óssea e epitélio, pelo que é recomendado a administração junto ao ácido folínico (41). Consequentemente, a terapia com S+P apresenta altas taxas de reações adversas, que requerem interrupção ou mudança da estratégia terapêutica (110, 132). Atualmente há novas propostas terapêuticas sendo estudadas (120, 133). Todavia, o tratamento com S+P em pacientes com AIDS, com encefalite toxoplásmica apresentam diminuição na taxa de mortalidade e no desenvolvimento de hernia cerebral (134). Assim mesmo, em crianças e bebês com toxoplasmose congênita o

tratamento combinado S+P garante um normal desenvolvimento e preservação da função intelectual (135).

A terapia S+P tem sido amplamente estudada em modelos murinos, desde as perspectivas de sobrevivência (136-138), alterações histopatológicas (139), e carga parasitária em diversos órgãos afetados (137, 138, 140). No **artigo 3,** avaliamos o tratamento etiológico como um possível mecanismo adjuvante no controle intrínseco imunomediado dos cistos (107), e o seu impacto nas alterações comportamentais e neurocognitivas e a relação com a carga de cistos, neuroinflamação, integridade da BHE e níveis séricos de citocinas, durante a infecção crônica experimental de longo prazo pelo *T. gondii*. Neste estudo fizemos também uma análise estatística de múltiplas variáveis, Análise de Componentes Principais, (PCA), que permitiu o reconhecimento de padrões dos animais NI, infectados tratados com veículo e infectados tratados com S+P, além de correlacionar alterações neurocognitivas e comportamentais com a carga parasitaria e níveis elevados de citocinas periféricas.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Investigar se as infecções crônicas por *Trypanosoma cruzi* ou *Toxoplasma gondii* causam alterações comportamentais e neurocognitivas em modelos murinos, buscando replicar aspectos das doenças humanas, e a relação entre as alterações destas com a presença de parasito e a inflamação no sistema nervoso central.

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2.2. OBJETIVOS ESPECÍFICOS

1. Investigar se a infecção crônica experimental por *Trypanosoma cruzi* em camundongos C57BL/6 causa alterações comportamentais e neurocognitivas;
2. Investigar se a infecção crônica experimental por *Toxoplasma gondii* em camundongos C57BL/6 causa alterações comportamentais e neurocognitivas;
3. Determinar se as alterações comportamentais e neurocognitivas nestes modelos de infecção crônica estão relacionadas à presença de neuroinflamação;
4. Investigar a contribuição da presença de parasitos no sistema nervoso central nas alterações comportamentais e neurocognitivas, através da intervenção terapêutica com medicamentos tripanossomicida ou toxoplasmicida em modelos murinos cronicamente infectados;
5. Avaliar o perfil inflamatório sistêmico durante as infecções crônicas por *T. cruzi* ou *T. gondii*, buscando correlacionar a presença destas à ocorrência de alterações comportamentais e neurocognitivas.

3. RESULTADOS

Artigo 1. Memory impairment in chronic experimental chagas disease: benznidazole therapy reversed cognitive deficit in association with reduction of parasite load and oxidative stress in the nervous tissue. Vilar-Pereira*, G., L. Castaño Barrios*, A. A. d. Silva, A. Martins Batista, I. Resende Pereira, O. Cruz Moreira, C. Britto, H. A. Mata Dos Santos and J. Lannes-Vieira (2021). PloS one 16(1): e0244710-e0244710.

***** Contribuiram igualmente para este trabalho

Neste artigo, inicialmente, buscamos na literatura aspectos de alterações cognitivas em portadores da doença de Chagas (DC), revisto na introdução. Assim, buscamos abordar nossos objetivos 1, 3, e 4. Nele, descrevemos a presença de alterações cognitivas da memória aversiva, memória de reconhecimento do novo objeto e memória de habituação ao campo aberto na infecção crônica experimental, que se estabelecem de forma progressiva. Mostramos a resposta à terapia etiológica da DC, indicando o efeito benéfico de benznidazol (Bz) nestas alterações e em alterações biológicas a elas associadas. Avaliamos se camundongos C57BL/6 fêmeas cronicamente infectadas com a cepa Colombiana tipo I do *T. cruzi* replicam algumas alterações observadas em humanos. A função neurocognitiva dos animais foi avaliada após a cronificação da infecção (120 dias pós-infecção, dpi), determinada por controle da parasitemia. Para verificar o efeito do medicamento tripanossomicida benznidazol (Bz) na função cognitiva, os animais foram tratados por 30 dias consecutivos (de 120 a 150 dpi) por gavagem com um quarto (25mg/kg) da dose recomendada ou com veículo (Veh, água apirogênica) e submetidos aos testes comportamentais. Ademais, buscamos esclarecer bases biológicas, pesquisando a relação entre as alterações neurocognitivas descritas, carga parasitária e estresse oxidativo no SNC. Em 120 dpi, animais infectados pelo *T. cruzi* têm preservação da capacidade muscular, podendo realizar os testes. Eles apresentam comprometimento da memória de reconhecimento de objetos, comparados a animais não infectados (NI), mas não de outros aspectos cognitivos (memória de habituação e aversiva). A análise em 150 dpi, mostrou que a terapia com Bz reverteu o prejuízo da memória no reconhecimento de objetos e impediu a instauração da alteração de memória de habituação e perda de memória aversiva, o que é observado em animais que receberam Veh. Análise histopatológica foi realizada para avaliar o status inflamatório do cérebro no modelo crônico da infecção. Como esperado, os cérebros dos animais infectados que receberam Veh estavam desprovidos de neuroinflamação. Somente raras células mononucleares perivasculares estavam presentes nas meninges e no plexo coróide. A terapia com Bz aboliu a presença destas células inflamatórias no SNC e reduziu a carga parasitária no hipocampo e no córtex cerebral. Os níveis de peroxidação lipídica mostraram-se aumentados em extratos cerebrais de córtex e hipocampo de animais cronicamente infectados (120 e 150 dpi) e, de modo importante, houve redução destes níveis no córtex após a terapia com Bz. Assim, mostramos que a terapia com Bz melhorou a perda de memória, associada à redução da carga parasitária e do estresse oxidativo no SNC na DC experimental. Nossos dados sugerem que a implementação da terapia com Bz na fase crônica da infecção pode proporcionar melhora na qualidade de vida dos portadores da doença de Chagas.

PLOS ONE

RESEARCH ARTICLE

Memory impairment in chronic experimental Chagas disease: Benznidazole therapy reversed cognitive deficit in association with reduction of parasite load and oxidative stress in the nervous tissue

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Abstract

Memory impairment has been associated with chronic Chagas disease (CD), a neglected tropical disease caused by the protozoan parasite *Trypanosoma cruzi*. In degenerative diseases, memory loss has been associated with increased oxidative stress, revealed as enhanced lipid peroxidation, in the cerebral cortex. Benznidazole (Bz), a trypanocidal drug efficient to reduce blood parasite load in the acute and chronic phases of infection, showed controversial effects on heart disease progression, the main clinical manifestation of CD. Here, we evaluated whether C57BL/6 mice infected with the Colombian type I *T. cruzi* strain present memory deficit assessed by (i) the novel object recognition task, (ii) the open field test and (iii) the aversive shock evoked test, at 120 days post infection (dpi). Next, we tested the effects of Bz therapy (25mg/Kg/day, for 30 consecutive days) on memory evocation, and tried to establish a relation between memory loss, parasite load and oxidative stress in the central nervous system (CNS). At 120 dpi, *T. cruzi*-infected mice showed memory impairment, compared with age-matched non-infected controls. Bz therapy (from 120 to 150 dpi) hampered the progression of habituation and aversive memory loss and, moreover, reversed memory impairment in object recognition. In vehicle-administered infected mice, neuroinflammation was absent albeit rare perivascular mononuclear cells were found in meninges and choroid plexus. Bz therapy abrogated the infiltration of the CNS by inflammatory cells, and reduced parasite load in hippocampus and cerebral cortex. At 120 and 150 dpi, lipid peroxidation was increased in the hippocampus and cortex tissue extracts. Notably, Bz therapy reduced levels of lipid peroxidation in the cerebral cortex. Therefore, in

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experimental chronic *T. cruzi* infection Bz therapy improved memory loss, in association with reduction of parasite load and oxidative stress in the CNS, providing a new perspective to improve the quality of life of Chagas disease patients.

1. Introduction

Chagas disease (CD), a neglected tropical disease caused by the intracellular protozoan parasite *Trypanosoma cruzi*, afflicts 7–8 million people worldwide, most of them born and resident in Latin America [1]. The 2nd Consensus on Chagas disease estimates that 1.9 to 4.6 million individuals are infected with *T. cruzi* in Brazil. Ten to thirty years after infection, 20 to 30% of the patients progress to the cardiac form and 5 to 10% to the digestive form of CD [2]. Although a nervous form has been proposed by Carlos Chagas [3], the involvement of the central nervous system (CNS) in CD remains a matter of debate, mainly due to the lack of histopathological evidence [4, 5]. The non-recognition of a nervous form of CD hampers the adoption of more specific approaches, therapeutic strategies, and proposal of consensus to treat CD patients with behavioral alterations. Several studies described CNS impairment and behavioral alterations in chronic CD patients, attributed to left ventricular heart dysfunction, resulting in brain ischemia by hypoperfusion and/or embolic events [6]. A higher frequency of cognitive changes has been observed in patients with Chagas' heart disease, when compared to patients with other cardiac diseases [7]. *Post-mortem* analysis of the CNS of CD patients showed cerebral and cerebellar atrophy without neuroinflammation [8]. Later, a computed tomography-based study revealed that the cerebral atrophy was independent of a structural cardiac disease, raising the idea that brain atrophy may represent the main anatomical substrate of cognitive impairment in CD patients [9]. An association between *T. cruzi* infection and cognitive abnormalities has been detected using the mini-mental state examination score to test elderly CD patients, which were not mediated by CD-related electrocardiographic alterations or digoxin medication [10]. Furthermore, cognitive dysfunction in chronic CD patients has been supported by deficiency in orientation, attention, non-verbal reasoning, information processing and learning [11, 12]. More import, memory loss has been described in chronic chagasic infection [13–15].

Acute and chronically *T. cruzi*-infected rats present memory deficit [16]. Previously, we have described that in chronically *T. cruzi*-infected C57BL/6 mice depressive-like behavior, anxiety and motor coordination disorder occur independently of sickness behavior and neuromuscular disorder [17, 18]. The present study was carried out to evaluate the presence of memory impairment in chronically *T. cruzi*-infected mice, using the (i) memory habituation test in the open field, (ii) novel object recognition memory task and (iii) aversive shock evoked test. Further, we challenged the contribution of parasite load in the CNS in behavioral alterations, treating chronically infected mice with the trypanocidal drug benznidazole (Bz). Lastly, we investigated the impact of chronic *T. cruzi* infection and Bz therapy on the grade of CNS tissue lipid peroxidation, a biomarker of oxidative stress associated with memory dysfunction in a neurodegenerative disorder [19, 20].

2. Materials and methods

2.1 Ethics statement

The experimental procedures were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of

Animal Experimentation (https://www.mctic.gov.br/mctic/opencms/institucional/concea/) and the federal law 11.794 (8 October 2008). The Ethical Commission on Animal Use of Fiocruz and Oswaldo Cruz Institute/Fiocruz (licenses LW10/14 and L006/2018) approved all the procedures used in this study. All presented data were obtained from three independent experiments (Experiment Register Book #53 and Book #73, LBI/IOC-Fiocruz).

2.2 Experimental groups, infection by *Trypanosoma cruzi*, clinical follow-up and obtaining of brain tissue

Experimental check list is described in Author´s Check List (S1 Checklist). Details of experimental protocol are described in Fig 1. Female of 5-7-week-old mice (total of 92 mice) of the C57BL/6 strain (H-2^b) were supplied by the Institute of Science and Technology in Biomodels (ICTB) of the Oswaldo Cruz Foundation (Fiocruz) and housed in the Experimental Animal Facility (CEA-CF unit/IOC). Immediately after arrival, mice were housed in polypropylene cages with Pinus sawdust, randomically grouped in 3–5 mice per cage (experiment 1) or 5 mice per cage (experiments 2 and 3). The cages were maintained in microisolators, and mice received grain-based chaw food and water *ad libitum*. To minimize stress, mice were kept in adaptation for 10–14 days in a plastic igloo-enriched cage, in conditions free of specific pathogens, with light and noise control. After adaptation, mice were infected, treated, and analyzed according to the experimental protocols (Fig 1). Groups were composed as follow: **Exp 1** – time point pre-therapy–analysis at 120 dpi (total 12 mice): non-infected controls (5 mice); *T. cruzi*-infected (7 mice); **Exp 2** (total 40 mice): Part 1– time point pre-therapy (analysis at 120 dpi; total 15 mice)—non-infected controls (5 mice); *T. cruzi*-infected (10 mice); Part 2 –time point post-therapy (analysis at 150 dpi; total 25 mice)—non-infected controls (5 mice); vehicle-treated *T. cruzi*-infected (10 mice); Bz-treated *T. cruzi*-infected (10 mice); **Exp 3** (total 40 mice): repetition of Exp 2. These groups were formed at mice arrival when cages were numbered and randomically sorted for the experimental infection, analysis at 120 dpi or for

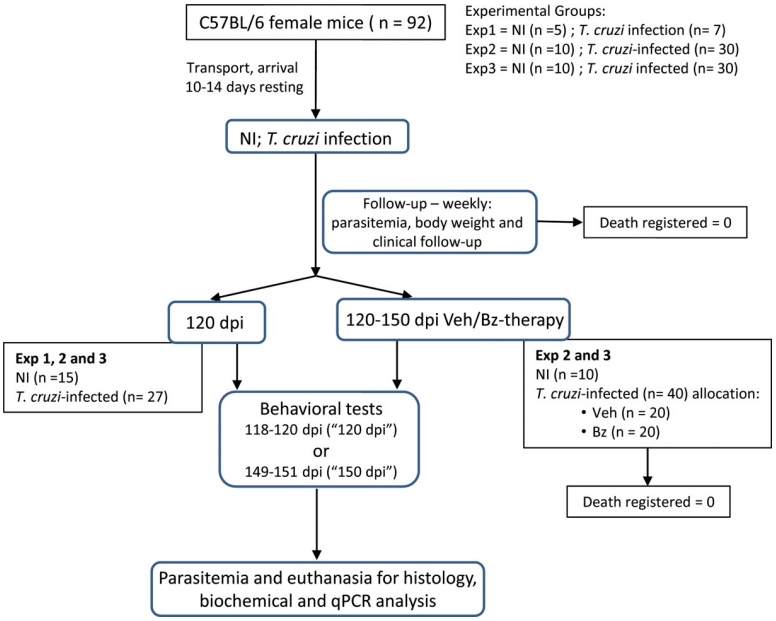

Fig 1. Flow chart showing the experimental protocol with the number of animals used, death registered, and mice included in 3 (endpoint registered as "120 dpi") or 2 (endpoint registered as "150 dpi") independent experiments.

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treatments. The experimental procedures were performed by different investigators (cited here as initials): housing, cage numbering and experimental group sorting: JLV and IRP; infection: JLV; drug treatments: IRP; mice follow-up and behavior tests: GVP and LCB; Tissue obtaining: GVP, LCB, IRP, JLV; Tissue analysis: AAS, AMB, OCM, HAMS; Data analysis: GVP, LCB, AAS, AMB, IRP, OCM, CB, HAMS, JLV.

Mice were infected intraperitoneally with 100 trypomastigote forms of the Colombian strain suspended in 0.2 mL of sterile saline buffer. The trypomastigote forms used were isolated from a chronic CD patient, currently classified as Type I *T. cruzi* strain [21] and kept by serial passages in C57BL/6 female mice each 35 days, at the Laboratory of Biology of the Interactions (LBI/IOC). Parasitemia was performed weekly, as previously described [22]. Weekly, death was recorded, and clinical signs analyzed. For clinical follow-up we evaluated external physical conditions (piloerection, apathy, prostration, mobility, posture, aggressive behavior, pain), and body weight loss (which reveals loss of appetite) was assessed using a rodent weighing scale (Sartorius scale, ED623S-OCE, USA). Negative parasitemia (35 to 45 dpi) and any death outside of planned euthanasia or humane endpoints were the exclusion criteria. The humane endpoints criteria were body weight loss ($\geq$ 30% of the initial weight), injuries from fights, pain, posture, ataxia and immobility. According to the experimental protocols, mice were euthanized at the endpoints (120 and 150 dpi), using CO_2 inhalation in an appropriate chamber allowing 70% of CO_2 saturation for 2–3 minutes, followed by decapitation. The encephala were collected and weighted. Cortex and hippocampus were dissected [23], RNA later (AM7021, Thermo Fisher Scientific, USA) was added, and the tissues immediately stored at -80C for neurochemical assays: lipid peroxidation by evaluation of tissue levels of thiobarbituric acid reactive species and parasite load determination by quantitative PCR.

2.3 Benznidazole treatment

Groups of 7–10 infected animals were treated daily, from 120 to 150 dpi, by gavage with 0.1 mL of vehicle (Veh) apyrogenic vaccine-graded water (BioManguinhos, Fiocruz, Brazil) or 0.1 mL of Veh containing 25 mg/Kg of the trypanocidal medicament Bz (LAFEPE, Brazil), previously shown to be effective in controlling parasitemia and parasitism [24]. As controls, 3–10 sex- and age-matched non-infected (NI) mice were kept concurrently and submitted to Veh administration.

2.4 Conditions of behavioral evaluation

All behavioral tests were conducted between 8:00 am-3:00 pm and recorded using a DSC-DVD810 video camera (Sony, USA). To minimize stress and increase familiarity, all behavioral tests applied to different experimental groups were performed in an environment provided with 12 hours of light and 12 hours of dark cycle at a room temperature of 22 ± 2˚C and noise level of approximately 40 dB produced by an air conditioner. Different groups of animals were submitted to behavioral tests from 118–120 dpi (referred as 120 dpi) or 149–151 dpi (referred as 150 dpi). No animals were re-tested. When possible, the animals were reused in different tests, aiming to reduce the number of animals used. Behavioral tests were performed from less stressful tests to the more stressful (habituation memory test, novel object recognition memory test, passive aversive shock evoked test, grip strength meter test).

2.5 Habituation memory test

To evaluate the habituation memory, we used the open field apparatus, a white acrylic arena measuring 60 cm x 60 cm. The floor of the apparatus was divided by black grid lines into 49 squares of approximately 8.5 cm each and two imaginary areas—the periphery (40 squares

along the walls) and center (9 squares in the central area of the apparatus). In the training session, the animals were carefully placed in the rear left square of the device and left to explore the environment freely for 5 min. Immediately after this time, the animals were returned to the housing cages. The long-term memory test was performed 24-hours after the training, in which the procedure was repeated, and the 5 min session was recorded using a digital video camera (Sony, USA). The apparatus was cleaned with 70% alcohol and dried with gauze between tests. The memory retention was evaluated by counting the number of total lines crossed on the test session [25] and the individual baseline differences were corrected using the change ratio score to compare behavior during initial and final sessions, as follows: number of crossed lines day 2 /(number of crossed lines day 1 + number of crossed lines day 2), as previously described [26].

2.6 Novel object recognition memory task

The one-trial learning or one-trial object recognition task consisted of a sample trial of 5 min duration, in which mice explore two equal objects in a 60 cm x 60 cm open field arena (the same used for the habituation memory test). After a 24-hours intersession interval, a novel object is presented together with one familiar object already explored during the sample trial, for a 5 min duration test session. The apparatus and objects were cleaned with a solution of 70% alcohol and dried to eliminate the odor and trace of the previously tested mouse. This test is based on the innate preference of non-infected normal rodents to explore the novel object rather than the familiar one [27]. This kind of test is derived from the "visual paired-comparison paradigm" used in human and non-human primates and may allow interspecies comparison [28]. The discrimination index (DI) was calculated as follows: time exploring the novel object/(time exploring the novel object + time exploring the familiar object), as previously shown [29].

2.7 Aversive shock evoked test

The procedure was modified from the previously described test [30]. Briefly, the inhibitory avoidance apparatus (EP 104MR, Insight, Brazil) consisted of a 35 x 28 x 50 cm epoxy-painted 2 mm aluminum box with acrylic front door, whose floor consisted of parallel stainless-steel bars (3 mm diam.) spaced 1 cm apart. A 7 cm wide x 2.5 cm high platform was placed on the floor of the box against the wall of the righthand side. In a pre-exposure session, animals were placed on the platform and allowed to explore the box freely for 5 min without foot shock. A training session was carried out 2 hours after pre-exposure, followed by a test session 24-hours after training. For training, animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. In training sessions, immediately after stepping down on the grid, the animals received a 3.0-sec scrambled foot shock (0.6 mA), and one to five stimulations were required for memory acquisition. In test sessions, no foot shock was administered, and the step-down latency (maximum 120 sec) was used as a measure of memory retention. An increase in step-down latency during test was taken as an index of improved memory and vice versa.

2.8 Grip strength meter test

Muscle strength was assessed using the grip strength meter (EFF 305, Insight, Brazil), according to manufacturer's instructions. This noninvasive method, which may disclose a neuromuscular disorder, is based on the natural tendency of mice to grab a horizontal metal bar when slightly pulled by the tail for 2–3 seconds. The bar is attached to a force transducer which

measures the traction peak (in gram-force) that is displayed on a digital screen. Data are presented as mean of strength intensity = gram-force (gf)/body weight (g).

2.9 Histopathology

At 150 dpi, non-infected and *T. cruzi*-infected mice were euthanized, as described in item 2.2. The encephalon was removed, fixed in buffered formalin 10%, dehydrated and embedded in paraffin. Five μm-thick sections were prepared, stained with hematoxylin and eosin and two sections per encephalon tissue were blindly examined using light microscopy. Representative images were digitized using a Sight DS-U3 color-view digital camera adapted to an Eclipse Ci-S microscope and analyzed with the digital morphometric apparatus NIS Elements BR version 4.3 software (Nikon Co., Japan).

2.10 Determination of parasite load in the CNS by quantitative PCR (qPCR)

Mice were euthanized and encephalon removed and dissected, as described in item 2.2. DNA was extracted from dissected cerebral cortex and hippocampus samples using 1 mL of TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA) for each tissue (20 mg), 200 μL of chloroform (Merck, USA) were added and incubated for 2 min. After centrifugation at 12,000 x g at 4˚C for 15 min, the organic phase and interface were obtained, and the DNA was precipitated by adding 300 μL of 100% ethanol (Merck, USA) and incubated for 3 min. The material was centrifuged at 2000 x g for 5 min at 4˚C. The supernatant was discarded, and the DNA pellet was washed twice in 1 mL of a solution containing 0.1 M sodium citrate (Sigma-Aldrich, St. Louis, MO, USA) in 10% ethanol: incubation for 30 min at room temperature with periodic stirring and centrifuged at 2000 x g for 5 min at 4˚C. The DNA pellet was suspended in 1.5 mL of 75% ethanol and incubated for 15 min at room temperature with periodic shaking. After centrifugation at 2000 x g for 5 min at 4˚C, the supernatant was removed, and the DNA was dried for 10 min. Next, the DNA was dissolved in 100 μL of 8 mM NaOH (Sigma-Aldrich, St. Louis, MO, USA). Centrifuged at 13000 x g for 10 min and the supernatant aliquoted. The qPCR assays were performed by absolute quantification to estimate the parasite load, based on a standard curve produced from DNA samples extracted from fragments of mouse cortex or hippocampus artificially contaminated with *T. cruzi*. For this, 20 mg of cortex or hippocampus samples (uninfected mouse) were spiked with 10^5 *T. cruzi* trypomastigotes, and DNA was extracted as described. Serial dilutions of 1:10 were carried out using Tris-EDTA buffer to produce the standard curve, ranging from 10^5 to 1 parasite equivalents, and from 20 to 2×10^{-4} mg mouse cortex or hippocampus equivalents. The qPCR assays were carried out with 5 μL of DNA, using the FastStart Universal Probe Master Mix (Roche Diagnostics, Mannheim, Germany) in a final volume of 20 μL. The amplifications were carried out in a ABI 7500 Fast Real Time PCR system (Applied Biosystems, USA) using 750 nM of Cruzi 1 (Sequence: `5 'AST CGG CTG ATC GTT TTC GA 3′`) and Cruzi 2 (Sequence: `5 'AAT TCC TCC AAG CAG CGG ATA 3'`) primers, 50 nM of Cruzi 3 probe (Sequence: `5′-FAM CAC ACA CTG GAC ACC AA- NFQ-MGB-3′`). Concurrently, a TaqMan assay targeting mouse GAPDH (Glyceraldehyde-3-phosphate dehydrogenase VIC/TAMRA-labelled, Mm99999915g1, Thermo Fisher Scientific, USA) was used to quantify the brain tissue mg equivalents, according to manufacturer´s instructions. Thus, the parasite load was normalized by the tissue mg equivalents, by dividing the *T. cruzi* quantity by the mass of mice tissue equivalents (cortex or hippocampus). PCR cycling conditions were:

95˚C for 10 min, followed by 40 cycles at 95˚C 15s and 58˚C for 1min. To analyze the results, the threshold was set at 0.02.

2.11 Lipid peroxidation evaluation in the CNS

Mice were euthanized and encephalon removed and dissected, as described in item 2.2. The tissue levels of thiobarbituric acid reactive species (TBARS) in the cortex and hippocampus were determined by Ohkawa's method [31]. Malondialdehyde (MDA) is one of several low-molecular-weight end products of degradation of hydroperoxides and lipid peroxides formed during the oxidation of fatty acids [19, 32]. MDA reacts with thiobarbituric acid (TBA) to form a pink-colored dimeric compound. Dissected cortex and hippocampus were homogenated. Ten percent (w/v) of tissue homogenate was mixed with 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid pH 3.5 and 0.8% TBA, and incubated at 95˚C for 1 hour. After incubation, the reaction product was extracted with n-butanol (1:1) and read using a spectrophotometer (Spectra Max M5, Molecular Devices, USA) at a wavelength of 532 nm.

2.12 Statistical analysis

The sample size was determined based on the experience of our group and previous studies using the model of experimental chronic chagasic cardiomyopathy; therefore, no formal sample size was calculated. The data of 3 independent experiments were grouped. Data are expressed as arithmetic means ± standard error (SE). Statistical comparisons between groups were carried out by analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. All statistical tests were performed with GraphPad Prism 8.0 (La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Muscular strength is preserved in C57BL/6 mice chronically infected with the Colombian *Trypanosoma cruzi* strain

Out of 92 C57BL/6 female mice, 25 were non-infected (NI) controls and 67 were infected with 100 trypomastigote forms of the Colombian strain of *T. cruzi* and weekly monitored for parasitemia, body weight and clinical follow-up (Fig 1). In the first set of experiments, mice were analyzed at 120 dpi (Fig 2A) when 100% of them were alive (Fig 2B), and no mouse was excluded by death outside of planned euthanasia or humane endpoint criteria. At 120 dpi, NI controls and infected mice presented similar physical characteristics and body weight (S1A Fig; 22.7 ± 0.70 in NI mice *vs* 20.6 ± 2.9 in *T. cruzi*-infected mice; $p > 0.05$). After 15 dpi, all infected mice showed circulating parasites, blood parasitism peaked at 42–45 dpi, and at 120 dpi parasitemia was controlled (Fig 2C). In comparison with sex- and age-matched NI controls, *T. cruzi*-infected mice showed no muscular strength alteration ($p > 0.05$; Fig 2D). Therefore, chronically Colombian-infected C57BL/6 mice were able to perform behavioral tests based on mobility and body activity.

3.2 Memory dysfunctions and brain atrophy are detected in chronically *Trypanosoma cruzi*-infected mice

Habituation memory was assessed using the open field test and comparing the performance in the first and second day of testing. At 120 dpi, *T. cruzi*-infected mice and NI controls showed similar discrimination index ($p > 0.05$; Fig 3A), supporting that habituation memory was preserved. However, the performance of *T. cruzi*-infected mice in the novel

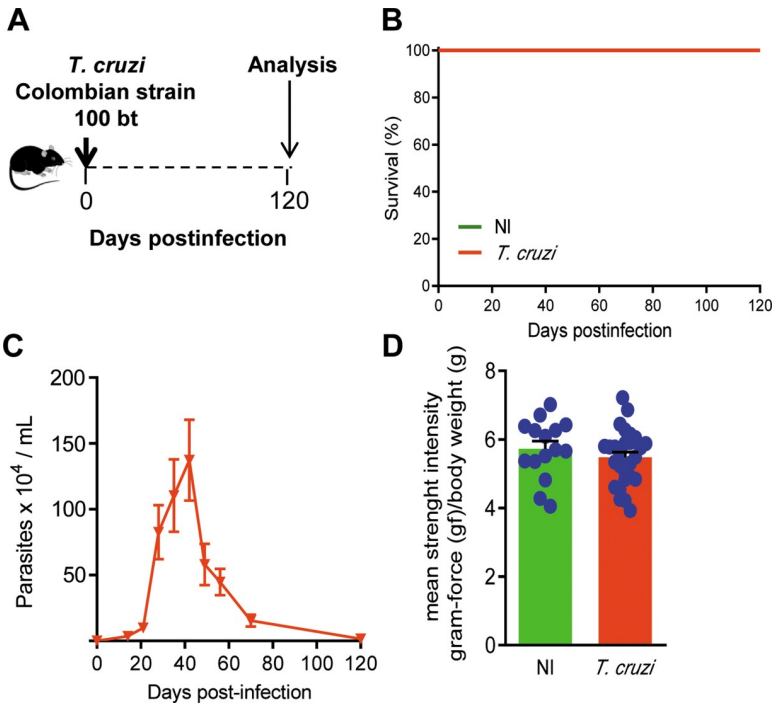

Fig 2. C57BL/6 mice infected with the Colombian *Trypanosoma cruzi* strain control parasitemia, survive, and develop chronic phase with preserved muscle strength. (**A**) Mice were infected with 100 blood trypomastigote forms, parasitemia and death were recorded weekly, and the animals were analyzed in the chronic phase of infection (120 dpi). (**B**) Survival curve (percentage of alive mice). (**C**) Parasitemia curve (Parasites x 10^4/mL). (**D**) The graph shows the results of muscle strength [gram force (gf)/body weight (g)] of *T. cruzi*-infected mice compared with sex- and age-matched noninfected controls (NI), at 120 dpi. Each experimental group consisted of 5 NI mice and 7–10 *T. cruzi*-infected mice. Each circle represents an individual mouse. Data are represented as means ± SE of three independent experiments (15 NI; 27 *T. cruzi*-infected mice). Data were analyzed using *t*-Student test.

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object recognition test revealed reduced discrimination index, supporting loss of object recognition memory (Fig 3B). At 120 dpi, similar numbers of stimulation were required for aversive memory acquisition in the training day (2.5 ± 0.34 in NI mice *vs* 1.9 ± 0.48 in *T. cruzi*-infected mice; $p > 0.05$). Interestingly, the aversive memory was preserved in most of the *T. cruzi*-infected mice (81%), though in 19% of them latency was reduced in the aversive shock evoked test (Fig 3C). At 120 dpi, no difference was detected in body weight of *T. cruzi*-infected mice, compared with NI controls (S1A Fig). However, reduced brain weight was observed in chronically *T. cruzi*-infected mice, in comparison with age-matched NI controls (Fig 3D), suggesting the presence of encephalon atrophy in chronic experimental chagasic infection.

3.3 Benznidazole therapy beneficially impacts on memory loss in chronically *Trypanosoma cruzi*-infected C57BL/6 mice

Chronically infected mice were treated with 25 mg/Kg/day of Bz from 120 to 150 dpi (Fig 4A) to evaluate the impact of Bz therapy on memory loss onset and progression. Survival was of 100% in the groups of Veh- and Bz-treated mice, and no mouse showed sickness scores for humane endpoint. At 150 dpi, Bz therapy did not impact body weight (S1A Fig), when compared to NI controls and Veh-treated infected and likened to *T. cruzi*-infected mice pre-therapy (at 120 dpi). At 120 and 150 dpi, *T. cruzi*-infected mice

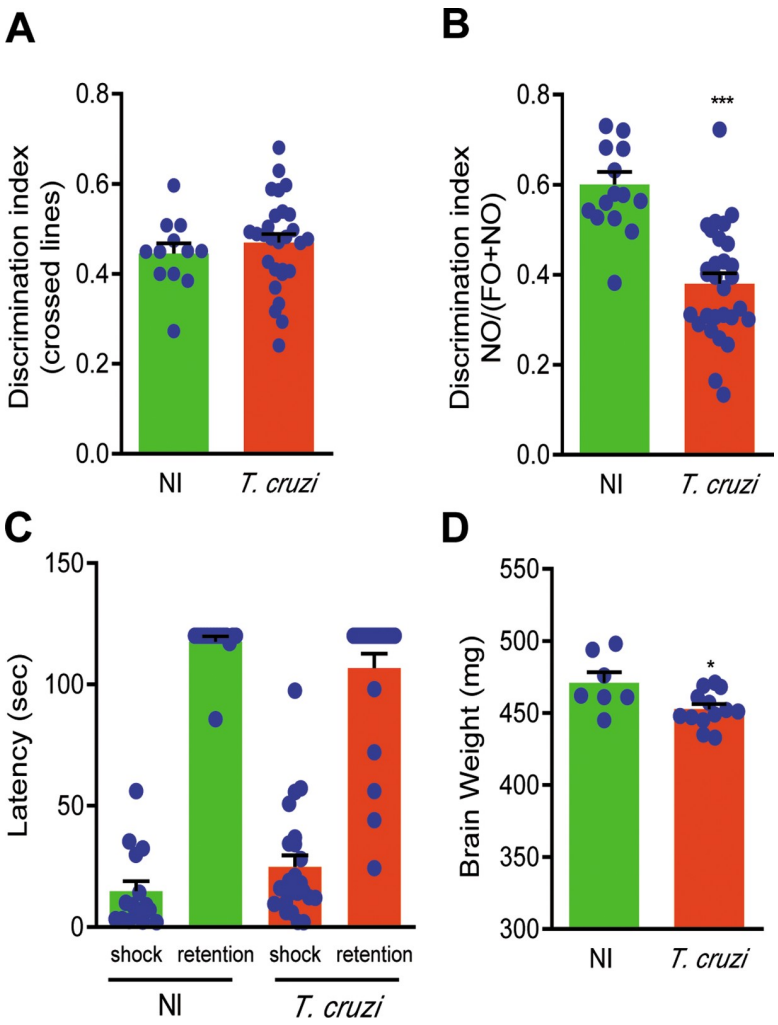

Fig 3. Memory loss in chronically *Trypanosoma cruzi*-infected C57BL/6 mice. Mice were infected with 100 blood trypomastigote forms of the Colombian *T. cruzi* strain and analyzed at 120 dpi, comparing with noninfected controls (NI). (**A**) The graph shows the discrimination index [number of crossed line day 2 /(number of crossed line day 1 + number of crossed line day 2)] assessed by the open field test used to evaluate habituation memory. (**B**) The graph shows the discrimination index [time exploring the novel object/(time exploring the novel object + time exploring the familiar object)] evaluated by the novel object recognition test object applied to assess recognition memory. (**C**) The graph shows the latency (second; sec) measured using the aversive shock evoked test to evaluate aversive memory. (**D**) The graph shows the encephalon weight (mg). Each experimental group consisted of 5 NI mice and 7–10 *T. cruzi*-infected mice. Each circle represents an individual mouse. Data are represented as means ± SE of three independent experiments (15 NI; 27 *T. cruzi*-infected mice). Data were analyzed using *t*-Student test (**A**, **B** and **D**) and ANOVA-Bonferroni posttest (**C**). *, $p<0.05$, ***, $p<0.001$, comparing *T. cruzi*-infected and NI mice.

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presented increased relative spleen weight, and Bz administration reduced this splenomegaly (S1B Fig), confirming the effectiveness of this therapy [24]. At 150 dpi, in comparison with sex- and age-matched NI controls, memory loss was detected in Veh-treated *T. cruzi*-infected mice submitted to the open field test (Fig 4B), novel object recognition task (Fig 4C) and shock evoked test (Fig 4D). Bz therapy initiated at 120 dpi prevented the habituation memory impairment (Fig 4B). Importantly, Bz treatment reversed the memory loss assessed by novel object recognition memory task (Fig 4C). The aversive memory loss already detected in a low number of mice (19%) at 120 dpi, progresses to a larger number of mice (34%) at 150 dpi, indicating progressive decline of aversive memory.

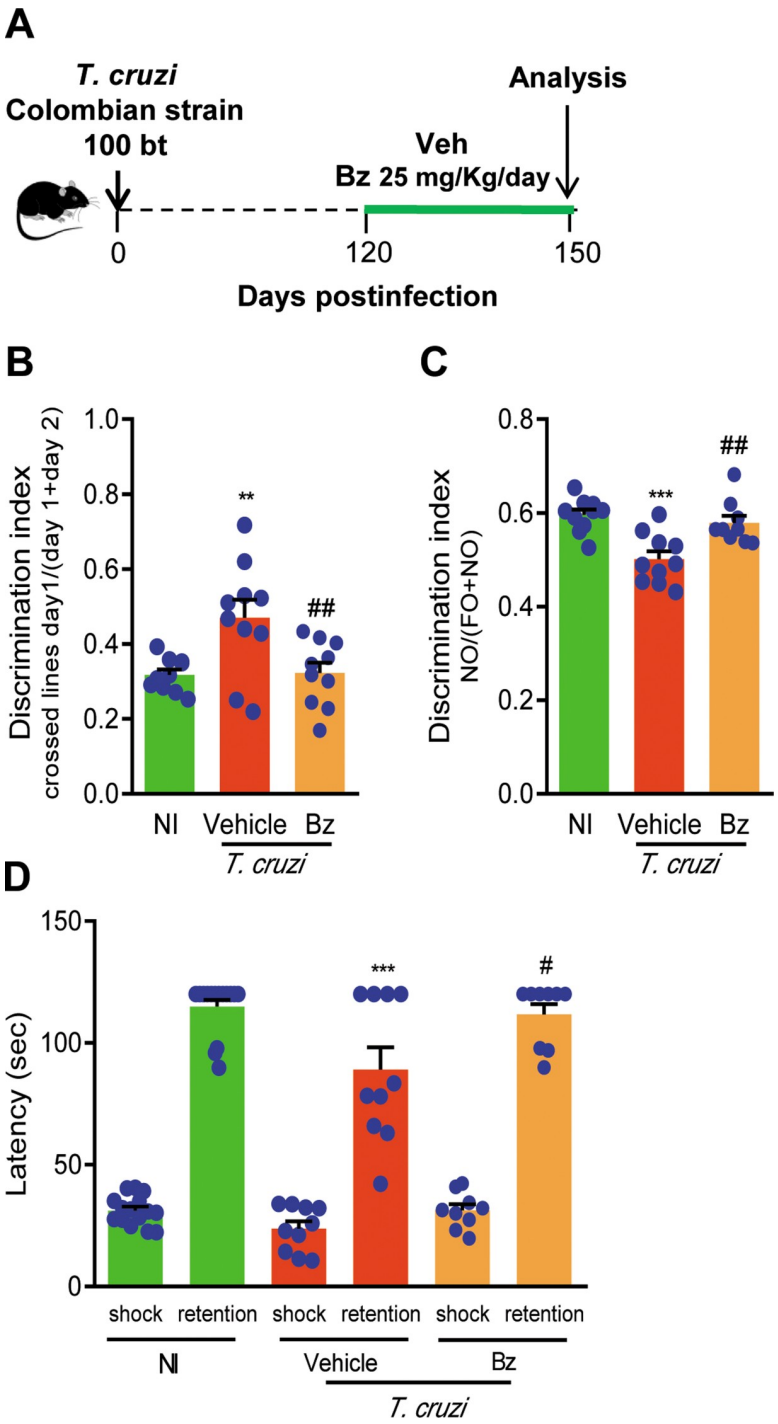

Fig 4. Memory loss is improved by benznidazole administration to chronically *Trypanosoma cruzi*-infected C57BL/6 mice. (**A**) Mice were infected with 100 blood trypomastigote forms of the Colombian *T. cruzi* strain and benznidazole (Bz, 0.1 mL gavage; 25 mg/Kg/day) or pyrogen-free water (Veh, 0.1 mL gavage) were administered for 30 consecutive days, from 120 to 150 dpi, when mice were tested. (**B**) The graph shows the discrimination index [number of crossed line day 2 /(number of crossed line day 1 + number of crossed line day 2)] assessed by the open field test used to evaluate habituation memory. (**C**) The graph shows the discrimination index [time exploring the novel object/(time exploring the novel object + time exploring the familiar object)] evaluated by the novel object recognition test. (**D**) The graph shows the latency (second; sec) measured using the aversive shock evoked test to evaluate aversive memory. Each experimental group consisted of 10 NI mice and 10 *T. cruzi*-infected mice. Each circle represents an individual mouse. Data are shown as means ± SE and represent two independent experiments. Data were analyzed

using ANOVA-Bonferroni posttest. **, $p<0.01$ and ***, $p<0.001$, comparing *T. cruzi*-infected and NI mice; #, $p<0.05$ and ##, $p<0.01$, comparing Bz-treated and Veh-treated *T. cruzi*-infected.

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Notably, Bz therapy initiated at 120 dpi hampered aversive memory loss at 150 dpi (Fig 4D). Therefore, Bz administration to chronically infected mice beneficially affected memory loss.

3.4 CNS tissue architecture is preserved in chronically *Trypanosoma cruzi*-infected C57BL/6 mice

At 150 dpi, the CNS structures were preserved in age-matched NI control mice. At 150 dpi, the CNS structures were also well-preserved and no signs of tissue damage as hemorrhage and parenchymal blood vessels inflammatory cuffs were detected, though rare inflammatory infiltrates were restricted to meninges in Veh-treated mice (Fig 5A). Importantly, the CNS tissue was devoid of neuroinflammation, as shown in tissue sections of brain areas as cortex, hippocampus, and cerebellum (Fig 5A). Further, Bz therapy did not modify the CNS tissue architecture in chronically *T. cruzi*-infected C57BL/6 mice and, moreover, abrogated

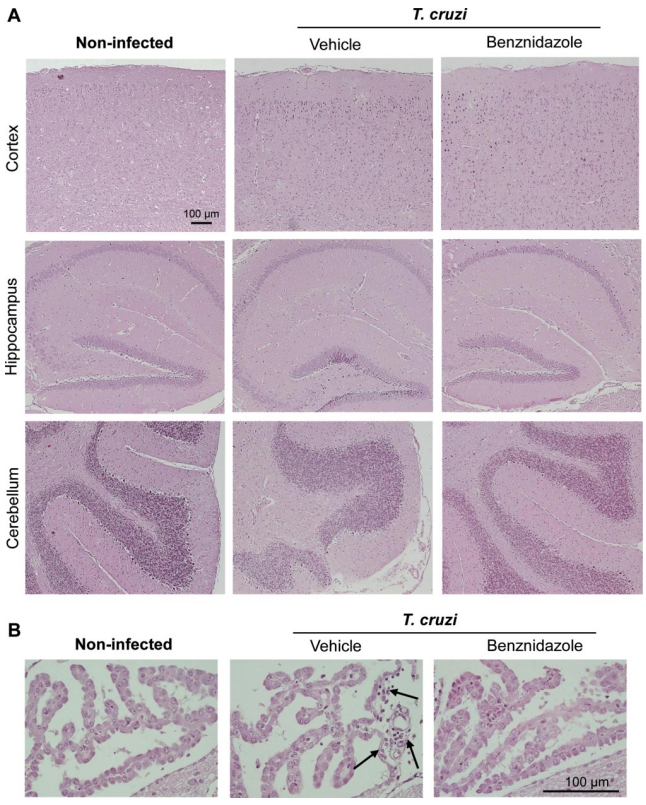

Fig 5. CNS tissue architecture is preserved in chronically *Trypanosoma cruzi*-infected C57BL/6 mice. Mice were infected with 100 blood trypomastigote forms of the Colombian *T. cruzi* strain and benznidazole (Bz, 0.1 mL gavage; 25 mg/Kg/day) or pyrogen-free water (Veh, 0.1 mL gavage) were administered for 30 consecutive days, from 120 to 150 dpi, when mice were euthanized, tissue collected and fixed. Tissue sections were stained with hematoxylin and eosin. (**A**) Representative sections of cerebral cortex, hippocampus, and cerebellum areas of noninfected, and Veh-treated and Bz-treated *T. cruzi*-infected mice. Bar = 100 μm. (**B**) Representative sections of choroid plexus areas of noninfected, and Veh-treated and Bz-treated *T. cruzi*-infected mice. Bar = 100 μm. Arrows show infiltration of rare mononuclear inflammatory cells in perivascular areas.

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the sporadic inflammatory infiltrates in meninges (Fig 5A). At 150 dpi, Veh-treated *T. cruzi*-infected mice showed blood vessels with a few mononuclear leukocytes and rare inflammatory cells infiltrating the choroid plexus, when compared with age-matched NI controls. Again, inflammatory cells were not detected in the choroid plexus of Bz-treated infected mice (Fig 5B).

3.5 Therapeutic intervention with benznidazole reduces parasite load in the CNS of chronically *Trypanosoma cruzi*-infected C57BL/6 mice

Next, we challenged the influence of parasite load in the CNS in behavioral alterations in chronically infected mice. As expected, 30 days of Bz administration (from 120 to 150 dpi) to chronically Colombian-infected C57BL/6 mice efficiently reduced parasitemia (Fig 6A). Parasite DNA was detected and quantified in the hippocampus and cerebral cortex of chronically *T. cruzi*-infected C57BL/6 mice (Fig 6B and 6C). Notably, in comparison with Veh administration, Bz therapy reduced parasite load in the hippocampus and cerebral cortex of infected mice (Fig 6B and 6C).

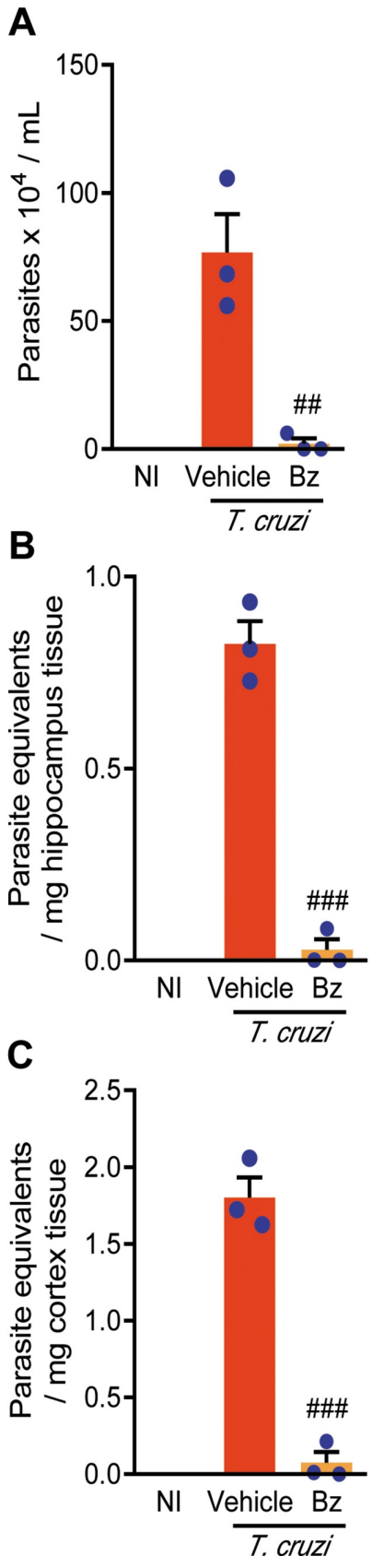
3.6 Therapeutic intervention with Bz decreases oxidative stress in the cerebral cortex of chronically infected mice

To provide additional mechanistic insights on the beneficial effects of Bz therapy on memory loss, we analyzed the presence of TBARS, as a biomarker of lipid peroxidation and oxidative stress, in the CNS of chronically *T. cruzi*-infected mice. At 120 dpi (pre-therapy point) and 150 dpi (end point of therapy) CNS were collected, dissected, and analyzed. At both time points, increased TBARS levels were detected in the hippocampus and cerebral cortex of infected mice, when compared with NI controls (Fig 7A–7B). A representative experiment shows that Bz therapy reduced TBARS levels in the extracts of hippocampus in 2 out of 3 tested infected mice (Fig 7A). Importantly, Bz therapy significantly reduced TBARS levels in the extracts of cerebral cortex of *T. cruzi*-infected mice (Fig 7B), supporting that Bz therapy reduced the abnormal lipid peroxidation in the CNS of chronically infected mice.

4. Discussion

In the present study, we showed that chronically *T. cruzi*-infected mice present deficits of spatial habituation, novel object recognition and aversive memory recall, in the absence of neuroinflammation but with *T. cruzi* parasite persistence and increased lipid peroxidation in the CNS hippocampus and cortex. Importantly, Bz therapy interfered beneficially with cognitive impairment in a way related to reduction of parasite load systemically and in the CNS. Further, Bz-therapy decreased the levels of lipid peroxidation in the cerebral cortex.

As consequence of successful interventions targeting vector transmission, most of the CD patients in Latin America are over 45-years old [2] and may be vulnerable to aging-borne behavioral abnormalities [20, 33]. The influence of *T. cruzi* infection on behavioral alterations, particularly on memory loss, is questioned. Importantly, cognitive and memory impairments have been described in chronic CD patients, mainly detected as disturbs of comprehension and perception, orientation and attention loss, as well as impaired capacity to answer to visual-paired comparison tests [11–15]. However, it is a controversial matter as immediate and delayed memory impairment were not detected in patients with Chagas' heart disease, the main clinical form of CD [7]. Herein, we questioned whether *T. cruzi* infection may impact memory/learning process in an experimental model of chronic CD. To approach our


A
Parasites x 10^4 / mL
150
100
50
0
##
NI
Vehicle
Bz
T. cruzi
B
Parasite equivalents
/ mg hippocampus tissue
1.0
0.5
0.0
###
NI
Vehicle
Bz
T. cruzi
C
Parasite equivalents
/ mg cortex tissue
2.5
2.0
1.5
1.0
0.5
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NI
Vehicle
Bz
T. cruzi

Fig 6. Benznidazole therapy reduces parasitemia and parasite load in the central nervous system of chronically *T. cruzi*-infected C57BL/6 mice. Mice were infected with 100 blood trypomastigote forms of the Colombian *T. cruzi* strain and benznidazole (Bz, 0.1 mL gavage; 25 mg/Kg/day) or pyrogen-free water (Veh, 0.1 mL gavage) were administered for 30 consecutive days, from 120 to 150 dpi, when parasitemia was analyzed, mice were euthanized, tissue collected, DNA extracted and qPCR performed. (**A**) Parasitemia levels. Group data for qPCR detection of *T. cruzi* satDNA in (**B**) hippocampus and (**C**) cerebral cortex. Each experimental group consisted of 3 NI mice and 3 randomly sorted *T. cruzi*-infected mice per experimental group. Each circle represents an individual mouse. Data are shown as means ± SE and represent two independent experiments. Data were analyzed using ANOVA-Bonferroni posttest. $^{\#\#}$, $p<0.01$ and $^{\#\#\#}$, $p<0.001$, comparing Bz-treated and Veh-treated *T. cruzi*-infected.

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questions, C57BL/6 mice were infected with low inoculum of blood trypomastigotes of the Colombian type I *T. cruzi* strain, which allowed immune response to control parasite, host survival and development of the chronic phase of infection [18, 22]. Further, in comparison with age-matched non-infected controls, chronically infected mice did not display neuromuscular disorders assessed by grip strength meter test, and, therefore, suitable as an experimental model to address our issues. Non-infected C57BL/6 mice preserved the abilities to establish and evoke habituation, novel object recognition and aversive shock-evoked memory. However, in age-matched Colombian-infected mice, object recognition memory was impaired, while habituation memory was preserved, at 120 dpi. At this moment, only a low frequency (19%) of mice showed aversive memory impairment, while at 150 dpi object recognition and habituation memories were disrupted, and the frequency of mice with aversive memory impairment was increased (34%). Dissociated memory loss has been reported as a traumatic brain injury outcome, with novel object recognition memory deficit without affecting habituation and aversive memory [34]. Moreover, long-term memory evocation tests showed that in chronically *T. cruzi*-infected mice disturbs in object recognition, habituation and aversive memory tends to be progressively established, mimicking aspects of memory loss in CD

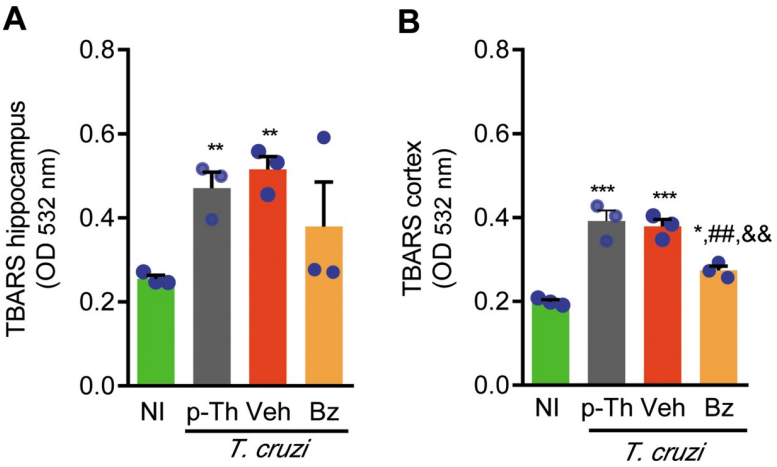

Fig 7. Therapeutic intervention with Bz decreases oxidative stress in cerebral cortex of chronically infected mice. Mice were infected with 100 blood trypomastigote forms of the Colombian *T. cruzi* strain and benznidazole (Bz, 0.1 mL gavage; 25 mg/Kg/day) or pyrogen-free water (Veh, 0.1 mL gavage) were administered for 30 consecutive days, from 120 to 150 dpi, when mice were euthanized, tissue collected, extracts prepared and TBARS revealed the oxidative stress marker MDA in (**B**) hippocampus and (**C**) cerebral cortex. Each experimental group consisted of 3 NI mice and 3 randomly sorted *T. cruzi*-infected mice per experimental group. Each circle represents an individual mouse. Data are shown as means ± SE and represent two independent experiments. Data were analyzed using ANOVA-Bonferroni posttest. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, comparing *T. cruzi*-infected and NI mice; $^{\#\#}$, $p<0.01$, comparing Bz-treated and Veh-treated *T. cruzi*-infected. $^{\&\&}$, $p<0.01$, comparing Bz-treated and *T. cruzi*-infected pre-therapy (p-Th; 120 dpi).

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patients [11–15]. This progressive decline of memory is a common feature of mnemonic deficits in aging and neurodegenerative disorders [20, 33]. It is important to note that these cognitive abnormalities were simultaneous with cerebral atrophy, as described in patients with chronic CD [9]. Therefore, *T. cruzi* infection may trigger and/or accelerate an aging-associated process of memory decline, here well reproduced in C57BL/6 mice chronically infected with type I parasite strain.

The association of cognitive impairment and CD is biologically plausible, as severe Chagas' heart disease frequently shows thromboembolism and congestive heart failure [2]. Conditions leading to oxygen deprivation and injury of the CNS have been associated with behavioral alterations and memory loss [35, 36]. The relation between cerebrovascular resistance evaluated by transcranial Doppler and worse cognitive scores suggests that microembolism may be responsible for a significant proportion of cognitive symptoms in chagasic and non-chagasic patients with congestive heart failure [37]. Acute *T. cruzi* infection induces cerebral microvasculopathy, with increased leukocyte-endothelium interaction (rolling and adhesion) and microvascular platelet-leukocyte aggregates [38, 39]. However, in chronically Colombian-infected mice, the CNS blood vessels are not activated, being refractory to cell adhesion [39]. Nevertheless, the contribution of heart disease to memory impairment cannot be ruled out as the Colombian-infected C57BL/6 mice present chronic chagasic cardiomyopathy with electrical abnormalities and reduced ventricular function [22, 40], thus limiting the interpretation of our findings. However, in a group of chronic CD patients cognitive impairment was associated with *T. cruzi* infection, but not mediated by CD-related electrocardiographic abnormalities [10], supporting that these could be dissociated processes.

Administration of the trypanocide drug Bz to chronically infected CD patients reduces parasite load in blood and does not aggravate the disease. However, Bz therapy presents controversial results, with beneficial or no measurable effects, in Chagas' heart disease outcome [41–45]. Bz therapy initiated after parasitemia control, in the early chronic phase of *T. cruzi* infection, reduced the severity of chronic heart disease at 8 months of infection [46]. When Bz was administered to chronically infected mice to reverse heart disease, the beneficial effects were restricted to improve average heart rate and reduce the frequency of mice with arrhythmias and second-degree atrioventricular blockage [24]. Importantly, administration of the full recommended dose of Bz (100 mg/Kg/day) in the acute phase of experimental Colombian infection prevented depressive-like behavior in the chronic phase of infection [17]. Here, we showed that memory deficits described in chronically infected mice were beneficially impacted by Bz therapy. The administration of reduced Bz dosage, one fourth of the recommended dose [24], initiated at 120 dpi for short period (30 consecutive days) prevented the habituation memory impairment. Moreover, Bz therapy reversed object recognition memory loss and hampered progression of the aversive memory impairment. Therefore, it is the first demonstration that Bz administration ameliorated and, even more, reversed cognitive abnormalities.

In CD patients, the cognitive impairment lacks histopathological evidence and occurs in the absence of neuroinflammation [8, 9]. Nonexistence of neuroinflammation is also a hallmark of experimental chronic *T. cruzi* infection [39, 47]. Here, we bring evidence that in Colombian-infected C57BL/6 mice, memory impairment occurs in the absence of inflammation in the CNS cortex, hippocampus, and cerebellum, crucial areas for memory functions [33, 48]. In the adopted experimental model of chronic infection of C57BL/6 mice, rare inflammatory cells were restricted to meninges and choroid plexus, corroborating studies using the Colombian *T. cruzi* strain for the acute infection of Swiss mice and the chronic infection of C3H/He mice [39, 47, 49, 50]. Although in low numbers and restricted to meninges and choroid plexus, one cannot rule out the participation of these infiltrating mononuclear cells and

their products, as cytokines and chemokines, in the cognitive impairment detected in chronically *T. cruzi*-infected mice.

The *T. cruzi* parasite DNA was detected in the CNS hippocampus and cortex areas of chronically Colombian-infected mice. Previously, low-grade parasitism was revealed as *T. cruzi*-antigen positive cells randomically scattered throughout the CNS parenchyma of acute and chronically infected mice [39, 47, 50]. Further, in HIV-infected immunosuppressed chronic CD patients the CNS is the main site of parasitism reactivation [51]. Thus, these data support parasite persistence in the CNS tissue in chronic *T. cruzi* infection in humans and experimental models, being able to contribute to behavioral alterations, as memory dysfunction. In another neuroinfection, caused by the intracellular parasite *Toxoplasma gondii*, behavioral changes as psychomotor alterations and decreased memory performance have been described and related with parasite persistence in the CNS [52]. Therefore, we asked the contribution of parasite persistence in the CNS to memory impairment in the chronic phase of experimental infection, using Bz as trypanocide drug. Previously, we have shown that Bz therapy in the acute *T. cruzi* infection prevented depressive-like behavior in early chronic infection [17], supporting the idea that *T. cruzi* infection may trigger behavioral alterations. Here, our data support that administration of a low dose of Bz for a short period to chronically *T. cruzi*-infected mice showed beneficial effects on memory loss, as prevented habituation memory impairment, reversed the object recognition memory loss, and hampered progression of aversive memory disruption. These beneficial effects were associated with decreased parasitemia, supporting the systemically action of Bz, and reduced parasite load in the hippocampus and cerebral cortex areas, showing the effectiveness of Bz in the CNS parasite control. In the BENEFIT study, in which chronic cardiac CD patients were treated with Bz or placebo, it was shown that the reduction in blood parasite load did not significantly affect the clinical deterioration of the patients [43]. Bz administration to chronically Colombian-infected C57BL/6 mice reduced parasitemia and heart parasite load but showed limited beneficial effects on cardiac disease [24]. Interestingly, in a model of septic shock Bz showed down-regulatory effect on tumor necrosis factor (TNF) production selectively linked to the nuclear factor NF-kB and mitogen-activated protein kinase (MAPK) pathways [53]. Further, Bz administration to chronically *T. cruzi*-infected mice also reduced TNF expression in the heart tissue [24]. Astrocyte-born TNF may contribute to *T. cruzi* persistence mainly in astrocytes, as parasite-positive spots in the CNS [47, 54]. However, in the CNS TNF expression may be restricted to these parasite-positive areas, where TNF may be rapidly consumed. Since TNF upregulation was undetectable in the CNS of the Colombian-infected mice [17], the effects of Bz on TNF expression in the brain tissue could not be explored. Therefore, besides the direct effect on intracellular parasite forms, the beneficial effects of Bz in reducing the CNS parasite load may reside in disrupting the autocrine cytokine production and, therefore, TNF signaling. Notably, the administration of anti-TNF and pentoxiphyline, a modulator of TNF receptor 1 (TNFR1) expression, to chronically *T. cruzi*-infected mice supports that signaling via TNF/TNFR1 may take part in depressive-like behavior [17]. Thus, a point to be further explored is the contribution of TNF/TNFR signaling in *T. cruzi* infection-associated memory loss. Crucially, there is an emerging comprehension of how dysregulation of cytokine networks is associated with neurodegenerative diseases and memory impairment in humans and animal models [55].

Multiple or sequential processes may contribute to memory deficits in chronically *T. cruzi*-infected mice. In Alzheimer disease, behavioral changes have been associated with oxidative stress, characterized as augmented lipid peroxidation in the cerebral cortex [19, 32]. Oxidative damage induces a cascade of downstream reactive oxygen species, some transient and other

downstream substances that accumulate in the CNS as malondialdehyde, a TBARS that may be detected in tissue extracts [56]. In mice acutely infected with type II *T. cruzi* strain, reduced response of the CNS endothelial cells to acetylcholine associated to enhanced detection of TBARS, was restricted to the parasitemia peak [38]. Further, in acutely *T. cruzi*-infected Swiss mice, aversive memory impairment has been shown in association with oxidative stress, revealed as increased cerebral acetylcholinesterase activity [57]. Here, we showed in chronically infected mice a relation of memory loss and enhanced oxidative stress, revealed as increased TBARS in the CNS hippocampus and cortex areas pre-therapy (120 dpi) and in vehicle-treated (150 dpi) mice. In the CNS, this increase in oxidative stress may result of the action of the parasite directly leading to cell death or indirectly inducing production of inflammatory mediators into this tissue. However, one could not exclude the leakage of reactive substances into the CNS parenchyma, since in chronically *T. cruzi*-infected rats increased TBARS in plasma may unveil a systemic oxidative stress [58]. Administration of Bz (40 mg/Kg/day) elicited oxidative stress and decreased antioxidant machinery in rat hepatocytes, which may contribute to Bz toxicity [59]. However, it does not seem to be the case in our study, since the levels of TBARS were not increased in the hippocampus and showed a significant reduction in the cerebral cortex after 30 consecutive days of Bz therapy, when memory deficits were beneficially impacted. These conflicting data may be explained as Bz is metabolized in liver, the brain is less permeable to orally administered Bz, we used a model of chronic *T. cruzi* infection in mouse and administered a low dose of Bz, which may reduce the toxic effects of this trypanocidal drug, in accordance with our initial goal [24, 60].

In conclusion, our results support that the nervous form of chronic CD occurs in the absence of neuroinflammation but associated with parasite persistence and increased oxidative stress in the CNS, showing as clinical outcome behavioral changes as long-term memory impairment. Although with some limitations, mostly related to the molecular levels of Bz action in the CNS, our present findings open a new pathway to be explored using Bz in the chronic phase of CD not only aiming to treat the cardiac form, but also to ameliorate cognitive disorders and improve the quality of life of CD patients.

Supporting information

S1 Checklist. The arrive essential 10: Author checklist.
(PDF)

S1 Fig. Beneficial effect of benznidazole administration on splenomegaly of chronically *Trypanosoma cruzi*-infected C57BL/6 mice. C57BL/6 mice were infected with 100 blood trypomastigotes of the Colombian strain of *T. cruzi*, and treated with Veh or Bz, as described in legend of Fig 3. (**A**) Body weight (g). (**B**) Relative spleen weight (mg/g). Data represent two independent experiments with 3 NI controls and 5 infected mice per group. Each circle represents an individual mouse. Data are shown as means ± SE and were analyzed using ANOVA--Bonferroni posttest. *, $p<0.05$ and ***, $p<0.001$, comparing *T. cruzi*-infected and NI mice; $^{\#\#}$, $p<0.01$, comparing Bz-treated and Veh-treated *T. cruzi*-infected.
(TIF)

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Supervision: Joseli Lannes-Vieira.

Validation: Leda Castaño Barrios.

Writing – original draft: Glaucia Vilar-Pereira, Leda Castaño Barrios.

Writing – review & editing: Joseli Lannes-Vieira.

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ARTIGO 2. Behavioral alterations in long-term *Toxoplasma gondii* infection of C57BL/6 mice are associated with neuroinflammation and disruption of the blood-brain barrier. Castaño Barrios L, Da Silva Pinheiro AP, Gibaldi D, Silva AA, Machado Rodrigues e Silva P, *et al*. (2021). PLOS ONE 16(10): e0258199. https://doi.org/10.1371/journal.pone.0258199

Após estudo da literatura, neste artigo trazemos dados que contribuem para atender aos nossos objetivos 2, 3, e 5. Descrevemos a presença de alterações comportamentais induzidas pela infecção crônica precoce e de longa duração em camundongos e procuramos estabelecer a relação entre as alterações observadas e a carga de cistos (número e tamanho) e localização nas diferentes áreas do sistema nervoso central (SNC), integridade da barreira hematoencefálica (BHE) e os níveis séricos de citocinas e expressão intracerebral. Inicialmente, estabelecemos o modelo de infecção crônica. Para tal, fêmeas de C57BL/6 ($H2^b$) foram infectadas por gavagem com 5 cistos da cepa cistogênica ME-49 do tipo II do *T. gondii.* A cinética da infecção foi avaliada em 30, 60 e 90 dias pós-infecção (dpi), evidenciando a presença de alteração semelhante à ansiedade, comportamento do tipo depressivo e hiperatividade na infecção crônica precoce (30 dpi) e de longa duração (60 e 90 dpi). Estas alterações foram associadas à presença de cistos do parasito, independentemente do tamanho e da localização no SNC. As alterações comportamentais foram relacionadas à neuroinflamação e à maior permeabilidade da BHE. Observamos que estas alterações comportamentais são paralelas ao aumento da expressão de TNF e CC-quimiocinas (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β e CCL5 RANTES) no tecido cerebral. Além disso, níveis aumentados de IFNγ, TNF e CCL2/MCP-1 foram detectados no soro, aos 30 e 60 dpi. Assim, propomos que neste modelo a persistência de cistos do parasito induz neuroinflamação sustentada e ruptura da BHE, permitindo o extravasamento de citocinas do plasma circulante para o tecido cerebral. Múltiplos fatores podem contribuir para as mudanças comportamentais na infecção crônica pelo *T. gondii* e abordagens terapêuticas devem considerar estas múltiplas facetas.

PLOS ONE

RESEARCH ARTICLE

Behavioral alterations in long-term *Toxoplasma gondii* infection of C57BL/6 mice are associated with neuroinflammation and disruption of the blood brain barrier

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Abstract

The Apicomplexa protozoan *Toxoplasma gondii* is a mandatory intracellular parasite and the causative agent of toxoplasmosis. This illness is of medical importance due to its high prevalence worldwide and may cause neurological alterations in immunocompromised persons. In chronically infected immunocompetent individuals, this parasite forms tissue cysts mainly in the brain. In addition, *T. gondii* infection has been related to mental illnesses such as schizophrenia, bipolar disorder, depression, obsessive-compulsive disorder, as well as mood, personality, and other behavioral changes. In the present study, we evaluated the kinetics of behavioral alterations in a model of chronic infection, assessing anxiety, depression and exploratory behavior, and their relationship with neuroinflammation and parasite cysts in brain tissue areas, blood-brain-barrier (BBB) integrity, and cytokine status in the brain and serum. Adult female C57BL/6 mice were infected by gavage with 5 cysts of the ME-49 type II *T. gondii* strain, and analyzed as independent groups at 30, 60 and 90 days postinfection (dpi). Anxiety, depressive-like behavior, and hyperactivity were detected in the early (30 dpi) and long-term (60 and 90 dpi) chronic *T. gondii* infection, in a direct association with the presence of parasite cysts and neuroinflammation, independently of the brain tissue areas, and linked to BBB disruption. These behavioral alterations paralleled the upregulation of expression of tumor necrosis factor (TNF) and CC-chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES) in the brain tissue. In addition, increased levels of interferon-gamma (IFNγ), TNF and CCL2/MCP-1 were detected in the peripheral blood, at 30 and 60 dpi. Our data suggest that the persistence of parasite cysts induces sustained neuroinflammation, and BBB disruption, thus allowing leakage of cytokines of circulating

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plasma into the brain tissue. Therefore, all these factors may contribute to behavioral changes (anxiety, depressive-like behavior, and hyperactivity) in chronic *T. gondii* infection.

1. Introduction

The protozoan parasite *Toxoplasma gondii* is the etiologic agent of toxoplasmosis [1, 2]. Currently, this infection afflicts a third of the world population, with a seroprevalence ranging from 0.8 to 92%, depending on the region and habits of the populations [3, 4]. For instance, in Brazil and USA the seroprevalence is of 92% and 22.5%, respectively [4, 5]. The parasite may invade any cell type and infect all body tissues showing tropism for the central nervous system (CNS) [3, 6]. The acute phase of *T. gondii* infection is characterized by presence of tachyzoite forms and the chronic phase is defined by the presence of encysted bradyzoite forms, called tissue cysts [7]. The drugs currently available for etiological treatment of *T. gondii* infection are more effective in the acute phase, however they do not offer a parasitological cure in the chronic phase [8]. Thus, the cysts cannot be eliminated, remaining in the CNS throughout the life of the host, apparently in silent state [7] and with sustained neuroinflammation during the acute and chronic phase in mouse models of infection [9, 10]. In humans, neuroinflammation has been linked to the development of neurodegenerative illnesses also associated with behavioral alterations as Alzheimer disease [11]. Several studies have investigated the role of *T. gondii* infection as a risk factor for neurological and behavioral disorders. In humans, infection by *T. gondii* has been related to mental illnesses such as schizophrenia, bipolar disorder, depression and obsessive-compulsive disorder, as well as mood, personality and other behavioral changes [12], as increased rate of involvement in traffic accidents [13], suicide attempts [14] and alteration of cognitive functioning [15]. Alike humans, mice are intermediate hosts of *T. gondii* [16] and show behavioral abnormalities. In experimentally infected mice, among the reported behavioral changes are loss of predator fear [17, 18], decreased anxiety [10], increased exploratory behavior [19, 20] and impairment of long-term memory during chronic infection [21]. All these findings contributed to the hypothesis of host manipulation by the parasite, contributing to exposition to definitive host predator and *T. gondii* cycle maintenance.

Behavioral alterations have been proposed to be independent of persistent neuroinflammation [18] and the apparent presence of *T. gondii* cysts [18, 22] in the CNS. In non-infectious and infectious experimental models, behavioral alterations have been linked to neuroinflammation or independent of it [23–25]. Further, systemic inflammatory profile associated with increased circulating cytokine levels raised as a contributor to underpin behavioral alterations in infectious diseases as hepatitis and Chagas disease [24, 26]. Therefore, in the present work we carried out a kinetics study of infection of C57BL/6 mice with the ME-49 type II strain of *T. gondii* to settle initially a model of long-term infection to study behavioral changes. For that, we used standardized tests to evaluate the presence of anxiety, depressive-like behavior, and hyperactivity. Further, trying to shed light on the biological factors associated with infection-induced behavioral abnormalities, we assessed cyst numbers and presence of neuroinflammation as well as their topographical localization in the CNS areas, cytokine expression in the brain tissue and serum, and BBB integrity.

2. Materials and methods

2.1 Ethics statement

The experimental procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Council for Animal

Experimentation. The Animal Use Ethics Committee of Oswaldo Cruz Institute/Fiocruz approved all procedures performed in this study (license L014/2018). All the data presented were obtained from two independent experiments registered in the Experience Record Book #73, LBI/IOC-Fiocruz.

2.2 Experimental design

Experimental check list is described in Author´s Check List (S1 Checklist). A total of 116 female mice of the C57BL/6 (H-2^b) lineage, 3-4-week-old, was provided by the Institute of Science and Technology in Biomodels (ICTB) of Oswaldo Cruz Foundation and housed in the Experimental Animal Facility (CEA-CF/IOC unit) under specific pathogen-free conditions, in polypropylene cages lined with pine sawdust and kept in microisolators, under noise and light controlled conditions (12 hours light/12 hours dark). Animals were randomly grouped into groups of 3–5 mice per cage and received water and grain-based *ad libitum*. To minimize the effects of stress and allow the adaptation process to the new environment, mice were kept without manipulation for 15 days in the cages, provided with environmental enrichment (igloo). After the adaptation period, the animals were infected and analyzed according to the experimental protocols (Fig 1).

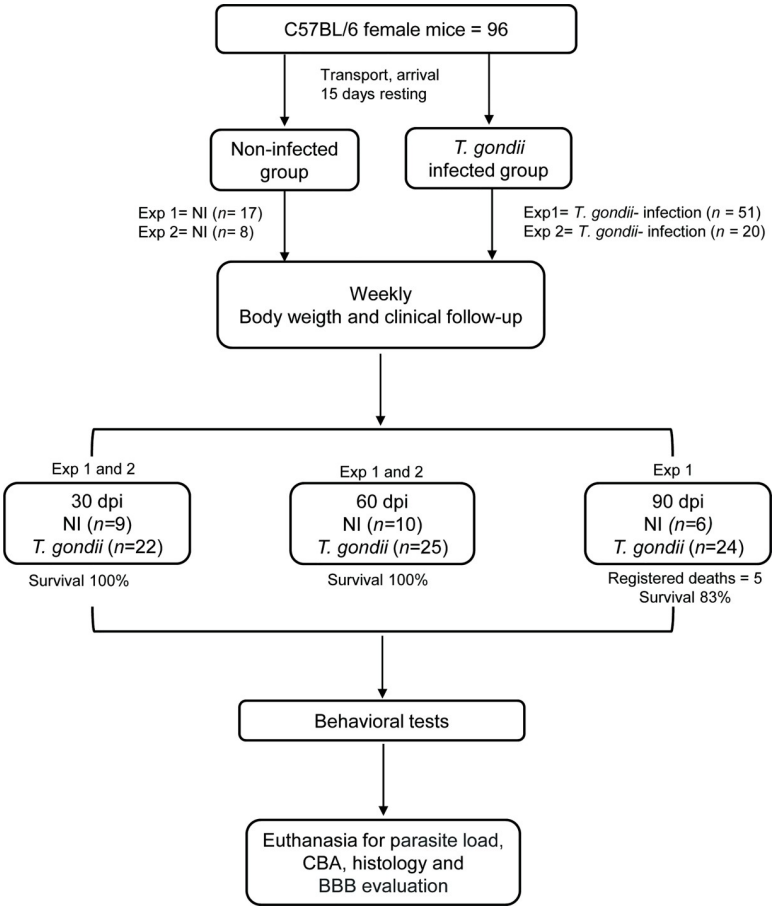

Fig 1. Flow chart showing the experimental protocol with the number of animals used. Death registered, and mice included in 2 (endpoint registered at "30 and 60 dpi") or 1 (endpoint registered at "90 dpi") independent experiments.

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At arrival, experimental groups were formed. The cages were numbered and randomly classified for experimental infection and analysis at the indicated timepoints. Experimental groups were formed with a total of 96 mice divided into two replications: Experiment 1 (i) 30 days postinfection (dpi): 5 non-infected (NI) controls and 12 infected; (ii) 60 dpi: 6 NI controls and 15 infected; and (iii) 90 dpi: 6 NI controls and 24 infected. Experiment 2 (i) 30 dpi: 4 NI controls and 10 infected; and (ii) 60 dpi: 4 NI controls and 10 infected. Additionally, 20 mice divided in two independent experiments of 10 mice each were used to study cytokine expression in the CNS at 20 dpi (2 NI, 3 infected) and at 45 dpi (2 NI, 3 infected), respectively, before and after neuroinflammation onset S1 Fig.

2.3 *Toxoplasma gondii* infection and clinical follow-up

Animals were infected orally with five cysts of the cystogenic ME-49 *T. gondii* strain [27], provided by Dra. Neide Maria da Silva (ICBIM, UFU) and kept in the Laboratory of Biology of the Interactions (LBI-IOC) by serial passages in C57BL/6 (H-2^b) female mice every 60 days. The clinical follow-up was carried out weekly, registering the following parameters: piloerection, apathy, prostration, mobility, posture, aggressive behavior, pain, mortality, and weight loss, assessed using a mouse precision scale (Sartorius ED623S Milligram Scale, OCE, USA) and used as an indicator of hyporexia. Signs of pain or suffering such as isolation from the group, loss of body weight greater than 30% of the initial weight, injuries from fights, ataxia, immobility, and any death outside of planned euthanasia or humane endpoints were the criteria established to guide the decision-making endpoint.

2.4 Behavioral tests

All behavioral tests were performed between 8:00 am and 4:00 pm and recorded on a DSC-DVD810 video camera (Sony, USA). To minimize stress and increase familiarity, all behavioral tests applied to the different experimental groups were performed in an environment provided with 12 hours of light and 12 hours of dark cycle at a temperature of 22 ± 2˚C and a noise level of approximately 40 dB produced by an air conditioner. The experimental groups (30, 60 and 90 dpi) were subjected to behavioral tests, no mouse was subjected to the same test more than once, but animals were reused in different tests to reduce the number of mice used in the research. Behavioral tests were performed from the least stressful to the most stressful: (i) open field test (OFT), (ii) grip strength meter test (GSMT) (iii) tail suspension test (TST), (iv) forced-swimming test (FST) and (v) footprint test [24]. After testing each mouse, the device was cleaned with 70% alcohol.

2.5 Open Field Test (OFT)

To assess anxiety, we used the OFT based on the exploratory profile of rodents denominated as thigmotaxis. Once exposed to a new environment, they will prefer to be close to the wall or peripheral areas, instead of being exposed in the central area of the field, which represents danger as it is the most exposed area. As time passes, anxiety levels decrease due to habituation and the mouse ventures to explore the central area, a shorter distance traveled in the central area. The less time exploring that area is an indication of anxious behavior [28, 29]. The open field test consists of a 60 cm acrylic cubic box, with white walls and soil divided by black lines into 49 equal squares, where the animal is exposed to an environment without aversive or rewarding stimuli. The mouse was allowed to freely explore the open field for 5 minutes. OFT is used to assess exploratory activity and/or locomotion; anxiety was evaluated as time expend in the central zone (s); the distance traveled (cm) was calculated by the number of lines crossed

during the total time; speed or velocity was estimated as time spent crossing the lines (cm/s); and immobility time (s) in total time.

2.6 Grip strength meter test (GSMT)

The grip strength meter apparatus (EFF 305, Insight, Brazil) was used to the GMST, a non-invasive method to assess the strength of the muscle of mice limbs [30]. It is composed of a metal bar fixed to a force transducer that measures the peak of traction (in gram-force) displayed on a digital screen. The test is based on the natural tendency of mice to grab a horizontal metal bar when slightly pulled by the tail for 2–3 seconds, consisting of three consecutive repetitions in 15s. Data are shown as mean of strength intensity = gram-force (gf)/body weight (g).

2.7 Tail suspension test (TST)

The test is based on the principle that mice placed in an unavoidable but moderately stressful situation will develop a motionless posture, indicative of depression-like behavior [31]. The device, called tail suspension apparatus (Insight, Brazil), consists of a large box measuring 61.10 cm wide by 55.40 cm high, divided into four identical 15.28 cm shares, allowing testing four animals at a time, and a bar of aluminum suspension placed horizontally at the top, where the animal hangs by the tail with the help of a ribbon. The division of the shares are arranged in such a way that, when the mice are hung, they cannot touch the other walls of the compartment or observe the others. The C57BL/6 strain has the ability to reach and climb using its own tail [32], thus we employed hollow transparent polycarbonate cylinders (4 cm long, 1.6 cm outside diameter, 1.3 cm inside diameter, 1.5 grams), placed around the tails to prevent climbing behavior. Shaking, reaching out, swinging vigorously, and body torsion or jerky are considered active movements. As the mice start to tire, the movements become more subtle until only the front legs move, which is considered immobility, as well as the balance of the body resulting from previous movements, passive oscillations, and total absence of movements. The test was recorded with a video camera (Sony, USA) for 5 minutes, and the total duration of immobility in 5 minutes was registered.

2.8 Forced swimming test (FST)

This test was used to evaluate depressive-liked behavior. The test consists of a cylinder (height 35 cm, diameter 25 cm) containing clean water (25 ± 1˚C), up to a level of 20 cm above the bottom, where the mice were gently placed on the surface. The test lasted a total of 6 min, the first 2 minutes was considered habituation and the total duration of immobility was recorded during the last 4 minutes [33]. Active movements were defined as the time during which the mouse made strong movements against the cylinder walls using the forelimbs. Immobility was defined as the time during which the mouse remained floating passively, and made no attempt to escape, thus showing only slow movements to keep its head above water. The water was changed before the introduction of each animal. After test, the animal was dried with gauze and replaced in its cage.

2.9 Footprint

The mouse gait analysis was performed using the footprint test. The paws were covered with non-toxic ink (red color to paint the forelimbs; blue color to the hindlimbs). The mouse was free to walk on a sheet of white paper (12 cm wide; 29.7 cm length) to generate a footprint pattern [34]. The following parameters were evaluated: stride length of the anterior and posterior limbs, width of the front and rear base, overlapping distance between the anterior and

posterior limbs and the spread of the fingers [35]. For analysis, we used the free software Image J (NIH). In order to generate reliable scoring data, footprints with 4–6 consecutive steps from each foot were analyzed. For each step parameter, three values were measured from each animal footprint, excluding footprints made at the beginning and at the end of the run where the animal was initiating and finishing movement, respectively. The mean value of each set of three values was used in subsequent analysis. The data are presented as the mean of the analyzed parameter considering the body weight as the correct factor = parameter (cm)/body weight (g), as previously described [36].

2.10 Determination of blood-brain barrier integrity, obtention of brain tissue and blood

To determinate the BBB integrity, we used the infusion of the Evans blue (EB) dye (113 mg/Kg), as shown previously [37]. Mice were sedated intraperitoneally with Diazepam (20 mg/Kg), and 200 μL of pyrogen-free saline (BioManguinhos, Fiocruz) containing EB dye (Sigma-Aldrich), administered via the orbital plexus. After 2 hours [37], mice were restrained physically and blood was collected by the orbital plexus, after anesthesia with the application of topical eye drops [38]. Exsanguination was practiced instead of perfusion, based on the efficiency of the technique [38] the shortest time needed per mouse, as we had a large number of animals per timepoint. Mice were euthanized at the end points (30, 60 and 90 dpi), using CO_2 inhalation, followed by decapitation. According to a previously described protocol, the brains were collected through a craniotomy, weighted, rinsed by immersion in saline, photographed (Samsung Note 10), sagittally sectionized [38], and photographed again. Hemi-brain was placed in 1.5 mL Eppendorf tubes containing 500 μL of 10% formalin [38] for 10 days, to extract the EB. The eluate from each hemi-brain was collected and analyzed by spectrophotometry at 620nm [37, 38]. The concentration of the dye in each sample was determined using a standard curve, with serial dilution with the following concentrations: 3 μg/mL; 1 μg/mL; 0.3 μg/mL; 0.1 μg/mL; 0.03 μg/mL and 0 μg/mL (diluent). The final concentrations were calculated for whole brain.

2.11 Evaluation of cysts number and diameter

As described above, mice were euthanized using CO_2 inhalation, followed by decapitation. Each brain collected was weighed, sagittally sectionized, and hemi-brain included in 1.5 mL of phosphate-buffered saline (PBS), then macerated initially using a 5ml syringe attached to a 18G hypodermic needle, making delicate movements (aspiration and disposal) until a homogenate is obtained. The process was repeated with a 21G hypodermic needle, to homogenize the smaller particles, until a homogenate was obtained. The number of cysts was determined by optical microscopy analyzing 20 μL of the homogenate in duplicate, and the total number calculated for the whole brain. The size of the cysts was determined using the digital software NIS Elements BR version 4.3 (Nikon Co., Japan), using images obtained with a Sight DS-U3 color vision digital camera adapted to an Eclipse Ci-S microscope. The diameter of the cysts was measured with the digital morphometric apparatus NIS Elements BR version 4.3 software (Nikon Co., Japan). Through a Sight DS-U3 color-view digital camera adapted to an Eclipse Ci-S microscope. The frequency distribution of the diameter length of the cysts was grouped into classes and the number of occurrences in each class was counted to know the behavior related to the size of the cysts, throughout the infection.

2.12 Histopathology

Encephala were collected, weighed, and sagittally cut, then fixed in 10% buffered formaldehyde in saline for 10 days, dehydrated and embedded in paraffin. Two sections of 4 to 6 μm thick

sagittal sections were prepared and stained with hematoxylin and eosin. The slides were scanned with the Motic infinity 100 Scanner and viewed using the VM-Motic Digital Slide Assistance software, version 1.0.7.46. Histopathological changes and the distribution of cysts were analyzed, as well as the presence of silent cysts (devoid of surrounding inflammation). Radius between the two points determined the distance between each cyst and foci of inflammation. The cyst was considered silent when in a 10X field it was not possible to observe the presence of inflammation. Representative maps of the topographical location of the cysts in the brain were constructed using the stereotaxic coordinates of the mouse brain bregma (Fig 7A). The precision of the coordinates allows a potential error of less than 0.5 mm in the location of any point in the brain [39]. The cyst location was plotted using the Open-Source Scalable Vector Graphics Editor Inskscape software, version 1.0.1 (3bc2e813f5, 2020-09-07). Representative images were constructed overlaying the images of all analyzed mice.

2.13 Determination of cytokines in sera by CBA

The blood collected of EB-injected mice was used to obtain serum, stored in a -80˚C freezer until CBA analysis. The levels of cytokines in sera were measured with the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (catalog 552364, BD Bioscience, USA). The kit was used for the simultaneous detection of interleukin 6 (IL-6), interleukin 10 (IL-10), interferon gamma (IFNγ), tumor necrosis factor (TNF), interleukin 12 (IL-12) and monocyte chemotactic protein (MCP-1/CCL2), in a single sample. The protocol was carried out according to the manufacturer's recommendations. Cytokine standards were diluted serially to construct the calibration curves and used to determine the cytokines concentrations. The samples were analyzed using the 13-Color CytoFLEX-S flow cytometer (Beckman-Coulter, USA). Individual cytokine concentrations were indicated by their fluorescent intensities and expressed in pg/mL, using the FCAP Array Software. The theoretical limits of detection were: 5 pg/mL for IL-6, 2.5 pg/mL for IFNγ, 7.3 pg/mL for TNF, 10.7 pg/mL for IL-12 and 17.5 pg/mL for IL-10.

2.14 RT-PCR assay for detection of cytokine mRNA

Mice were anesthetized (300mg/Kg ketamine and 30mg/Kg of xylazine), blood was obtained by cardiac perfusion for 10 minutes with cold-saline and encephala were collected, at 20 and 45 dpi. RNA was isolated from CNS tissue of mice by acid guanidinium thiocyanate-phenol-chloroform extraction: RNA STAT-60^{TM}. Reverse transcriptase-polymerase chain reaction conditions have been published elsewhere [40]. The PCR product and molecular weight marker were electrophoresed in a 6% polyacrylamide gel and stained with silver nitrate. Densitometry of gels was carried out on a Densitometer CS-9301PC (Shimadzu, Japan). The PCRs were standardized using hypoxanthine-guanine phosphoribosyl transferase (HPRT). Data are shown as relative IFNγ and TNF expression. Primers: **HPRT**: GTTGGATACAGGCCAGACTTTGTTG, GATTCAACTTGCGCTCATCTTAGGC, 30 cycles; **IFNγ**: AACGCTACACACTGCATCTTGG, GACTTCAAAGAGTCTGAGG, 32 cycles; **TNF**: GATCTCAAAGACAACCAACTAGTG, CTCCAGCTGGAAGACTCCTCCCAG, 28 cycles; **MIP-1α/CCL3**: CGCGGATCCCGGAAGATTCCACGCCAATTC, CGCGGATCCGGTTGAGGAACGTGTCCTGAAG, 32 cycles; **MIP-1β/CCL4**: CGCGGATCCCCCACTTCCTGCTGTTTCTCTTAC, CGCGGATCCAGCAGAGAAACAGCAATGGTGG, 33 cycles; **RANTES/CCL5**: CGCGGATCCCCACGTCAAGGAGTATTTCTACACC, CGCGGATCCCTGGTTTCTTGGGTTTGCTGTG, 26 cycles; **MCP-1/CCL2**: CCGGAATTCCACTCACCTGCTGCTACTCATTCAC, CCGGAATTCGGATTCACAGAGAGGGAAAAATGG, 30 cycles.

2.15 Statistical analysis

The sample size was determined based on the experience of our group and previous studies using the model of experimental toxoplasmic encephalitis; therefore, no formal sample size was calculated. To assess the normality of the data, the Kolmogorov-Smirnov and Shapiro-Wilks tests were used. To determine whether there were any significant statistical differences between the infected groups compared with the NI control groups, we applied the Student t-test with a 95% confidence level for data with normal distribution and the Mann Whitney test for data without normal distribution or ANOVA, when applicable. Correlation was analyzed using Pearson's correlation coefficient. Statistical tests were performed using GraphPad Prism version 8.0. Differences were considered statistically significant when $p < 0.05$. The data were expressed as mean and standard error of the mean (SEM).

3. Results

3.1 Long-term chronic *Toxoplasma gondii* infection in C57BL/6 leads to loss of muscle strength with preservation of locomotor capacity

Female C57BL/6 mice were infected with 5 cysts of the ME-49 *T. gondii* strain. Kinetic of infection was evaluated at 30, 60 and 90 dpi (Fig 2A). The survival rate of infected mice was 100% up to 60 dpi, and 83% (5/24) survival was registered at 90 dpi (Fig 2B), compared to 100% in the NI control group. The analysis of all studied parameters showed no difference among the NI control groups run concurrently to *T. gondii*-infected mice, at 30, 60 and 90 dpi. Thus, for

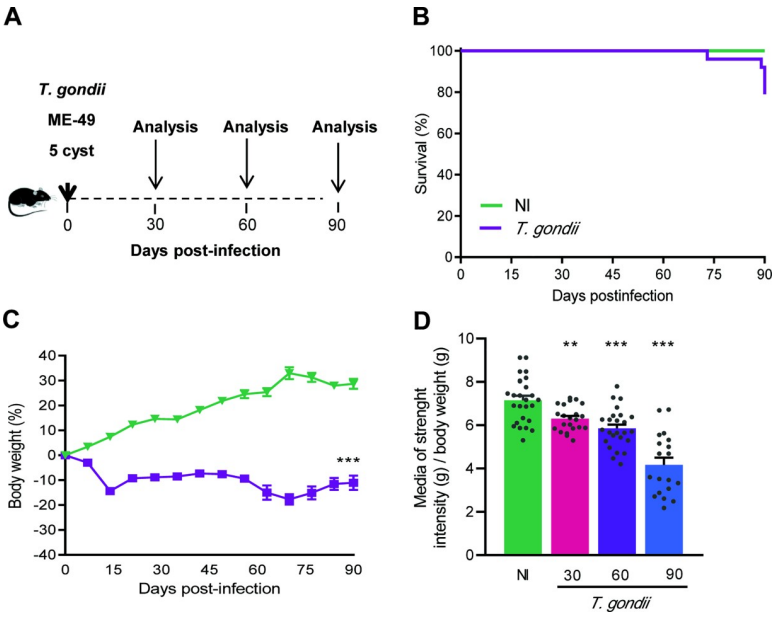

Fig 2. C57BL/6 mice chronically infected with the ME-49 *Toxoplasma gondii* strain survive and show weight and muscle strength loss. (**A**) Mice were infected with 5 cysts of the ME-49 *T. gondii* strain, the clinical follow-up and the mortality were assessed and recorded weekly, and the kinetics of infection was evaluated at 30, 60 and 90 dpi. (**B**) The survival curve shows a survival of 100% at 30 and 60 dpi, and 83% (19/24) at 90 dpi, compared to 100% in the age- and sex-matched NI control group. (**C**) Infected mice showed body weight loss during the acute phase of the infection (up to 15 dpi), after this period, the weight loss ceased. (**D**) Muscle strength was compromised in infected mice; values of muscle strength are shown as gram force (gf) / body weight (g). Each experimental group consisted of 4–6 NI mice and 10–19 *T. gondii*-infected mice. Each circle represents an individual mouse. Data are expressed as means ± SEM, and were analyzed using Welch's test (**C**), and ordinary one-way ANOVA (**D**). **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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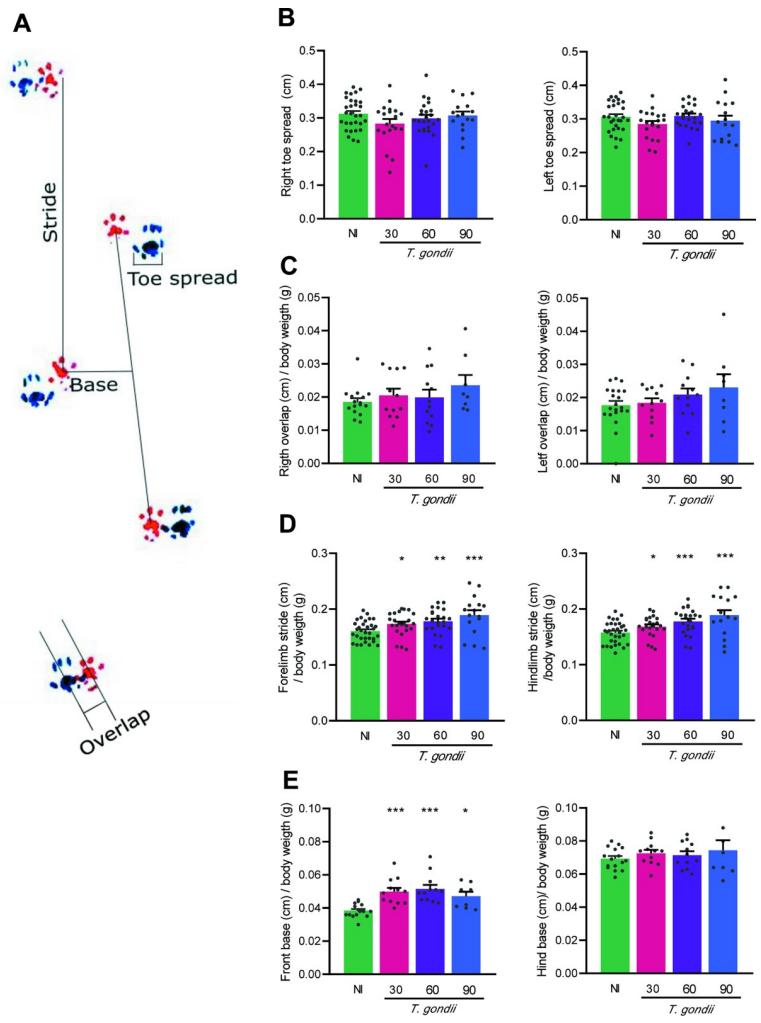

Fig 3. Mice with long-term chronic *Toxoplasma gondii* infection show preserved locomotor capacity. (**A**) The gait analysis was performed using the footprint test and were evaluated as such: the spread of the fingers and overlapping distance between the anterior and posterior limbs, right and left, and the stride length of limbs, width of the front and rear base. (**B**) The toe spread was normal in the right and left limb. (**C**) |No alteration was observed in the right and left step overlap of infected mice. (**D**) The forelimb and hindlimb stride were increased as the infection progresses. (**E**) The width of the front base was enlarged in infected mice, but the hind base was preserved. Each experimental group consisted of 4–6 NI mice and 8–12 *T. gondii*-infected mice. Each circle represents an individual mouse. Data are expressed as means ± SEM, and were analyzed using ordinary one-way ANOVA. *, $p<0,05$, **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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simplification, the collected data of all NI mice were gathered and referred as NI in graphs and figures. Body weight and clinical evolution were monitored weekly. The infected mice showed piloerection accompanied by body weight loss during the acute phase of the infection (up to 15 dpi). After this period, the weight loss ceased, nevertheless the body weight gain in infected mice remained lower than NI control mice (Fig 2C). In addition, the groups of infected mice showed loss of muscle strength at all timepoints of analysis (Fig 2D).

To explore whether the locomotor capacity was preserved, the gait pattern was also assessed using the footprint test (Fig 3A). At the three studied timepoints, no differences were detected in the width of the right and left toe spread (Fig 3B) and in the right and left step overlap (Fig 3C), when compared to NI mice. However, the forelimb and hindlimb stride were altered as

the infection progresses (Fig 3D). Lastly, the width of the front base increased at 30, 60 and 90 dpi, contrasting with the preservation of the width of the hind base (Fig 3E). Therefore, the locomotor capacity of *T. gondii*-infected mice was preserved.

3.2 Long-term chronically *Toxoplasma gondii*-infected mice exhibit anxiety, depressive-like behavior, and hyperactivity

To assess anxiety, we used the OFT and analyzed the time expended in the central zone of the apparatus [29]. Our data showed that when compared to NI controls chronically infected mice exposed to the OFT remained reduced time in the central zone, at 30, 60 and 90 dpi (Fig 4A). More, increased time was expended in the peripheral area of the apparatus, revealed the intensity of the registered lines near the apparatus' walls (Fig 4B). The presence of depressive-like behavior was evaluated using TST and revealed as enhanced time of immobility [31]. Consistently, when compared to NI controls chronically *T. gondii*-infected mice showed increased immobility time at early (30 dpi), and long-term (60 and 90 dpi) chronic infection (Fig 4C). Therefore, considering the standardized parameters in the early (30 dpi) and long-term (60 and 90 dpi) chronic *T. gondii*-infected mice showed depressive like-behavior and anxiety, compared with sex- and age-matched NI controls.

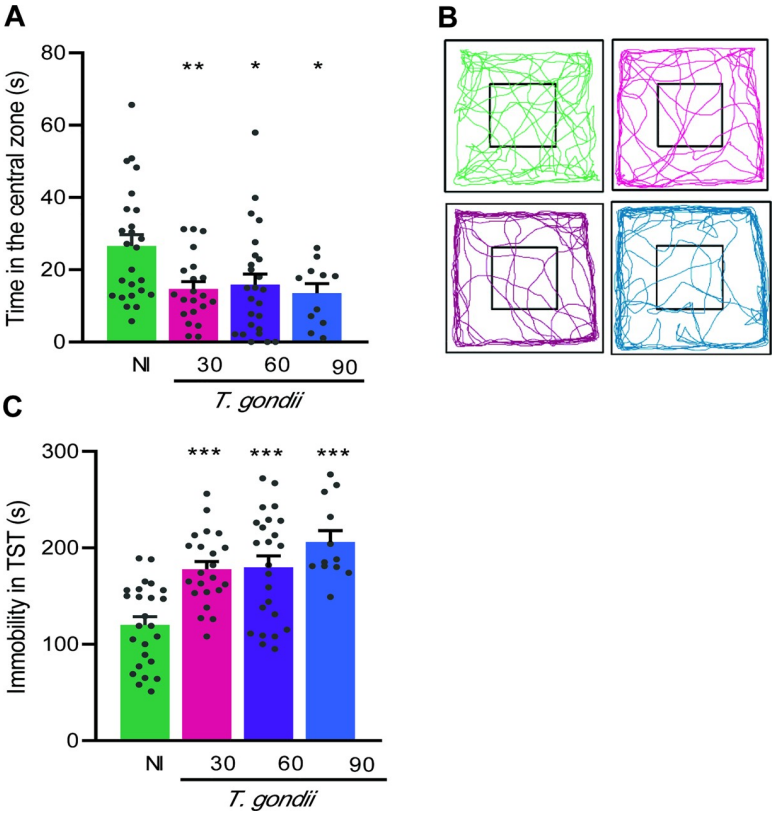

Fig 4. Anxiety and depressive-like behavior are detected in long-term chronically *Toxoplasma gondii*-infected C57BL/6 mice. (**A**) Chronically infected mice showed reduced time in the central zone in OFT, and (**B**) increased time exploring the peripheral area of the open field. (**C**) Depressive-like behavior was revealed as enhanced time of immobility in TST. Each experimental group consisted of 4–6 NI mice and 10–15 *T. gondii*-infected mice. Each circle represents an individual mouse. Data are expressed as means ± SEM, and were analyzed using *t*-Student test. *, $p<0,05$, **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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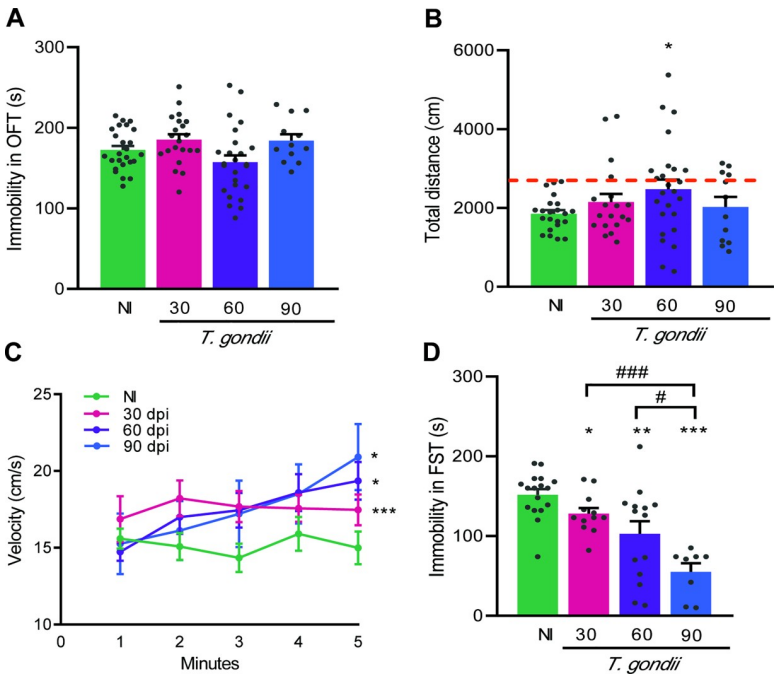

Fig 5. Hyperactive behavior is observed in chronically *Toxoplasma gondii*-infected C57BL/6 mice. To determine hyperactivity, we evaluated the time spent immobile, the distance and the speed of travel in OFT, and the activity in FST. (**A**) Infected mice did not show differences in immobility time when in the OFT. (**B**) At 60 dpi, mice showed increased distance traveled in OFT. At 30 (18%) and 90 (21%) dpi only a reduced percentage of mice showed increase in the distance traveled. Red dot line shows mean distance of NI group + 2 standard deviation. (**C**) The walk velocity in OFT was increased as infection progressed. (**D**) In FST, immobility time decreased as infection progressed. Each experimental group consisted of 4–6 NI mice and 8–15 *T. gondii*-infected mice. Each circle represents an individual mouse. Data are expressed as means ± SEM, and were analyzed using *t*-Student test (**A-C**) and the Mann-Whitney test (**D**). *, $p<0.05$, **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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Curiously, we observed that *T. gondii*-infected mice were apparently more active than NI mice. Thus, we refined our analysis and evaluated the time that infected mice spent immobile, and the distance and speed of travel inside the OFT. Initially, we did not notice differences in terms of immobility time in the OFT when chronically infected mice were compared to NI controls (Fig 5A). The analysis of the walked distance revealed that compared with NI controls 18%, 32% and 21% of infected mice traveled longer distances at 30, 60 and 90 dpi, respectively (Fig 5B). Indeed, the group of long-term chronically infected mice (at 60 dpi) walked longer distances than NI controls (Fig 5B). Moreover, compared with NI controls the walk speed was increased in early (30 dpi) and long-term (60 and 90 dpi) chronically infected mice (Fig 5C), thus suggesting aggravation of this change as the infection progresses. Altogether, these findings support an increase in the locomotor activity in chronically *T. gondii*-infected mice. Next, we tested the performance of infected mice in FST, a test to evaluate mice activity in adverse environments [33]. Compared to NI controls, we detected a significant decrease in the immobility time in all groups of infected mice submitted to FST. Further, this behavioral alteration is visibly aggravated with the course of infection, comparing 60 dpi with 90 dpi ($p < 0.01$) and 30 dpi with 90 dpi ($p < 0.001$) (Fig 5D). Altogether, increase in locomotor activity in OFT and reduced time of immobility in FST, also registered as increased time of activity, are suggestive of impulsive hyperactivity in early and, mainly, in long-term chronic *T. gondii* infection in C57BL/6 mice.

3.3 The number of cysts in the CNS decreases significantly throughout *Toxoplasma gondii* infection

Herein, the parasite load was determined by analyzing the number and size of *T. gondii* cysts present in the CNS of ME-49-infected mice. The number of cysts decreased as the early (30 dpi) chronic infection coursed to long-term chronic infection at 60 dpi ($p < 0.05$ compared with 30 dpi) and at 90 dpi ($p < 0.001$ vs 30 dpi; $p < 0.01$ vs 60 dpi), suggesting gradual control of infection (Fig 6A). However, the size of the cysts increased as infection progressed from early (30 dpi) to long-term (60 and 90 dpi) chronic phase (Fig 6B). Indeed, a more detailed analysis of the cysts size using the digital morphometric apparatus NIS Elements BR version 4.3 software (Nikon Co., Japan), revealed 18 size classes to be considered. Further, these data showed increase in the relative frequency of cysts > 42–46 μm in 60 and 90 dpi, compared with 30 dpi (Fig 6C).

Next, we tried to establish associations between the number of cysts in the CNS with the behavioral features analyzed, and no correlation was found in most of the analysis performed. Correlation ($p < 0.05$) was detected between the number of cysts in the CNS and immobility time in FTS S2A Fig. Further, correlation was observed between the number of cysts and the left and right forelimb strides ($p < 0.05$) and left and right hindlimb strides ($p < 0.01$) in the footprint test S2B and S2C Fig.

3.4 Cysts of *Toxoplasma gondii* prevailed in some regions of the encephalon

Next, to evaluate a putative differential accumulation of *T. gondii* cysts in specific areas of the CNS, we analyzed two hematoxylin-eosin-stained histological sections per mouse. We divided the brain areas into: Olfactory areas (OLF), Isocortex (ICTX), Cerebral Nuclei (CNU), Hippocampal formation (HPF), Thalamus (TH), Hypothalamus (HY), Midbrain (MB), Pons (P), Medulla (MY) and Cerebellum (CB), according to Allen Institute for Brain Science [41], as shown in Fig 7A. At all timepoints evaluated (30, 60 and 90 dpi), the cysts were found in all areas of the CNS except for HPF at 90 dpi, when no cyst was observed in this area of any of the studied mouse. A larger number of cysts was detected in the ICTX, TH and MB areas, and this

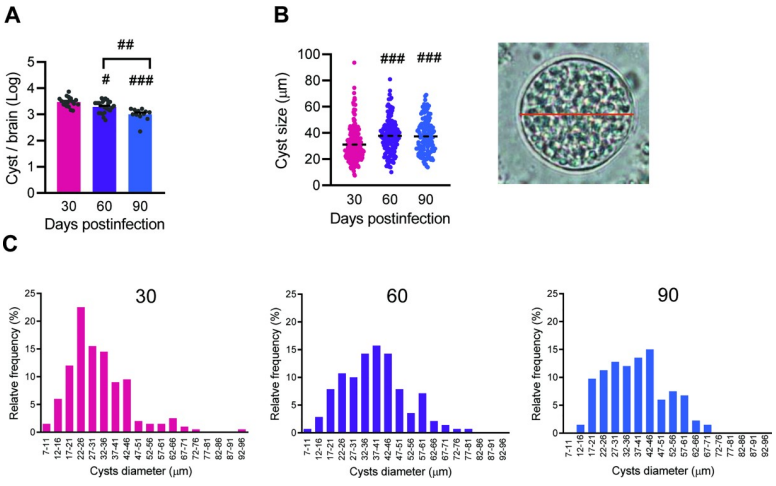

Fig 6. The numbers of parasite cysts in the brain decrease and the sizes increase as *Toxoplasma gondii* infection progresses in mice C57BL/6. (**A**) The number of cysts decreased as the early (30 dpi) infection coursed to long-term chronic infection (60 and 90 dpi). (**B**) The size of the cysts increased as infection progressed from early to long-term chronic phase. (**C**) The graphs show the relative frequencies of cysts in each class at the three timepoints analyzed. Each experimental group consisted of 8–12 *T. gondii*-infected mice. Each circle represents the number of cysts in the brain of each animal (**A**), and the diameter of each cyst (**B**). Data are expressed as means ± SEM, and analyzed using the Mann-Whitney test. #, p <0.05, ##, p <0.01. ###, p <0.001, comparing different timepoints.

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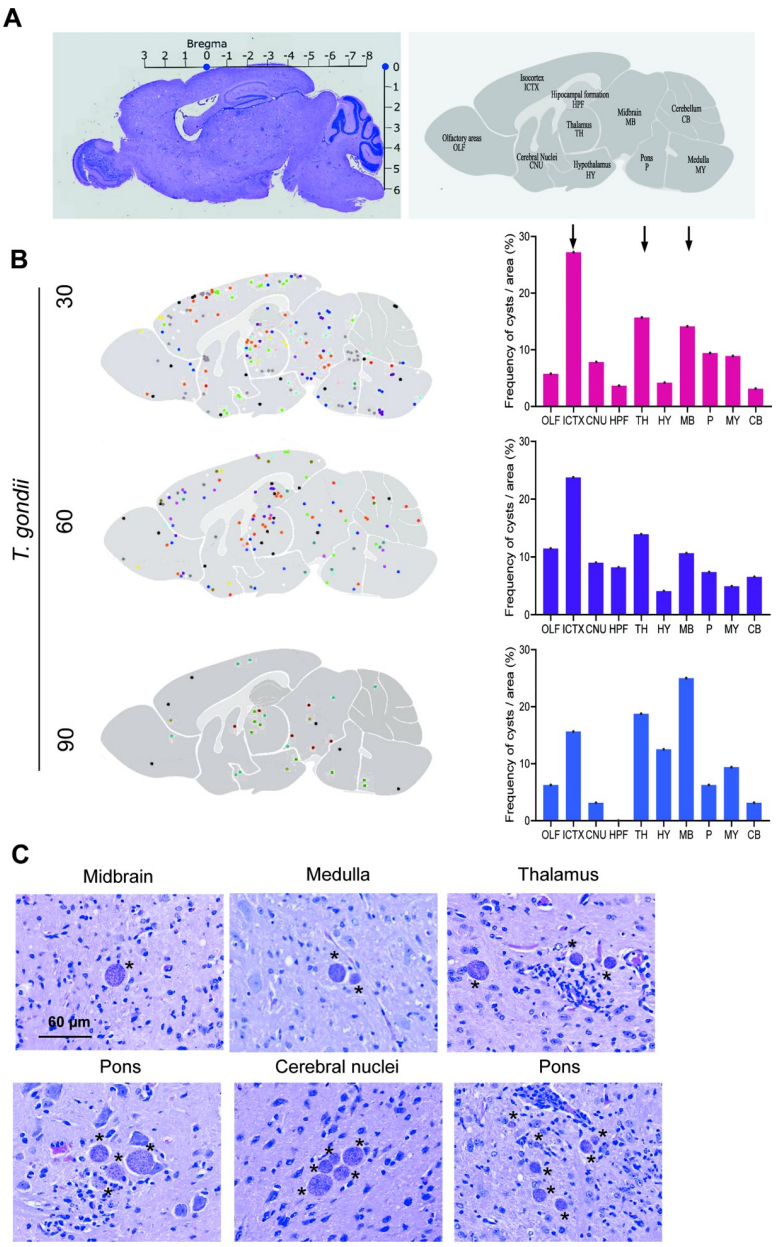

Fig 7. Some regions of the brain of C57BL/6 *Toxoplasma gondii*-infection mice are intensely infected. (**A**) The stereotaxic coordinates of the bregma of the mouse´s brain were used to construct representative maps of the topographic location of the cysts in each brain area: Olfactory areas (OLF), Isocortex (ICTX), Cerebral Nuclei (CNU), Hippocampal formation (HPF), Thalamus (TH), Hypothalamus (HY), Midbrain (MB), Pons (P), Medulla (MY) and Cerebellum (CB). (**B**) The cysts were localized in all brain regions, but the isocortex, thalamus and midbrain were colonized more intensely in the early (30 dpi) and long-term (60 and 90 dpi) chronic infection (each color represents an individual mice). The histograms show the percentage of cysts per area studied. (**C**) Representative pictures show individual cysts or multiple cysts per microscopic field in different brain areas, associated or not with inflammatory foci. Bar = 60 μm. Each experimental group consisted of 4–10 *T. gondii*-infected mice. Data were analyzed using ordinary one-way ANOVA.

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pattern was preserved in the three analyzed timepoints (Fig 7B and S3A Fig), supporting the existence of differential accumulation of *T. gondii* cysts in these CNS tissue areas. The size of the cysts does not vary due to the region in which they are located S3B Fig. Further, most cysts

were observed isolated, but groups of 2 to 7 cysts were also found, regardless the studied areas of the brain (Fig 7C).

3.5 Behavioral changes are concomitant with generalized neuroinflammation in long-term chronic *Toxoplasma gondii* infection

In the three analyzed timepoints, infected mice presented inflammatory foci with meningoencephalitis and perivascular inflammatory cuffs composed of mononuclear inflammatory cells in all evaluated areas of the CNS (Fig 8A and S3C Fig). Rare apparently silent cysts devoid of

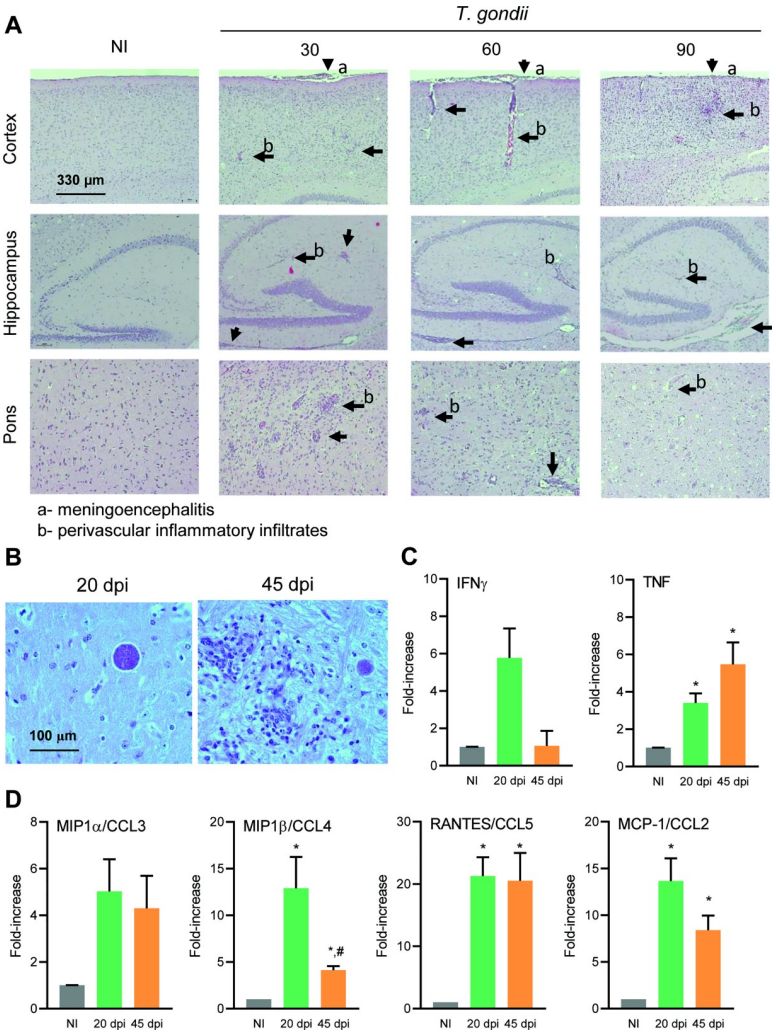

Fig 8. *Toxoplasma gondii*-infected C57BL/6 mice showed generalized neuroinflammation in early and long-term chronic phase of infection. (**A**) Infected mice presented inflammatory foci with meningoencephalitis and perivascular inflammatory cuffs in all evaluated areas of the CNS in early (30 dpi) and long-term (60 and 90 dpi) chronic infection. Bar = 330 μm. (**B**) Cysts devoid of inflammation were firstly detected in the CNS at the acute phase (20 dpi, left panel), and the inflammation is already settled in the chronic phase (45 dpi, right panel). Bar = 100 μm. (**C**) Increased expression of the pro-inflammatory cytokines IFNγ and TNF was detected in the acute phase, and TNF expression was sustained at the chronic phase of infection. (**D**) In the acute phase, the expression of MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and MCP-1/CCL2, was increased. The upregulation of the expression of TNF and CC-chemokines were sustained at 45 dpi. Each experimental group consisted of 2–4 NI mice and 2–10 *T. gondii*-infected mice, in two independent experiments. Data are expressed as means ± SEM, and were analyzed using ordinary one-way ANOVA. *, $p<0,05$ comparing *T. gondii*-infected and NI mice, and #, $p<0.05$ comparing acute and chronic groups of *T. gondii* infected mice.

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inflammatory process were observed at 30 dpi (4.71%; 9/191) and at 60 dpi (4.09%; 5/122), while at 90 dpi no silent cyst was observed. Most of the cysts were surrounded or closer to inflammatory foci. A similar pattern was observed in the three evaluated timepoints in a way that the average distance between cysts and inflammatory foci ranged from 61.4 to 195.2 μm at 30 dpi, 104.9 to 182 μm at 60 dpi group, and 46.5 to 254.3 μm at 90 dpi S3C Fig. In addition, the association of cysts and inflammatory foci patterns was similar in different CNS areas.

Considering that mononuclear cells prevailed in neuroinflammatory processes at 30, 60 and 90 dpi, we settled an experiment to shed light on the pattern of cytokines and CC-chemokines driving putatively the migration of these cells. For that, a group of mice was analyzed at 20 dpi, when cysts devoid of inflammation were firstly detected in the CNS, and at 45 dpi, when inflammation was already settled (Fig 8B). Compared to NI controls, increased expression of proinflammatory cytokines IFNγ and TNF was detected at 20 dpi, therefore, preceding neuroinflammation. Further, TNF expression was sustained at 45 dpi (Fig 8C). At 20 dpi, the expression of the four CC-chemokines analyzed namely MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and MCP-1/CCL2, was enhanced, when compared to NI controls. Although at 45 dpi the expression of MIP1β/CCL4 and JE-MCP-1/CCL2 was reduced in comparison with the expression at 20 dpi, these CC-chemokines remained upregulated in this timepoint of chronic infection compared to NI controls (Fig 8D).

3.6 Systemic cytokine expression is upregulated at early and long-term chronic *Toxoplasma gondii* infection

To assess systemic cytokine serum levels, blood of NI controls and *T. gondii*-infected mice was collected. The obtained sera were stored and submitted to simultaneous detection of cytokines, using the Mouse Inflammation CBA kit (IL-12, IL-6, TNF, IFN, MCP-1/CCL2, IL-10). Representative data plots of the FACS analysis are shown (Fig 9A), supporting that at 60 dpi all analyzed cytokines were upregulated, when compared to NI controls. In general, chronically *T. gondii*-infected mice increased serum levels of pro-inflammatory cytokines. The levels of TNF, IFNγ and MCP-1/CCL2 were elevated significantly at 30 and 60 dpi. At 90 dpi, levels of all cytokines showed a tendency to decrease or, even, cytokines levels were alike those found in sera of NI controls, except for IFNγ and MCP-1/CCL2 levels that remained upregulated at this timepoint (Fig 9B).

3.7 *Toxoplasma gondii* infection induces disruption of the blood-brain barrier and brain edema

Based on the property of EB dye that binds proteins, mainly plasma albumin and on the physiological ability of the preserved BBB to be impervious to this protein, NI controls and infected mice were injected with EB and the encephala analyzed for EB extravasation, assumed as a biomarker of BBB disruption [42]. Representative images of brains depict localized and spotted EB extravasation at 30 dpi, while it is more evident in the whole brain at 60 dpi, and less noticeable at 90 dpi (Fig 10A). Representative sagittal sections of the encephala at 30 and 60 dpi corroborated this description (Fig 10B). When compared to NI controls, the quantitative data disclosed increased concentrations of EB in the encephala of infected mice at the three analyzed timepoints, with maximum levels achieved at 60 dpi, therefore, revealing BBB significant vascular permeability in *T. gondii*-infected (Fig 10C). Further, infected mice also presented a significant increase in relative brain weight in all evaluated timepoints (Fig 10D). Thus, associated with increased EB extravasation, these data are suggestive of cerebral edema.

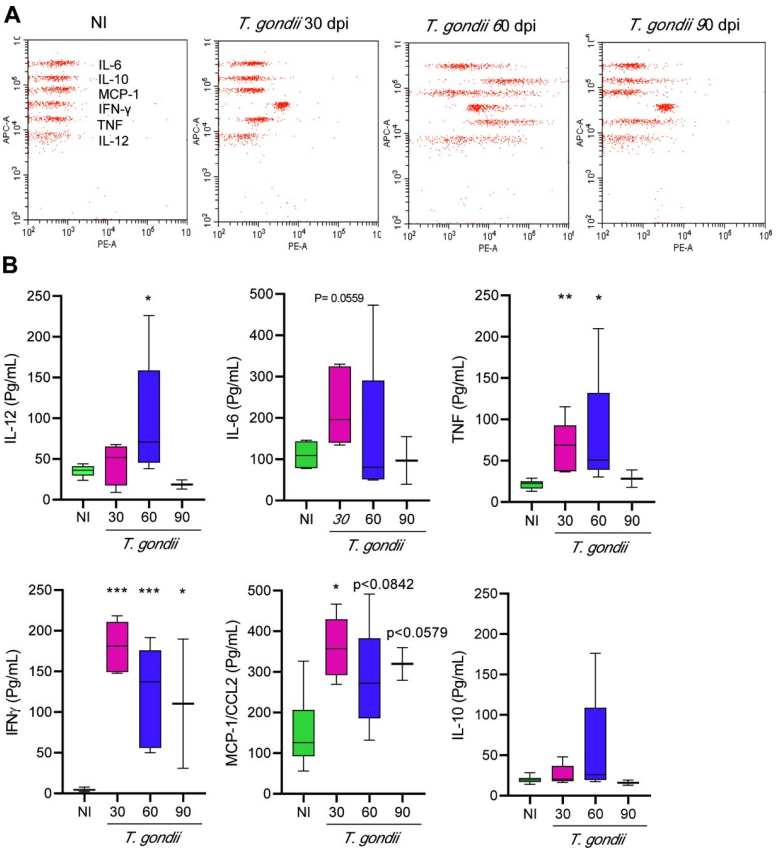

Fig 9. Systemic cytokine expression is upregulated in the early and long-term chronic *Toxoplasma gondii* infection. (**A**) The images show representative data plots of the FACS analysis of CBA. (**B**) Chronically *T. gondii*-infected mice showed increased levels of pro-inflammatory cytokines at 30 and 60 dpi. At 90 dpi, all cytokine levels showed a tendency to decrease or, even, exhibited cytokines levels like those found in sera of NI controls, except for IFNγ and MCP-1/CCL2 levels. Each experimental group consisted of 2–3 NI mice and 2–5 *T. gondii*-infected mice, in two independent experiments. Data are expressed as means ± SEM, and were analyzed using ordinary one-way ANOVA. *, $p<0,05$, **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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4. Discussion

The present study evaluated the kinetics of the behavioral alterations of *T. gondii*-infected C57BL/6 mice, using a battery of standardized behavioral tests. Our data revealed that during the early (30 dpi) and long-term (60 and 90 dpi) chronic infection mice showed anxiety, depressive-like behavior and hyperactivity, concomitant with neuroinflammation, systemic inflammatory environment and BBB disruption. Further behavioral changes occurred regardless of the progressive decrease in the number of cysts and were independent of the localization of the cysts and inflammatory foci in the CNS areas. Thus, multiple factors may contribute to the observed behavioral abnormalities.

The experimental model we used, consisting of C57BL/6 mice infected with the ME-49 strain, allowed survival and chronic phase onset. Although our focus is on chronic infection, we monitored the clinical evolution of mice after the first day postinfection up to the end point. The mice showed a decline in body weight and the presence of piloerection in the first 15 days of infection, features proposed to be associated with the period of widespread parasite multiplication, typical of the acute *T. gondii* infection [43]. In the chronic phase, weight loss ceased, and body weight was steadied, alike previously described in other studies [9, 20, 44,

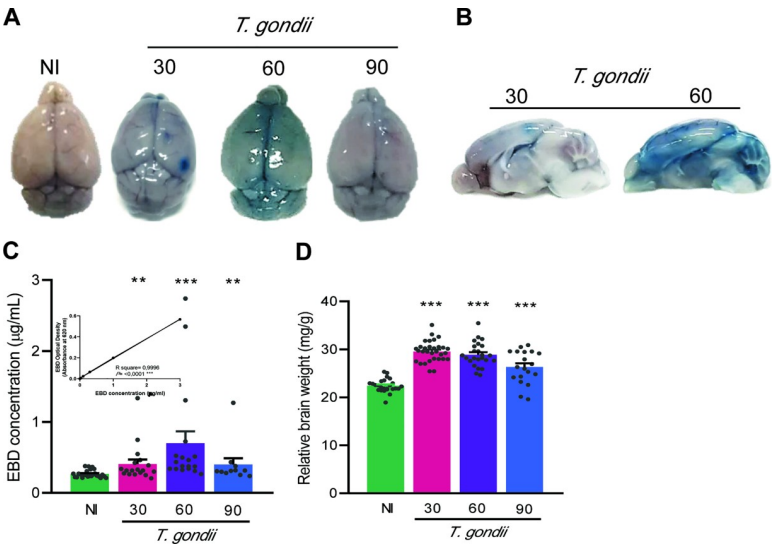

Fig 10. The increase in BBB permeability is concomitant with edema in *Toxoplasma gondii*-infected C57BL/6 mice. (**A**) Representative images of brains depicting EB extravasation at the timepoints assessed. (**B**) Representative sagittal sections of the encephala, at 30 and 60 dpi. (**C**) The concentrations of EB in the brain tissue of infected mice increased at the three studied timepoints, indicating the increase in BBB permeability. (**D**) Cerebral edema, manifested by a significant increase in the relative brain weight (brain weight in milligram / whole body weight in gram), was present in the timepoints evaluated. Each experimental group consisted of 4–6 NI mice and 8–19 *T. gondii*-infected mice, in two independent experiments. Each circle represents an individual mouse. The data are expressed as means ± SEM, and were analyzed using Mann-Whitey test (**C**) and *t*-Student test (**D**). **, $p<0.01$. ***, $p<0.001$, comparing *T. gondii*-infected and NI mice.

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45]. Neuromuscular strength decreased in ME-49 strain-infected C57BL/6 mice, thus corroborating previous data that showed reduced neuromuscular strength in this mode [45] and in C57BL/6 mice infected with 10^3 bradyzoites of ME49 strain [46]. Also, in chronically ME-49-infected Swiss Webster mice, decreased muscle strength was associated with myositis and reduction in muscle mass, being proposed that the inability to recover the weight during the chronic phase (> 30 dpi) is due, in part, to the continuous damage of the skeletal muscle [9, 45]. Likewise, muscle loss in the anterior tibialis, gastrocnemius and quadriceps muscles have been shown in chronically (37 dpi) ME-49-infected CBA/J mice [43]. Therefore, these data support that chronic infection characterized by cyst formation in the CNS, was associated with body weight loss, as reproduced in our experimental model.

Gait changes can represent a pathological state in many neurological diseases and disorders [47]. To analyze the locomotor capacity, the footprint test was performed, this is a gait analysis method, sensitive to neurodegenerative changes, and can be used in locomotor assessment of different disorders [48]. Here, we evaluated the spatial components of gait: stride length, width of the front and rear base, overlapping distance of the paw and spreading of the toes of right and left limbs. Stride length has been described as a reliable index of motor disorders caused by dysfunction of the basal ganglia in mice [49]. The increase in stance width generally indicates a compensatory gait pattern to avoid gait instability resulting from pain [50]. The overlapping distance of the limbs assesses the existence of greater flexibility of hip rotations or strength of the limbs to reach longer strides [47] as well as the uniformity of the alternation of steps [34] and, the toe spread can be used to form functionality indices for the sciatic, tibial, and peroneal nerves [50]. Here, the *T. gondii*-infected mice showed increase in the forelimb and hindlimb stride length at early and long-term chronic infection. The increase in stride length is influenced by the speed at which the mouse walks or runs, without affecting its gait

[48, 49, 51]. The reduction in this parameter has been described in patients with neurodegenerative diseases such as Parkinson disease [52], and in murine models of Huntington disease [34, 53] and arthritis as a manifestation of pain [48, 54], but unbalanced gait was not observed in the model we described here. *T. gondii*-infected mice showed increase in the stance forelimb width. The increase in the width of the rear base, accompanied by a decrease in the length of the steps, was reported in a murine model of antigen-induced arthritis, maintained even after intra-articular treatment with AMPA/kainate glutamate receptors antagonist, which prevents pain and reduces pathology [55]. Thus, wider stance widths generally indicate a compensatory gait pattern used to protect a member of a painful movement and avoid gait instability [50]. In our infected mice, there were no signs of ataxia or foot drag detectable by the footprint test, and rear base width, toe spread and overlap of step were preserved. More, when compared to NI controls, the infected mice showed similar exploratory behavior, similar immobility time, and covered similar or longer distances in the OFT, besides the higher speed. So, we consider that these features support that our model maintains preserved locomotor capacity. In a previous study, chronically *T. gondii*-infected C57BL/6 mice with motor coordination deficits, impaired gait (mis-steps, stride length short, foot dragging in the rear paws), and reduced muscle strength, showed a reduction in exploration in the OFT, but no changes in social behavior or impaired cognitive function were observed, as object recognition test and spatial memory tests require displacement in the open field [46]. Interestingly, *Trypanosoma cruzi*-infected C57BL/6 mice show preserved muscle strength but alterations in cognitive function, also evaluated in tests that require displacement in the open field [25], supporting that these are dissociated features. Thus, our data suggest that decreased neuromuscular strength did not affect the motor function and the general locomotor activity of chronically ME-49-infected C57BL/6 mice.

Mice tend to explore a new environment, however anxiety behavior reduces time spent expended in central area and increases time consumed closer to the wall or peripheral area of the OFT apparatus [28]. The presence of anxiety, revealed as decreased time expended in the central area of the OFT, was detected in the early and long-term chronic infection of C57BL/6 mice with the ME-49 strain. Recently, similar results were shown in the model of early (30 dpi) chronic *T. gondii* infection of Swiss Webster mice [19]. In experimental models, TST and FST are widely used to assess depressive-like behavior in infectious and non-infectious diseases [24, 56–58]. It is expected that, when compared to their controls, afflicted mice subjected to both tests will increase immobility time as an indication of a depressive-like behavior [31, 33]. Here, in early (30 dpi) chronic *T. gondii* infection, mice showed an increase in the immobility time in TST, supporting a depressive-like behavior that is sustained as the infection progresses to the long-term chronic phase (60 and 90 dpi). The FSL rats, a lineage susceptible to behavioral alterations, showed depressive-like behavior when infected with the ME-49 *T. gondii* strain, and evaluated by sucrose preference test and FST [44]. Contrasting with the finding in FSL rats assessed in FST and added to our results of increased immobility in TST, we bring evidence that ME-49-infected C57BL/6 mice showed a decrease in the immobility time in FST, when compared to the NI controls. Thus, these conflicting results obtained in the two behavioral tests initially proposed to study depressive-like behavior applied to the same experimental groups, leading us to query the applicability of the FST to describe other behavior alterations. Truly, FST has been used previously to examine hyperactivity [59], stress coping behavior [60], while other authors suggest that the FST can be used to assess learning capability [61] and anxiety [62]. Discordant results have also been reported in non-infectious models, when no changes were exhibited in TST and reduced immobility time was shown in FST [57, 58]. Again, in a model of schizophrenia in Swiss Webster mice treated with a NMDA antagonist in the acute (single dose) or the chronic (10 consecutive days) process and submitted to FST and

TST, the results were dissenting. In the acute protocol, increased immobility in TST was observed, and no changes were detected in FST. Conversely, in the chronic protocol immobility was increased in FST, whereas decreased immobility was detected in TST [56], supporting that these behavioral tests reveal different physiological mechanisms underlying the immobility process [56–58]. Chronic infection with *T. gondii* has been noticed as an anxiety reducer in mice exposed to elevated plus-maze test (EPMT) where they visited the open arms more frequently, even showing a decrease in the exploration of the central area of the OFT [10]. However, similar results in EPMT were also interpreted as changes in risk behavior, in model of chronic *T. gondii* infection [20]. We consider that like the FST, the results obtained in the EPMT may be misinterpreted, whenever EPMT allows to explore the impulsive behavior, one of the symptoms of attention-deficit/hyperactivity disorder (ADHD), which is expressed by the increase in exploratory activity in the open arms [63–67]. Likewise, we propose that the FST test can be a valid tool in the study of impulsivity in a murine model, though further studies are necessary. Anyhow, the reduced immobility in the FST, added to the increase in exploratory behavior and increased velocity in the OFT, led us to conclude that ME-49-infected C57BL/6 mice show a complex pattern of behavioral alterations with depressive-like behavior and hyperactivity in the early and long-term chronic infection, with aggravation of the process as the infection progresses. Interestingly, a recent study showed a relation between the severity of ADHD and IgG positivity for *T. gondii* infection in 6 to 18-year-old children, suggesting a role for the parasite infection in the exacerbation of ADHD [68]. Therefore, our results supporting hyperactivity in the OFT, depicted as increased velocity and total distance covered, corroborate the description of hyperactivity in the type II Prugniaud strain-infected BALB/cJ mice [22, 69], C57BL/6J infected with genetically modified ME-49 tachyzoites [20], and Swiss Webster infected with the ME-49 strain [19]. Notably, in our model regardless of the reduction in the number of brain cysts, the described behavioral changes were sustained at 60 and 90 dpi. In contrast, a relationship between the load of cysts and the severity of behavioral changes has been reported [10]. Indeed, our results revealed that the reduction in the number of brain cysts as infection progresses was not associated with reduction of hyperactivity, corroborating data in BALB/cJ mice chronically infected with the Prugniaud strain and treated with the antiparasitic guanabenz [22].

To shed light on possible biological contributors to the observed behavioral changes and knowing that the positioning of cysts in specific areas of the brain could give rise to certain behavioral changes [20], we investigated the topographical distribution of cysts and the proximity with inflammatory foci in the brain tissue. Since the cortex occupies 56% of the brain area [39], we divided it into different regions: olfactory areas, isocortex and hippocampal formation, to avoid bias in our results. Our model showed an accumulation of cysts in the isocortex, thalamus and midbrain in the three timepoints analyzed. The existence of tropism of *T. gondii* cysts to specific regions of the CNS is not entirely clear [70] and previous studies reported the heterogeneous distribution of cysts in the brain of mice. In C57BL/6 mice chronically infected with 30 cysts of the ME-49 strain, a larger infection of the hippocampus and telencephalon was observed, an area comprising the cerebral cortex and the cerebral nucleus [71]. In chronically ME-49-infected B6CBAF1/J mice, a heterogeneous distribution of brain cysts was observed, with enrichment of cysts in the frontal cortex and brain stem structures [10], as well as a higher parasite load in diencephalon, cortex and hippocampus in ME-49-infected Swiss Webster mice [9]. In Long-Evans rats infected with tachyzoites of the Prugniaud strain, a random distribution was observed throughout the forebrain, with enrichment in the preoptic area and the paraventricular hypothalamic nucleus [72]. CD1 mice infected with the HIF strain also showed an accumulation of cysts in the telencephalon [73]. These data led us to the support the existence of tropism of ME-49 strain to specific regions of

the CNS, especially the cerebral cortex, and that tropism is dependent on the murine model and the *T. gondii* strain, indicating that further studies are required to explore this point. Cortical areas, limbic regions and basal ganglia are implicated in patients with depressive disorders [74], anxiety [75] and hyperactivity [76]. In addition, changes in the cerebellar brain region have been reported in patients with ADHD [76]. Despite the higher frequency of cysts in the isocortex, thalamus and midbrain showed in *T. gondii*- infected mice, the cysts were found to colonize all areas evaluated, although less frequently. Thus, it is not possible to link the location of the brain cysts detected in our model with the behavioral changes reported here. Once the influence of cysts´ localization in the brain on behavioral changes has been ruled out, we evaluated the influence of the size of ME-49 strain cysts, as cyst diameter, evaluating the kinetics of progression of the infection, and the influence of the size of the cyst in the affected brain area. We observed the presence of brain cysts of various sizes in all groups, indicative of an alternation of the growth and rupture phases underlying the population renewal of cysts [10], which did not allow correlation with behavioral changes. In addition, the size of the cysts by area of the brain was homogeneous, so it was not possible to observe any correlation. The variable observed in the three timepoints was neuroinflammation, detected in all brain regions, independently of the presence of cysts. Indeed, neuroinflammation was consistently described in previously analyzed experimental models of chronic toxoplasmosis [9, 10, 22]. Hyperactivity in a murine model of chronic *T. gondii*-infection was reduced with the use of guanabenz, a drug with potency to reduce neuroinflammation and perivascular cuff, but not the number of cysts in the CNS [22]. In addition, in ME-49-infected BALB/c mice, anxiety and short- and long-term memory impairment of new object recognition were reversed in mice treated with rosuvastatin. This therapy reduced the burden of tissue cysts in the brain and attenuated, but not resolve, the signs of neuroinflammation, including meningitis, perivascular cuffs, microglial proliferation, inflammatory cell infiltration and tissue damage [77]. The combination of sulfadiazine and pyrimethamine, the conventional therapy against toxoplasmosis [8], prevents the presence of parasites in the brain and the development of toxoplasmic encephalitis in murine models, but not the development of mild inflammatory lesions [78], which could still contribute to behavioral abnormalities, a question to be further explored. In humans, there are studies that demonstrate the efficacy of combined therapy in cognitive function in infants [79] and children [80] that developed normally the CNS and preserved intellectual function after treatment. However, the efficacy of this combined therapy regardng the cognitive functions of murine models of *T. gondii* infection is a matter to be further explored. In our study, mononuclear cells prevailed in the neuroinflammatory processes at 30, 60 and 90 dpi, thus we evaluated the profile of expression of cytokines and CC-chemokines, that could be involved in cell migration to the CNS, at 20 dpi (before neuroinflammation onset) and at 45 dpi (in the presence of neuroinflammation). We observed increased intracerebral expression of the proinflammatory cytokines IFNγ and TNF, and of the CC-chemokines, MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and MCP-1/CCL2 at 20 dpi, preceding neuroinflammation. At 45 dpi, when neuroinflammation is present, although the expression of IFNγ, MIP1β/CCL4 and MCP-1/CCL2 were reduced, the intracerebral expression of TNF and other CC-chemokines remained upregulated. Similar results were described in a model of toxoplasmic encephalitis in BALB/c mice, during the acute (10 dpi) and chronic (30 dpi) phases of the infection. In this model, increase in intracerebral mRNA of MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and MCP-1/CCL2 was dependent on IFNγ expression, as leukocyte recruitment to brain tissue was impaired in IFNγ-deficient mice [81]. Astrocytes, microglial cells and inflammatory leukocytes can produce intracerebral chemokines, that might act as facilitators of the recruitment, adherence and transendothelial migration of leukocytes through the BBB to the brain parenchyma, thus influencing the composition of the inflammatory infiltrate [81]. On the other hand, the

peripheral inflammation can cause changes in cytokine levels in the brain through several mechanisms and, therefore, control inflammatory cell invasion of the CNS. Macrophages residing the CNS can be activated through the vascular endothelium [82] or through the circumventricular organs of brain regions devoid of BBB [83]. Also, circulating cytokines can be actively transported across the BBB [82], and may, therefore, activate glial cells to produce cytokines. Thus, leakage of cytokines of systemic plasma, together with the intracerebral cytokines and chemokines, could contribute to the maintenance of neuroinflammation. In our study, most of the assessed inflammatory cytokines (IL-12, IL-6, TNF, IFNγ and CCL2/MCP-1) showed high serum levels in the early chronic infection (at 30 dpi), with peak at 60 dpi and control at 90 dpi, except for IFNγ and CCL2/MCP-1, that persisted elevated. IL-10 serum levels, however, tended to increase only at 60 dpi. Thus, as the specific immune response is established, it may contribute to control parasite growth, leading to decrease the number of parasite cysts in the CNS, and contributing to reduce the stimulus to production of pro-inflammatory cytokines [84]. Previous kinetics study (7 to 70 dpi) in ME-49-infected mice has shown increased IL-12 and IFNγ levels in the acute phase with a decrease in the chronic phase of *T. gondii* infection, but without returning to baseline levels [85]. Molecules of *T. gondii* stimulate innate Toll-like receptors, which lead to the production of IL-12 that with TNF synergistically act to induce IFNγ production, as part of a robust Th1 immune response, crucial to establish an efficient antiparasitic response in the acute phase [86]. In addition, in chronic infection IFNγ is pivotal to control multiplication and dissemination of the parasite within the brain [81]. Indeed, depletion of this cytokine in the chronic phase leads to reactivation of the infection and inflammatory foci in the CNS [87]. Therefore, continuous systemic Th1 immune response, crucially IFNγ, is necessary to control *T. gondii* parasitism in the brain in the chronic phase of infection [84, 88]. IL-6 is traditionally described as a pro-inflammatory cytokine. However, it has been shown that increased IL-6 levels may regulate the production of IL-12 and IFNγ, resulting in an anti-inflammatory signal [89]. On the other hand, IL-6 plays a protective role in chronic infection, as IL-6-deficient ME-49-infected mice show high numbers of cyst and mortality, with severe toxoplasmic encephalitis with areas of necrosis [90]. IL-10 downregulates the expression of IL-12 and Th1 cytokines, but not the CC-chemokine CCL2/MCP-1 [91]. The anti-inflammatory response triggered by IL-10 may act favoring tissue repair but also contributing to the maintenance of cysts in the CNS [92]. The expression of the CC-chemokine CCL2/MCP-1 can be induced by inflammatory stimuli, such as TNF [93]. Moreover, intracerebral CCL2/MCP-1, acting via CCR2, plays a role in activating microbicidal mechanisms that control *T. gondii* parasitism in the CNS [93]. Altogether, our data suggest that the C57BL/6 mice model infected with the ME-49 strain triggered an efficient effector immune response, involving cytokines and CC-chemokines, that contributes to parasite control and reduction in the number of cysts in the CNS. Further, neuroinflammation, which may lead to behavioral changes, could be maintained through positive regulation of the intracerebral and peripheral pro-inflammatory cytokines and CC-chemokines, attracting inflammatory cells. A variety of neuroendocrine, neurochemical and behavioral changes are proposed to be consequence of peripheral immune activation, through the release of pro-inflammatory cytokines [11]. It has been shown that anxiety and depression can be induced by the systemic administration of IFNα in patients with hepatitis C [26]. Even so, mild peripheral inflammation in non-infected humans leads to impaired spatial memory [94]. In C57BL/6 mice infected with the ANKA *Plasmodium berghei* strain, which causes cerebral malaria, and cognitive deficits can be triggered by the early migration of mononuclear cells to the brain, facilitating the increase of chemokine levels in the brain [95]. The transient systemic inflammation induced by intraperitoneal administration of LPS to a mouse model of chronic neurodegenerative disease, can exacerbate inflammation in the CNS and accelerate disease progression as impaired

motor coordination and cognitive function [96]. In absence of neuroinflammation, the role of TNF levels in serum in inducing depression-like behavior was demonstrated in a murine model of experimental chronic Chagas disease [24]. Thus, we suggest that the increased levels of cytokines and CC-chemokines in plasma and their leakage in the CNS may sustain neuroinflammation, which may result in the observed behavioral changes, anxiety, depression, and hyperactivity in chronic *T. gondii* infection.

The disruption of the BBB resulting from the establishment of a vascular or cerebral pathology will result in the leakage of serum-derived components to the CNS, which can lead to brain dysfunction affecting the thinking processes, mood and behavior, and generate psychiatric disorders [97]. A hypothesis has been proposed that the high rate of psychiatric diseases is associated with the breakdown of BBB thus assuming a relationship of peripheral inflammatory processes in psychiatric disorders [11]. In the last decades, data have sustained that disruption of the BBB allows the extravasation of pro-inflammatory cytokines and immune cells that can activate the CNS resident cells and, therefore, induce neurodegeneration, underpinning behavioral alteration [11]. Here, our data show that in the three timepoints BBB integrity was impaired and the relative brain weight was raised, suggestive of brain edema, supporting the leakage of blood-born molecules into the CNS putatively contributing to neuroinflammation. Similar data were obtained in chronically ME-49-infected Swiss Webster [98]. Inflammatory processes in the CNS may contribute to determine the severity and prognosis of neurological and cognitive disorders and can both cause and result from BBB dysfunction [99]. Truly, BBB impairment is associated with brain pathophysiology in several neurological disorders in humans and experimental mice models, including traumatic brain injury [100], stroke [101], epilepsy [102], autoimmune encephalitis, schizophrenia [103], Alzheimer disease [102] and depression [103, 104]. Similarly, psychiatric disorders are related to changes in levels of pro-inflammatory cytokines in serum [105–107]. Patients with bipolar disorder have elevated peripheral levels of IL-6 and TNF [105]. Increased IL-1β, IL-10 and TNF levels are present in patients with depression [106]. Likewise, IL-12, IL-6, TNF and IFNγ peripherical levels are enhanced in patients with schizophrenia [107], and IL-6 and IL-10 levels elevated in children with ADHD [108, 109]. In acute and chronic *T. cruzi* infection, C57BL/6 mice are refractory to neuroinflammation and upregulation of cytokine expression in the CNS but show depressive-like behavior. This behavioral change was associated with increased TNF levels in serum and abolished by anti-TNF antibody [24], reinforcing that in an infectious situation peripheral blood cytokine may overflow into the CNS and contribute to behavioral alterations.

Altogether, our data indicate that persistence of *T. gondii* cysts in the CNS may stimulate intracerebral cytokine and CC-chemokine production, contributing to recruit inflammatory cells, thus sustaining neuroinflammation and BBB disruption, which may allow the leakage of inflammatory mediators into the brain tissue. Hence, in chronic toxoplasmosis the systemic and brain-born inflammatory milieu may contribute to behavioral changes, as anxiety, depression, and hyperactivity (S1 Graphical abstract). Therefore, multifactorial components shall be considered when proposing a therapeutic approach to hamper progression or to reverse mental disorders associated with chronic *T. gondii* infection.

Supporting information

S1 Checklist.
(PDF)

S1 Graphical abstract. In the chronic phase of *T. gondii* infection, the persistence of parasite cysts in the brain may sustain neuroinflammation and BBB disruption, permitting leakage of serum cytokines into the CNS. The CNS inflammatory milieu may contribute to

anxiety, depressive-like behavior, and hyperactivity.
(TIF)

S1 Fig. Flow chart showing the experimental protocol with the number of animals used to assess the expression of intracerebral CC-chemokines and cytokines in non-infected (NI) and *T. gondii*-infected C57BL/6 mice, at 20 dpi and 45 dpi, in two independent experiments.
(TIF)

S2 Fig. Correlation between the number of cysts in the CNS with the behavioral features. (**A**) Correlation between the number of cysts and the immobility time in FTS. (**B**) Correlation between the number of cysts and the left and right forelimb stride in the footprint test. (**C**) Correlation between the number of cysts and the left and right hindlimb stride in the footprint test. Data were analyzed using Pearson's correlation coefficient.
(TIF)

S3 Fig. C57BL/6 mice showed generalized neuroinflammation with a greater presence of cysts in the isocortex, thalamus and midbrain, without influence of the size of the cysts. The analyses are shown the brain areas: Olfactory areas (OLF), Isocortex (ICTX), Cerebral Nuclei (CNU), Hippocampal formation (HPF), Thalamus (TH), Hypothalamus (HY), Midbrain (MB), Pons (P), Medulla (MY) and Cerebellum (CB). (**A**) The histograms show the total number of cysts for each area in the brain in the three analyzed timepoints. Large numbers of cyst were found in the isocortex, thalamus and midbrain areas. (**B**) The histograms show that the size of the cysts was not influenced by the brain region where they were localized. (**C**) The histograms show that most of the cysts were found surrounded or close to inflammatory foci, and a similar pattern was observed in the three evaluated timepoints. Each experimental group consisted of 4–10 *T. gondii*-infected mice. Data were analyzed using ordinary one-way ANOVA.
(TIF)

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ARTIGO 3. Sulfadiazine plus pyrimethamine therapy reversed multiple behavioral and neurocognitive changes in long-term toxoplasmosis by reducing brain cyst load and inflammation-related alterations. Castaño Barrios L *et al*., Submetido (Front. Immunol.)

Neste artigo buscamos abordar questões relacionadas aos objetivos 2, 3, 4 e 5. Primeiramente replicamos as alterações comportamentais anteriormente descritas, e desafiamos a ideia de que o tratamento etiológico na fase crônica pode acrescentar vantagem ao controle intrínseco imuno-mediado dos cistos cerebrais e, impactar as mudanças comportamentais e neurocognitivas. Assim, combinamos sulfadiazina com pirimetamina (S+P), a terapia de primeira escolha clínica para toxoplasmose, para pesquisar a associação da carga de cistos cerebrais e processos biológicos relacionados à resposta imune (neuroinflamação, integridade da barreira hematoencefálica -BHE e níveis séricos de citocinas), com alterações comportamentais e neurocognitivas na infecção crônica de longo prazo. Para tal, fêmeas de camundongos C57BL/6 ($H2^b$) foram infectadas com 5 cistos da cepa ME-49 e tratados com S+P ou Veh por 30 dias consecutivos, 30 a 60 dias após a infecção. Os grupos foram testados nos pontos finais (pré-terapia, 30 dpi; terapia S+P, 60 dpi; e após interrupção da terapia, 90 dpi). Alterações comportamentais e neurocognitivas foram detectadas na infecção crônica precoce (30 dpi) e de longo prazo (60 e 90 dpi). A terapia S+P resolveu alterações locomotoras, ansiedade e comportamento parecido com depressão, resolveu parcial ou transitoriamente a hiperatividade e a perda de memória de habituação, mas não teve efeito nas alterações da memória aversiva. Ademais, melhorou o controle da carga de cistos cerebrais, alterações histopatológicas, ruptura da BHE e níveis séricos de citocinas Th1 nos animais tratados com a terapia, quando comparados com animais que receberam veículo. Observou-se associação entre os níveis séricos de IFNγ, TNF e MCP-1/CCL2, carga de cistos cerebrais e alterações comportamentais e neurocognitivas. Além disso, a análise do componente principal (projeções PCA-2D e 3D) destacou a distinção entre três *clusters* (não infectados; infectados tratados com Veh e infectados com S+P). Em conjunto, nossos dados apoiam a ideia de que a carga de cistos cerebrais pode, direta ou indiretamente, desencadear alterações relacionadas à inflamação, que estão crucialmente associadas a distúrbios comportamentais e neurocognitivos. Portanto, a terapia S+P, adicionando ganho ao controle de cistos cerebrais imuno-mediados, pode impactar a saúde mental de pessoas cronicamente infectadas com *T. gondii* e proporcionar uma melhora na qualidade de vida.

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Sulfadiazine plus Pyrimethamine Therapy reversed Multiple Behavioral and Neurocognitive Changes in Long-Term Chronic Toxoplasmosis by Reducing Brain Cyst Load and Inflammation-Related Alterations

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Author contribution statement

Conceived and designed the experiments: LCB, JLV. Performed the experiments: LCB, ASP, DG, JLV. Analyzed the data: LCB, LLHV, DG, JLV. Wrote the paper: LCB, JLV. Discussed data and revised the manuscript: LCB, AAS, LLHV, JRM, NMS, JLV.

Keywords

Toxoplasma gondii, Anxiety, Depression, hyperactivity, memory loss, Neuroinflammation, Cytokines

Abstract

Word count: 325

Toxoplasma gondii infects one-third of the world population. For decades, it has been considered a silent lifelong infection. However, chronically T. gondii-infected persons may present psychiatric and neurocognitive changes as anxiety, depression, and memory loss. In a model of long-term chronic infection, behavioral alterations parallel neuroinflammation and systemic high cytokine levels, and may reflect brain cyst load. Recent findings support that in chronic infection an active parasite-host interplay involves an immune-mediated control of tissue cysts. Here, we challenged the idea that etiological treatment in chronic phase may add advantage to intrinsic immune-mediated cyst control and impact behavioral changes. Thus, we combined sulfadiazine-plus-pyrimethamine (S+P), the first-choice therapy for toxoplasmosis, to study the association of brain cyst load and the biological processes related to the immune response (neuroinflammation, blood-brain barrier -BBB- disruption and serum cytokine levels), with behavioral and neurocognitive changes of the long-term chronic infection. Female C57BL/6 mice (H-2b) were infected (5 cysts, ME-49 strain) and treated with S+P from 30 to 60 days postinfection (dpi), compared with vehicle (Veh)-treated and noninfected controls. At endpoints (pre-therapy, 30 dpi; S+P therapy, 60 dpi; after ceased therapy, 90 dpi), independent groups were subjected to behavioral tests, and brain tissues and sera were collected. Multiple behavioral and neurocognitive changes were detected in the early (30 dpi) and long-term (60 and 90 dpi) chronic infection. S+P therapy resolved locomotor alterations, anxiety, and depressive-like behavior, partially or transiently ameliorated hyperactivity and habituation memory loss, with no effect on aversive memory changes. S+P therapy improved control of brain cyst load, reduced neuroinflammation and BBB disruption, and the upregulated systemic Th1-cytokine levels. Correlation analysis revealed association between IFNγ, TNF and MCP-1/CCL2 serum levels, brain cyst load and behavioral and neurocognitive alterations. Moreover, the principal-component analysis (PCA-2D and 3D projections) highlighted distinction between clusters (noninfected; Veh-treated and S+P-treated infected). Thus, S+P therapy added gain to immune-mediated brain cyst control and, direct or indirectly, ameliorated inflammation-related alterations, traits crucially associated with behavioral and neurocognitive disorders.

Contribution to the field

Toxoplasma gondii infects one-third of the world population. Although considered a silent lifelong infection, chronic seropositive persons may present psychiatric and neurocognitive changes. In a model of long-term chronic infection, we showed that behavioral and neurocognitive alterations parallel inflammation-related changes (neuroinflammation, blood-brain barrier disruption and systemic high Th1-cytokine levels), and may reflect brain cyst load. Recent data support that in the chronic infection an active parasite-host interplay involves an immune-mediated control of tissue cysts. Here, we challenged the idea that etiological treatment in chronic phase may add advantage to intrinsic immune-mediated cyst control and impact behavioral changes, combining sulfadiazine-plus-pyrimethamine (S+P), therapy for T. gondii infection. Correlation analysis revealed association between IFNγ, TNF and MCP-1/CCL2 serum levels, brain cyst load and behavioral and neurocognitive alterations. The principal-component analysis (PCA-2D and 3D projections) highlighted distinction between clusters (noninfected; Veh-treated and S+P-treated infected), supporting beneficial impact of S+P. In the present scenario of virus infections (zika, chikungunya, Covid-19) of unknown long-lasting consequences, our study may interest the readers of Frontiers in Immunology as chronic infections may cause in situ and systemically inflammation-related alterations, which may contribute to psychiatric and neurocognitive changes.

Ethics statements

Studies involving animal subjects

Generated Statement: The animal study was reviewed and approved by The experimental procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Council for Animal Experimentation. The Animal Use Ethics Committee of Oswaldo Cruz Institute/Fiocruz approved all procedures performed in this study (license L014/2018). .

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Sulfadiazine plus Pyrimethamine Therapy reversed Multiple Behavioral and Neurocognitive Changes in Long-Term Chronic Toxoplasmosis by Reducing Brain Cyst Load and Inflammation-Related Alterations

Running title: Reduction of *T. gondii* brain cyst load improved behavioral and inflammation-related changes

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Key words: *Toxoplasma gondii*, Anxiety, Depression, Hyperactivity, Memory Loss, Neuroinflammation, Cytokines.

ABSTRACT

Toxoplasma gondii infects one-third of the world population. For decades, it has been considered a silent lifelong infection. However, chronically *T. gondii*-infected persons may present psychiatric and neurocognitive changes as anxiety, depression, and memory loss. In a model of long-term chronic infection, behavioral alterations parallel neuroinflammation and systemic high cytokine levels, and may reflect brain cyst load. Recent findings support that in chronic infection an active parasite-host interplay involves an immune-mediated control of tissue cysts. Here, we challenged the idea that etiological treatment in chronic phase may add advantage to intrinsic immune-mediated cyst control and impact behavioral changes. Thus, we combined sulfadiazine-plus-pyrimethamine (S+P), the first-choice therapy for toxoplasmosis, to study the association of brain cyst load and the biological processes related to the immune response (neuroinflammation, blood-brain barrier -BBB- disruption and serum cytokine levels), with behavioral and neurocognitive changes of the long-term chronic infection. Female C57BL/6 mice (H-2^b) were infected (5 cysts, ME-49 strain) and treated with S+P from 30 to 60 days postinfection (dpi), compared with vehicle (Veh)-treated and noninfected controls. At endpoints (pre-therapy, 30 dpi; S+P therapy, 60 dpi; after ceased therapy, 90 dpi), independent groups were subjected to behavioral tests, and brain tissues and sera were collected. Multiple behavioral and neurocognitive changes were detected in the early (30 dpi) and long-term (60 and 90 dpi) chronic infection. S+P therapy resolved locomotor alterations, anxiety, and depressive-like behavior, partially or transiently ameliorated hyperactivity and habituation memory loss, with no effect on aversive memory changes. S+P therapy improved control of brain cyst load, reduced neuroinflammation and BBB disruption, and the upregulated systemic Th1-cytokine levels. Correlation analysis revealed association between IFNγ, TNF and MCP-1/CCL2 serum levels, brain cyst load and behavioral and neurocognitive alterations. Moreover, the principal-component analysis (PCA-2D and 3D projections) highlighted distinction between clusters (noninfected; Veh-treated and S+P-treated infected). Thus, S+P therapy added gain to immune-mediated brain cyst control and, direct or indirectly, ameliorated inflammation-related alterations, traits crucially associated with behavioral and neurocognitive disorders.

INTRODUCTION

Toxoplasma gondii was first described in rabbits by Splendore in Brazil (1) and in rodents of the genera Gundii by Nicolle and Manceaux, in Tunisia (2). Currently one-third of the world population is seropositive for *T. gondii* infection (3). This extremely successful parasite is the etiological agent of toxoplasmosis, a disease of two phases: (i) the acute phase characterized by the presence of circulating tachyzoite forms and (ii) the chronic phase, when cysts containing bradyzoite forms are detected in tissues (4). *T. gondii* may invade any cell type as well as any body tissues, showing, however, tropism for the central nervous system (CNS) (5, 6). Although the success of therapy is guaranteed during the acute phase, there is no etiological treatment capable of eliminating the parasite in the chronic infection, as cysts are mostly resistant to the currently available drugs (7). The folate pathway, involved in DNA synthesis with participation of the enzymes dihydrofolate reductase and dihydrofolate synthetase, is the main target of the available anti-*Toxoplasma* drugs (7). Combined sulfadiazine (S) plus pyrimethamine (P) therapy (S+P) is the first-choice treatment for toxoplasmosis (8). P acts on the parasite dihydrofolate reductase but is unable to distinguish it from the human enzyme. More, P acts synergistically with S, blocking dihydrofolate synthetase, an enzyme indispensable for folate biosynthesis (7, 8). The pathways underlying the S therapeutic effect are still unknown, but changes related to the host metabolism are likely to occur (9). Corticosteroids used in encephalic and ocular toxoplasmosis to minimize tissue damage caused by inflammation (10), may, however, suppress the specific immune response against the parasite and increase the severity of the disease, thus highlighting the role of intrinsic immune response to parasite control (10). Indeed, fulminant ocular toxoplasmosis may occur after application of corticosteroids in monotherapy or combined with antiparasitic treatment (11).

In the acute infection, the robust Th1 immune response, characterized by production of proinflammatory cytokines, as interferon-gamma (IFNγ) and tumor necrosis factor (TNF) allows an efficient antiparasitic response (12, 13). Although the proinflammatory response is sustained during the chronic phase of infection (14, 15), the parasite evades the immune response, remaining in tissues as cysts, mainly in the CNS throughout the host life, in an apparent silent state considered harmless to the host (13). Studies carried out in the chronic phase of infection revealed an active protective innate and adaptive immune response, mediated by phagocytes and perforin-dependent $CD8^+$ T cells, against

mature cysts, thus opening the debate on the concept of quiescent infection, accepted for many years (15). Moreover, infection by *T. gondii* has never been a silent condition. Psychiatric and behavioral changes such as suicide attempts (16), schizophrenia, depression, obsessive-compulsive disorders, personality and bipolar disorders (17), and alteration of neurocognitive functioning (18) have been related to chronic *T. gondii* infection. Several studies investigated the influence of *T. gondii* infection in behavioral and neurocognitive disorders in mouse models, replicating aspects as locomotor alteration (19), anxiety-like disorder (20-25), hyperactivity (20, 25-28), and also alterations in aversive memory consolidation (29, 30), spatial memory loss (31), and long-term memory impairment (24).

The mechanisms underpinning behavioral and neurocognitive changes are not unveiled. In humans, mental disorders with behavioral and neurocognitive alterations have been linked to neuroinflammation and systemic inflammation in infectious and noninfectious diseases (32, 33). Although mostly linked to neuroinflammation, as in Alzheimer disease (32), behavioral and neurocognitive alterations can also be detected in the absence of neuroinflammation (34, 35). Importantly, behavioral changes were described after administration of IFNα to hepatitis virus-infected patients (36). More, systemic inflammatory profile has been associated with behavioral abnormalities in major psychiatric illnesses (33). In chronically *Trypanosoma cruzi*-infected mice, depressive-like behavior was associated with increase in systemic TNF levels and, moreover, reversed by anti-TNF therapy, but independent of neuroinflammation (34). Thus, a more complex network of biological interactions may underpin the onset and progression of behavioral and neurocognitive changes in noninfectious and infectious conditions. In infection by *T. gondii*, neuroinflammation was raised as determinant factor for hyperactivity independent of brain cyst load (26). In ME-49 *T. gondii*-infected male B6CBAF1/J mice, behavioral alterations were associated with inflammation-related processes and a continuous increase in cyst load in the CNS (21). Conversely, a recent study showed that in female C57BL/6 mice ME-49 infection evolves from the early to the long-term chronic phase with reduction of brain cyst load, thus supporting an intrinsic immune-mediated cyst control, and multiple behavioral changes (anxiety-like disorder, depressive-like behavior and hyperactivity) (25). Further, these behavioral alterations occur in a scenario of upregulation of inflammatory cytokines and chemokines in the CNS, neuroinflammation, blood-brain barrier (BBB) disruption, and increase in systemic Th1 cytokine levels (25). Altogether, these findings support the participation of

inflammation-related processes in behavioral changes, probably reflecting brain cyst load, still undisclosed. In the present work, we challenge the idea that the use of the combined etiologic S + P therapy when *T. gondii* infection progresses from the initial to its long-term chronic phase may be advantageous to the intrinsic control of the immune-mediated cyst and impact behavior and neurocognitive changes. To test this idea, female C57BL/6 mice infected with the cystogenic ME-49 type II strain received S+P therapy from early to late chronic phase, from 30 to 60 days postinfection (dpi), and were compared with matched vehicle (Veh)-treated and noninfected (NI) controls. Based on previous work (25), mice were analyzed at endpoints pre-therapy (30 dpi), S+P therapy (60 dpi) and after ceased therapy (90 dpi). We used standardized tests to evaluate locomotor/exploratory activities, anxiety-like disorder, depressive-like behavior, and spatial and aversive memory. Further, we addressed possible biological factors involved, thus evaluating brain cyst load, neuroinflammation, BBB disruption and systemic cytokine levels. Finally, correlation study and principal-component analysis (PCA-2D and 3D projections) were performed.

MATERIAL AND METHODS

Ethics Statement

The experimental procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Council for Animal Experimentation. The Animal Use Ethics Committee of Oswaldo Cruz Institute/Fiocruz approved all procedures performed in this study (license L014/2018). All the data presented were obtained from two independent experiments registered in the Experiment Record Book #73, LBI/IOC-Fiocruz.

Experimental Design

A total of 153 female mice with 3 to 4 weeks of age of the lineage C57BL/6 (H-2^b), was provided by the Institute of Science and Technology in Biomodels (ICTB) of the Oswaldo Cruz Foundation and housed in the Experimental Animal Facility (CEA-CF/IOC unit), under specific pathogen-free conditions. Mice were randomly grouped into groups of 3 to 5 animals and placed in a polypropylene cage lined with pine sawdust and enriched with an igloo, kept in microisolators, and received water and grain-based *ad libitum*. Upon

arrival at the Experimental Animal Facility, the animals remained unhandled in the cages for 15 days to facilitate adaptation to the new environment. The environmental conditions were controlled with temperature of 22 ± 2 °C and a 12-hours cycle of light and dark. After the adjustment period, mice were infected and analyzed according to the experimental protocols (**Supplementary Figure S1**). Upon arrival, the experimental groups were randomly formed. The cages were numbered and classified for experimental infection, and mice were analyzed at the indicated endpoints. The following groups were defined as Experiment 1: (i) Pre-therapy, 30 dpi: 5 NI controls and 12 infected; (ii) Therapy, 60 dpi - mice treated between 30 and 60 dpi, subdivided into two groups (a) Veh-treated: 6 NI controls and 15 infected and (b) S+P-treated: 6 NI controls and 12 infected; and (iii) Ceased Therapy, which consisted of mice evaluated 30 days after therapy cessation (90 dpi), subdivided into 2 groups (a) Veh-treated ceased: 6 NI controls and 24 infected and (b) S+P-treated ceased: 6 NI controls and 21 infected. Experiment 2 (i) Pre-therapy (30 dpi): 4 NI controls and 10 infected; and (ii) Therapy (60 dpi), subdivided into 2 groups (a) Veh-treated: 4 NI controls and 10 infected (b) S+P-treated: 4 NI controls and 8 infected.

Toxoplasma gondii Infection and Clinical Follow-up

Mice were infected by gavage with 0.2 mL of pyrogen-free saline (BioManguinhos, Fiocruz) containing five cysts of the cystogenic ME-49 *T. gondii* strain (37), provided by Dra. Neide Maria da Silva (ICBIM, UFU) and maintained at the Laboratory of Biology of the Interactions (LBI-IOC) by serial passages in female C57BL/6 mice every 60 days. Mice were monitored daily. Weight was evaluated weekly using a mouse precision scale (Sartorius ED623S Milligram Scale, OCE), and following clinical signs were registered: piloerection, apathy, prostration, mobility, posture, aggressive behavior, pain, weight loss and mortality. Signs of pain, isolation from the group, loss of body weight greater than 30% of initial weight, fight injuries, ataxia and immobility were the criteria established to guide the decision-making endpoint for ethical recommendations.

Therapy

Groups of 4 to 6 sex- and age-matched NI controls were submitted to Veh administration by gavage with 0.1 mL of apyrogenic vaccine-graded water (BioManguinhos, Fiocruz). Groups of 8 to 24 *T. gondii*-infected mice were treated for 30 consecutive days using 0.1 mL of Veh or 0.1 mL of Veh containing sulfadiazine (S, 100 mg/Kg, Sulfazina®, Sobral)

and pyrimethamine (P, 4 mg/Kg, Daraprim, Farmoquímica S/A), as described previously (38, 39).

General Conditions for Behavioral Tests

In order to increase familiarity and minimize stress, the environment where the behavioral tests were performed remained under controlled light conditions (cycles of 12-hours of light and 12-hours of dark) at a temperature of 22 ± 2 °C and a noise level of approximately 40 dB produced by an air conditioner. All behavioral tests were performed between 8:00 am and 4:00 pm and recorded using a DSC-DVD810 video camera (Sony). Independent experimental groups (pre-therapy, therapy and ceased therapy) were subjected to behavioral tests at the endpoints. No mouse was subjected to the same test more than once, but a mouse was subjected to different tests to reduce the number of mice used in the present study. Behavioral tests were performed from the least stressful to the most stressful: (i) open field test (OFT), (ii) habituation memory test (iii) grip strength meter test (GSMT), (iv) tail suspension test (TST), (v) forced-swimming test (FST) and (vi) aversive shock evoked test (34). After testing each mouse, the device was cleaned using 70% alcohol and dried with gauze between sessions to eliminate odors and traces of the mouse tested previously.

Open Field Test and Habituation Memory Test

The open field apparatus consists of a 60 cm acrylic cubic box, with white walls and the floor divided by black lines into 49 equal squares, where the animal is exposed to an environment without aversive or rewarding stimuli. The OFT is a straight-forward test to investigate activity or exploratory behavior and assess locomotor impairment in animal models of neuromuscular disease (40), anxiety-related behavior (41), and memory of habituation (42) in rodents. The mouse was allowed to explore freely the open field for 5 minutes for two consecutive days (day one = training; day two = test session). The training session in the open field is used to assess exploratory activity and/or locomotion and anxiety. The exploratory activity was evaluated as the number (units) of vertical activity (rearing behavior). Immobility time (s) in total time was registered. The distance traveled (cm) was calculated by the number of lines crossed during the total time. Speed or velocity was estimated as time spent crossing the lines (cm/s). To assess anxiety, we assayed the time taken exploring the central area (s). Once mice are exposed to a new environment, they prefer to be close to walls or peripheral areas, rather than being

exposed to the central, more exposed area of the field, which means danger. As time elapses, anxiety levels decrease due to habituation and the mouse ventures to explore the central area. A shorter distance traveled in the central area and or less time exploring that area is an indication of an anxious behavior (41, 43). The test session is used to evaluate the habituation memory after the training session (24 hours later). The long-term memory test was performed, in which the procedure was repeated. The memory retention was evaluated by counting the number of total lines crossed on the test session (42) and the individual baseline differences were corrected using the discrimination index (DI) to compare behavior during training and test sessions, as follows: number of crossed lines crossed at day 2 /(number of crossed lines at day 1 + number of lines at day 2), as described (44). DI > 0.6 indicates impairment of memory habituation. The analyzed data were discarded or considered outlier when the animal did not interact with the test in any of the sessions. The 5 min of the training and test session were recorded using a digital video camera (Sony).

Grip Strength Meter Test

The grip strength test is a non-invasive method used to measure neuromuscular function as maximum muscle strength of the mouse limbs (45). We used the grip strength meter apparatus (EFF 305, Insight) to the GMST that consist in a grid connected to a sensor that measures the peak of traction (in gram-force). Three attempts were made in a succession of 15s, when the mouse was lightly pulled by the tail for 2-3 seconds. All grip strength values obtained are normalized by the mouse body weight.

Tail Suspension Test

The tail suspension apparatus (Insight) consists of a box measuring 61.10 cm wide by 55.40 cm high, divided into four equal compartments (15.28 cm) and an aluminum suspension bar placed horizontally at the top, where the animal is hung by the tail with the help of a paper tape. This internal compartment division allows the animals not to touch the other walls of the compartment or observe each other. Hollow cylinders of transparent polycarbonate (4 cm long, 1.6 cm outside diameter, 1.3 cm inside diameter, 1.5 grams) are placed in the mouse's tail to prevent climbing behavior (46). The test is based on the principle that animals placed in a moderately stressful or uncomfortable situation and without escape, will develop an immobile posture or apathetic behavior, indicative of depressive-like behavior (47). The test lasted for 5 min, once the mouse is

suspended from the tail it reacts with active movements such as shaking, swinging vigorously, torsion or jerking the body, and reaching out in an attempt to escape the circumstances. When they begin to tire out, their movements become subtler. The immobility time was recorded. The movements of their front legs alone, body sway from previous movements, passive oscillations and total absence of movement are considered immobility.

Forced Swimming Test

This test was used to assess behavioral impulsivity (48). The forced swimming test consists of a cylinder of 35 cm high and of 25 cm in diameter, which is filled with 20 cm of clean water (25 ± 1 °C). Mice were individually placed inside the cylinder carefully on the surface. The animal was checked for its ability to float or not, then floating mice were maintained for 6 min in the test and non-floating mice were excluded from the test. The first 2 minutes of the test were considered habituation and the total duration of immobility was recorded during the last 4 minutes (49). Immobility was defined as the time during which the mouse remained passively floating, made no attempt to escape and showed only slow movements to keep its head above water. Strong movements with the forelimbs against the cylinder walls and swimming were considered active movements. The water was changed before introducing each animal. After the test, the animal was dried with gauze and returned to its cage.

Aversive Shock Evoked Test

The inhibitory avoidance apparatus (EP 104MR, Insight) consisted of an aluminum box (35 x 28 x 50 cm) epoxy-painted, with acrylic front door (2 mm), whose floor consisted of parallel stainless-steel bars (3 mm diameter) spaced 1 cm apart. A 7 cm wide x 2.5 cm high platform was placed on the floor of the box against the wall on the right-hand side of the compartment. The procedure was modified from the test above (50) and consisted of 3 sessions: (i) pre-exposure session, mice were placed on the platform and allowed to explore the box freely for 1 min with no aversive stimuli; (ii) training session, which was held 2 hours after pre-exposure. Mice were placed on the platform and the latency to descend to the grid with all four paws was measured with a stopwatch. Immediately after stepping on the grid with all four paws, the animals received an aversive stimulus, produced by a 3-second electrical stimulus (0.6 mA) to the paws. The process was repeated until the aversive memory was acquired, which was considered as a descent

latency greater than 120 sec. The number of stimuli needed for memory acquisition was counted to assess memory consolidation; (iii) 24 hours after the training session, the test session took place. The animal was placed again on the platform and the latency to descend was timed, no aversive stimuli were administered, and the reduction latency (maximum of 120 seconds) was used to measure memory retention. An increase latency during testing was considered an improved memory index and vice-versa.

Determination of Blood-Brain Barrier Integrity

Permeability of the BBB was evaluated using the Evans blue (EB) dye, as described previously (51). For this, mice were intraperitoneally sedated with Diazepam (20 mg/Kg). After 15 or 20 min of sedation, 200 µL of Evans Blue dye (Sigma-Aldrich) diluted in a 1% pyrogen-free saline solution (BioManguinhos, Fiocruz) were administered via the orbital plexus, anesthetized previously with the application of topical eye drops. After 2 hours, mice were physically restrained and the maximum blood volume was collected by the orbital plexus, after local anesthesia with topical eye drops. Mice were euthanized at endpoints (pre-therapy, therapy and ceased therapy) using CO_2 inhalation, followed by decapitation. According to a previously described protocol (52), the brains were collected, weighed and sagittally sectioned. Hemi-brain was placed in 1.5 mL Eppendorf tubes containing 500 µL of a 10% formalin for 10 days to extract the EB and the eluate from each hemi-brain was collected and analyzed by spectrophotometry at 620 nm (52). The dye concentration in each sample was determined by means of a standard curve, with serial dilution in the following concentrations: 3 µg/mL; 1 µg/mL; 0.3 µg/mL; 0.1 µcg/mL; 0.03 µg/mL and 0 µg/mL (diluent). The final concentrations were calculated for the whole brain.

Evaluation of Cysts Number and Diameter

Mice were euthanized at the corresponding analysis points. Each brain collected was weighed, sagittally sectionized, and hemi-brain included in a 1.5 mL of phosphate-buffered saline (PBS), and then initially macerated using a 5 mL syringe connected to an 18G hypodermic needle, making gentle movements of aspiration and discarded after tissue disintegration. In order to homogenize the smaller particles, the process was repeated using a 21G hypodermic needle, until a homogenate was obtained. A 20 µL aliquot of the homogenate was evaluated in duplicate by light microscopy to determine the number of cysts present in the brain. The diameter of the cysts was measured using

the digital morphometric device NIS Elements BR version 4.3 of the software (Nikon Co., Japan). The measurements obtained were grouped by classes to determine the frequency distribution of the length of the diameter of the cysts. The number of occurrences in each class was counted to understand the behavior related to the size of the cysts throughout the infection.

Histopathology

Encephala were collected, weighed and sagittally cut, and then a cerebral hemisphere was fixed in a 10% buffered formalin in saline solution for 10 days, dehydrated and embedded in paraffin. Two sections of 4 to 6 μm thick sagittal sections were prepared and stained with hematoxylin and eosin. The slides were scanned using the Motic infinity 100 Scanner and viewed using the VM-Motic Digital Slide Assistance software, version 1.0.7.46. Histopathological changes were analyzed in blind by two independent observers.

Determination of Cytokines in Sera by CBA

After injection of EB dye to analyze BBB integrity (described above), blood was collected through the orbital plexus, after local application of anesthesia eye drops. The collected blood was centrifuged to obtain serum, divided into aliquots, and stored in a freezer at -80°C until use. To measure serum cytokine levels, the BD Cytometric Bead Array (CBA) Mouse Inflammation kit (catalogue 552364, BD Bioscience) was used, according to the manufacturer's recommendations. The kit was used for the simultaneous detection of interleukin 6 (IL-6), interleukin 10 (IL-10), interferon gamma (IFNγ), tumor necrosis factor (TNF), interleukin 12 (IL-12) and monocyte chemotactic protein (MCP-1/CCL2), in a single sample. The cytokine standards were diluted serially to construct the calibration curves, used to determine the concentrations of the cytokines. The samples were analyzed using the 13-Color CytoFLEX-S flow cytometer (Beckman-Coulter, USA). Individual cytokine concentrations were indicated by their fluorescent intensities and expressed in pg/mL, using the FCAP Array Software. The theoretical limits of detection were: 5 pg/mL for IL-6, 2.5 pg/mL for IFNγ, 7.3 pg/mL for TNF, 10.7 pg/mL for IL-12 and 17.5 pg/mL for IL-10.

Statistical Analysis

To assess the normality of the data, the Kolmogorov-Smirnov and Shapiro-Wilks tests were used. To evaluate significant statistic differences between infected groups and NI control groups. When applicable, the Student *t*-test or ANOVA was applied with a 95% confidence level for data with normal distribution and the Mann Whitney test for non-parametric data. Correlation was analyzed using Pearson's correlation coefficient. Statistical tests were performed using the GraphPad Prism version 8.0. Data were expressed as mean and standard error of the mean (SEM). Differences were considered statistically significant when $p < 0.05$.

To visualize simultaneously the most important variables involved in our analysis, we computed principal-component analysis (PCA). This statistical method allows us to identify graphically which cytokines and behaviors are more related with the number of cysts and, therefore, recognizes the values of the variables associated with the performance of the treatment groups. PCA was performed on scaled normalized expression values using the built-in R function PCA() from package FactoMineR (v1.34), and the plots were generated using the ggplot2 (v3.3.3), factoextra (v1.0.7) and pca3d (v0.10.2) R packages. All R analysis was carried out using the RStudio environment (v1.4.1103) (53).

RESULTS

Combined Sulfadiazine plus Pyrimethamine Therapy Increases Weight Gain and Restores Muscle Strength in the Long-term Chronic *Toxoplasma gondii* Infection

Based on the previous kinetic study (25), female C57BL/6 mice were infected and treated with S+P therapy for 30 consecutive days (from 30 to 60 dpi), i.e., from early to late chronic infection. The independent groups were evaluated at three timepoints: (i) in the early chronic phase, pre-therapy (30 dpi); and in the late chronic infection (ii) therapy group (60 dpi) and (iii) 30 days after therapy cessation (90 dpi), as described (**Figure 1A**). A preliminary statistical analysis of the three NI control groups run concurrently with *T. gondii*-infected groups showed no difference ($p > 0.05$) between the NI controls at the three timepoints. For simplicity, and when possible, data collected from all NI mice were pooled and referred as NI in graphs and figures.

Clinical evaluation revealed loss in body weight during the acute phase of infection (up to 15 dpi). After this period, the weight loss stopped. Nevertheless, the bodyweight gained in Veh-treated infected mice remained lower than the NI controls. In the second

week of S+P therapy, an increase in body weight gain in infected mice was noticed, when compared with Veh-treated mice. However, S+P-treated mice did not reach bodyweight comparable to the bodyweight of NI mice (**Figure 1B**). This effect was sustained one month after cessation of S+P treatment (at 90 dpi). Muscle strength progressively decreased in *T. gondii*-infected mice from the early (30 dpi) to the long-term (60 and 90 dpi) chronic infection. Although the loss of muscle strength was reversed by S+P treatment, this effect was transient as 30 days after therapy cessation the loss of muscle strength was evident in S+P-treated mice, alike the Veh-treated group (**Figure 1C**).

Behavioral and neurocognitive tests require interaction with the environment, thus firstly we evaluated exploratory and locomotor activities to check for a possible influence of loss in muscle strength detected in *T. gondii*-infected C57BL/6 mice on test performance. Motor function was assessed in the open field, broadly used to study murine models of muscle diseases as muscular dystrophy (40). The number of rearing and the immobility time were analyzed. No changes in exploratory and locomotor activities were detected in the early chronic phase (pre-therapy group), but a decrease in the number of rearings (**Figure 2A**) was detected in the long-term chronic infection (60 and 90 dpi). Treatment with S+P hampered the onset of this alteration, an effect sustained one month after cessation of therapy (**Figure 2A**). At 30 and 60 dpi, infected mice showed similar time of immobility in the OFT, compared with NI matched controls (**Figure 2B**). At 90 dpi, Veh-treated mice remained immobile longer than NI controls, supporting progression of behavioral changes as infection evolved from 60 to 90 dpi. Nonetheless, S+P therapy hampered the onset of this behavior (**Figure 2B**). Thus, our results indicate that *T. gondii*-infected mice are able to perform the proposed behavioral and neurocognitive tests. Further, there were beneficial effects of S+P therapy on weight gain, muscle strength as well as exploratory and locomotor changes, even after cessation of therapy.

Anxiety-like Disorder and Depressive-like Behavior Present in the Early and Long-term Chronic *Toxoplasma gondii* Infection Are Reversed by S+P Therapy

The anxiety-like disorder was assessed using the OFT, and determined as the time spent in the central zone of the apparatus (41). Compared with NI controls, infected mice remained shorter time in the central area of the open field, at the three timepoints analyzed (**Figure 3A**). Compared with Veh, S+P therapy reversed anxiety-like disorder, and such effect was maintained even after cessation of therapy (**Figure 3A**). Depressive-like behavior was revealed as enhanced time of immobility in the TST (47). When compared

with NI controls, *T. gondii*-infected mice showed increased immobility time at early (30 dpi) and long-term (60 and 90 dpi) stages of the chronic infection. This behavioral alteration was also reversed by S+P therapy. Again, the beneficial effect of S+P was sustained one month after suspension of therapy (**Figure 3B**).

Hyperactive Behavior in C57BL/6 Mice Chronically Infected with *Toxoplasma gondii* Is Responsive to S+P therapy

The FST was used to assess the hyperactive behavior, detected as reduced time in immobility (48). At the early chronic phase (30 dpi), hyperactive behavior was not detected. However, hyperactivity was present in long-term infected Veh-treated mice (60 and 90 dpi), as they remained immobile for shorter time in the FST, compared with NI controls. At 60 dpi, the combined S+P therapy hampered the onset of the hyperactive behavior. This effect was maintained in 58% of the S+P-treated infected mice, even 30 days after therapy discontinuation (**Figure 3C**).

Chronically *Toxoplasma gondii*-infected C57BL/6 Mice Show Transient or Permanent Memory Impairments that Are Selectively Affected by S+P therapy

We evaluated two types of long-term memory during chronic *T. gondii* infection. Habituation memory was assessed using the OFT, comparing the performance in the first and second days of the test (44), shown as discrimination index. In the early (30 dpi) and long-term (60 and 90 dpi) chronic phase of infection, the Veh-treated mice showed habituation memory loss (**Figure 4A**). The S+P therapy reversed habituation memory impairment; however, the beneficial effect was not sustained after cessation of therapy (**Figure 4A**). Aversive memory was assessed by the passive avoidance task (50). At 30 and 60 dpi, ME-49-infected female C57BL/6 mice showed impairment of the aversive memory retention. At 60dpi, S+P therapy had no effect on this long-term memory loss. In the ceased therapy group (90 dpi), 67% of the Veh-treated and 82% of the S+P-treated infected mice presented intact aversive memory (**Figure 4B**), suggesting a transient impairment of this memory and a slight beneficial effect of the S+P therapy. In addition, loss of aversive memory consolidation, shown as an increase in the number of stimuli needed to acquire memory, was detected throughout the infection (**Figure 4C**). At 90 dpi, the S+P therapy showed a partial effect on this alteration, as 67% of the infected mice showed improvement in memory consolidation (**Figure 4C**).

Therapeutic Intervention with S+P Reduces the Number and Size of Brain Cysts

Next, we challenged the relation of *T. gondii* brain cysts with behavioral and neurocognitive changes. The Veh-treated mice showed a significant decrease in the number of cysts in the encephalon as the infection evolved from early (30 dpi) to long-term (60 and 90 dpi) chronic phase (**Figure 5A**), indicating gradual infection control. At the end of the S+P therapy, the decrease in the number of cysts was even greater than in the Veh group, a process sustained after cessation of therapy (**Figure 5A**). A significant correlation was observed between the number of cysts in the CNS and anxiety-like disorder ($p < 0.001$), depressive-like behavior ($p = 0.0026$), and habituation memory loss ($p < 0.0001$). Further, these data reinforced the beneficial role of the S+P therapy leading to reduction in the number of cysts in the CNS associated with improvement of behavioral alterations (**Figure 5B**). Further, the cysts were classified according to diameters into 18 classes and expressed as relative frequency of each class (**Figure 5C**). The size of cysts increased in the Veh-treated mice as infection evolved from early (30 dpi) to long-term (60 and 90 dpi) chronic phase. Indeed, at 60 and 90 dpi, most of the cysts were >42-46 µm, therefore, larger in comparison with cysts size in the early (30 dpi) chronic phase of infection (<37-41nm). After the S+P therapy, the size of the cysts decreased, showing a higher relative frequency of cysts <12-26 µm, a feature that is sustained after cessation of therapy (**Figure 5C**), reinforcing the effect of therapy on control of brain cyst load.

Combined S+P Therapy Reduces the Severity of Neuropathological Alterations in Chronically *Toxoplasma gondii*-infected C57BL/6 Mice

In the early (30 dpi) and long-term (60 and 90 dpi) chronic infection mice showed meningoencephalitis and perivascular inflammatory infiltrates, mainly composed of mononuclear cells, with rare hemorrhagic events (**Figure 5D**). S+P therapy reversed meningoencephalitis and conspicuously reduced inflammatory and hemorrhagic foci. Crucially, these beneficial effects were maintained one month after therapy discontinuation (**Figure 5D**).

Next, we used EB dye to assess BBB integrity (51, 52). Compared with NI controls, the BBB of infected mice was disrupted in the early (30 dpi) and long-term (60 and 90 dpi) chronic phase of the infection. The CNS tissue tonality turned blue due to extravasation of the EB dye into the tissue, which can be observed macroscopically (**Figure 6A**). When compared to age-matched NI controls, increased EB concentrations were detected in the brain of infected mice, at all analyzed timepoints (**Figure 6B**). S+P

therapy improved BBB disruption, detected macroscopically (**Figure 6A**) and colorimetrically (**Figure 6B**), restoring the BBB integrity in 80% (12/15) of treated mice. Even one month after therapy discontinuation, the effect was maintained in 91% (10/11) of the S+P-treated infected mice (**Figure 6B**). In addition, non-treated or Veh-treated infected mice of the three timepoints showed an important increase in relative brain weight (**Figure 6C**). At 60 dpi, S+P therapy reduced the relative brain weight in comparison with the Veh administration. However, this effect was not sustained as the Veh- and the S+P-treated mice presented similar increase in the relative weight of the brain compared with age-matched NI controls, at 90 dpi (**Figure 6C**). Thus, indicating that the S+P therapy transiently reversed the cerebral edema present in chronically *T. gondii*-infected female C57BL/6 mice.

Upregulated Systemic Levels of Cytokines in the Early and Long-term Chronic *Toxoplasma gondii* Infection Are Decreased by S+P Therapy

Lastly, we assessed the systemic immune response evaluating the serum levels of cytokines (IL-6, IL-10, MCP-1/CCL2, IFNγ, TNF, IL-12) in NI controls and *T. gondii*-infected mice. At 30 dpi (pre-therapy) and 60 dpi, among the Veh-treated infected mice IL-6, IL-10, MCP-1/CCL2, IFNγ, TNF, IL-12 levels in serum were upregulated, compared with NI controls (**Figure 7A-B** and **Table 1**). S+P therapy affected the levels of all cytokines (**Figure 7A-B**); however, significant reduction was detected only in IFNγ and TNF serum levels (**Figure 7A-B** and **Table 1**). The cytokine levels in serum of the Veh- and the S+P-treated infected mice after cessation of therapy (90 dpi) tended to decrease. Cytokine levels were alike those found in serum of NI controls, except for IFNγ, MCP-1/CCL2 and IL-12 levels that remained upregulated (**Table 1**).

Systematization of Data

Table 2 shows a summary of the obtained data, independently rated from 0 to 4 for each analyzed parameter. Thus, two independent observers considered a feature (eg, levels of cytokine) or performance in a test in NI controls as absence of alteration (0 = absent). Alterations detected in *T. gondii*-infected mice were graded from mild to severe (1= mild; 2= moderate; 3=severe). Prior scoring, a putative exacerbation of an alteration due to therapy was also considered (4= aggravation). In the early (30 dpi) and long-term (60 and 90 dpi) chronic *T. gondii* infection, behavioral and neurocognitive changes were detected, reproducing relevant aspects of the long-term human toxoplasmosis. Importantly, the S+P

administration to infected mice in the early chronic phase brought selective beneficial effects that were transient or sustained 30 days after cessation of therapy. No aggravation of any detected abnormality was observed in the S+P-treated groups, compared with their matched Veh-treated groups. Indeed, the S+P therapy resolved locomotor alterations, anxiety-like disorder, and depressive-like behavior, partially or transiently solved hyperactivity and habituation memory loss, but presented no effect on aversive memory changes. The beneficial effects of the S+P therapy were paralleled by reduction of brain cyst load, neuroinflammation, BBB disruption and brain edema. Lastly, the favorable impact of the S+P therapy was associated with a decline of the pro-inflammatory Th1 cytokines IFNγ and TNF levels in serum. Altogether, our data support that although acting selectively or transiently on neurocognitive impairments, the S+P therapy resolved locomotor and behavioral (anxiety and depression) alterations.

A correlation analysis clearly revealed significant correlation between IFNγ, TNF and MCP-1/CCL2 levels in serum with brain cyst load. Likewise, the correlation showed impairment of habituation memory and depression behavior associated with brain cyst load and elevated systemic levels of IFNγ and TNF. The CC-chemokine MCP1/CCL2 showed a relationship with impairment of habituation memory. Finally, a weak association between TNF levels and impairment of consolidation of aversive memory was also observed (**Figure 8A**). Further, to visualize the similarities between groups and identify putative clusters of differentiation, we performed a PCA using data of behavioral and neurocognitive abnormalities and proinflammatory cytokine levels in serum, comparing NI controls, the Veh-treated and the S+P-treated infected mice. The four first principal components were used in order to explain 73.5% of the variance. The 2D projection of the samples using the two first principal components, which explain 49.81% of the variance, confirmed the association between brain cyst load, serum levels of the cytokines IFNγ, TNF and MCP-1/CCL2 and behavioral and neurocognitive abnormalities (**Figure 8B**). PC3 and PC4 showed similar behavior as shown in PC1 and PC2 (**Figure 8C-D**). We found that 3D projections, with the three first principal components explaining the 63.25%, emphasized the differences between the three groups, distinguishing the Veh-treated from the NI control cluster and the S+P-treated from the Veh-treated cluster. Moreover, the NI control and the S+P-treated clusters presented a similar behavior (**Figure 8E**). In summary, PCA indicates that brain cyst load and elevated systemic proinflammatory cytokine levels are associated with behavioral/neurocognitive changes in the Veh-treated infected mice. Conversely, low

levels of cytokines and absence or reduction of brain cyst load is associated with the absence of behavioral/neurocognitive alterations in the NI controls and resolution/amelioration of these changes in the S+P-treated mice. Altogether, these data support that the S+P therapy adds significant advantage to intrinsic immune-mediated cyst control in chronically *T. gondii*-infected female C57BL/6 mice, thus impacting behavioral and neurocognitive changes.

DISCUSSION

In order to shed light on the mechanisms underlying behavioral alterations in *T. gondii* infection in the present study, firstly we showed that in C57BL/6 mice chronically infected with the ME-49 strain some behavioral and neurocognitive changes described in human patients are replicated. In the early and long-term chronic infection, female C57BL/6 mice showed locomotor disorders, behavioral alterations (anxiety-like disorder, depressive-like behavior, and hyperactivity) and neurocognitive impairments (habituation and, partially, aversive memory loss). Mostly, these abnormalities were related with brain cyst load, neuroinflammation, BBB disruption, and systemic inflammatory profile. Although in ME-49-infected female C57BL/6 mice brain cyst load is reduced as infection evolves from early to long-term chronic phase, it was not associated with improvement of behavioral and immune-related abnormalities. Depending on the studied feature, a selective (total, partial or transient) beneficial effect of the S+P therapy was detected on behavioral and neurocognitive alterations, which were paralleled by drastic reduction of brain cyst load and neuroinflammation, partial restoration of the BBB disruption and decrease in the systemic inflammatory cytokine levels. Correlation analysis revealed association between IFNγ, TNF and MCP-1/CCL2 serum levels, brain cyst load and behavioral and neurocognitive alterations. Moreover, PCA 2D and 3D projections clearly highlighted the existence of three distinct clusters (NI controls, Veh-treated and S+P-treated) and, particularly, distinguished the clusters representing the groups of the Veh- and the S+P-treated infected mice.

T. gondii-infected mice showed weight loss during the acute phase (15 dpi), which ceased in the early and long-term chronic infection. This dynamic of weight loss during the acute phase of *T. gondii* infection has been widely documented in murine models (14, 28, 54-56). Here, mice treated with the S+P in the early chronic phase showed increase of body weight. The S+P treatment in the acute phase of infection of C57BL/6 mice with

601 the Fukaya (type II) strain hampered weight loss, supporting that the combined therapy
602 controlled parasite dissemination and, as consequence, suppressed body weight loss (56).
603 Decreased muscle strength, another common feature of murine *T. gondii* infection (14,
604 19, 55), was replicated in ME-49-infected female C57BL/6 mice (25). S monotherapy
605 administered to chronically ME-49-infected Swiss Webster mice did not restore muscle
606 strength (14). Presently, we showed that even though it is transiently, the S+P therapy
607 restored neuromuscular strength, supporting the need of the combined therapy, as here,
608 proposed.

609 In the early (30 dpi) and long-term (60 and 90 dpi) chronic infection, alterations in
610 locomotor and exploratory activities, behavioral changes (anxiety-like disorder,
611 depressive-like behavior, hyperactivity), and neurocognitive impairments (habituation
612 memory, and retention and consolidation of aversive memory) were concurrently
613 recorded in the ME-49-infected female C57BL/6 mice. Similarly, decreased numbers of
614 rearing (19), anxiety (20-25), hyperactivity (20, 25-28), impaired spatial memory (31)
615 and alteration in the aversive memory consolidation (29, 30) have been reported
616 independently in different studies using murine models of *T. gondii* infection. In contrast,
617 the absence of behavioral changes or controversial findings have also been reported in *T.*
618 *gondii*-infected mice. In contrast, locomotor alterations with increased number of rearings
619 (23) or the absence of any type of locomotor alteration (24, 30), the absence of anxiety-
620 like disorder (19, 28, 30) and even decreased of general anxiety (21) have also been
621 described. These controversial results may be attributed to differences between the host
622 (murine lineages and gender), the parasite (*T. gondii* strains, infective stages and
623 inoculum) and settings to assess behavioral changes (methodologies, environmental
624 conditions, timepoints of evaluation after infection) (57). Therefore, the here adopted
625 experimental model using C57BL/6 mice infected with low inoculum (5 cysts) of the ME-
626 49 strain simultaneously replicates crucial behavioral and neurocognitive changes
627 detected in the long-term human toxoplasmosis (17, 18), and therefore is appropriate to
628 challenge the effects of the intrinsic immune response controlling brain cysts (25) and the
629 effects of the S+P therapy on these changes.

630 The effects of the intrinsic brain cyst control as infection evolves from early to long-
631 term chronic infection in ME-49-infected female C57BL/6 mice (25) was replicated here,
632 contrasting with the increase in cyst load in ME-49-infected male B6CBAF1/J mice (21).
633 A relationship between the parasite load and the severity of behavioral changes, such as
634 loss of predator fear, the exploratory behavior and anxiety has been observed (21). In

C57BL/6J mice infected with genetically modified tachyzoites of the ME-49 strain only mice with brain cysts, but not the ones devoid of cysts in the CNS, exhibited behavioral changes (28), supporting that parasite cysts in the CNS are crucial for these abnormalities. The here described reduction in brain cyst load from early to late chronic infection observed in the Veh-treated infected mice was not enough to impact behavioral and neurocognitive changes. Thus, we proposed the use of the S+P therapy to add advantage to the immune-mediated cyst control (15), thus challenging the effects on behavioral and neurocognitive changes. Here, we described a positive correlation between the number of cysts in the CNS and behavioral and neurocognitive alterations. Furthermore, reduction of brain cyst load with the S+P therapy was related with transient resolution of muscle strength loss, hyperactivity and habituation memory, partial restoration of BBB disruption and of impaired aversive memory consolidation, and total resolution of the anxiety-like disorder and depressive-like behavior. In addition, the S+P scheme drastically reduced the number of brain cysts, an effect sustained after ceased therapy. Despite that, alterations of retention and consolidation of aversive memory are still present. Notably, there was a positive correlation between the number of cysts and the anxiety-like disorder, the depressive-like behavior, and habituation memory impairment. Beneficial effects of the combined S+P therapy have been shown previously. Children and infants, who were born with severe involvement of the CNS because of congenital toxoplasmosis and treated for one year with the S+P plus folinic acid presented normal development and preserved intellectual function (58). In a randomized study carried out with AIDS patients with toxoplasmic encephalitis, the S+P therapy offered a better primary outcome in terms of mortality and brain herniation compared to trimethoprim-sulfamethoxazole (TMP-SMX) (59). Crucially, in *T. gondii*-seropositive AIDS patients treated with the S+P scheme experienced complete resolution or partial improvement of toxoplasmic encephalitis, which, however, relapsed within 6 weeks after discontinuation of treatment (60). Effectiveness of the S+P therapy has been tested in murine models, exploring features as survival (61-63), histopathological tissue damage (64) and parasite load in distinct organs (62, 63, 65). Combined S+P administered in the acute phase to ME-49-infected CF1 mice resulted in reduction of brain cysts (63). Nevertheless, effectiveness of the S+P therapy on behavioral and neurocognitive functions in chronic infection has been neglected, despite alternative treatments have been tested. Recent report shows that chronically ME-49-infected BALB/c mice treated with rosuvastatin, which reduces the burden of brain cysts and attenuate neuroinflammation, showed reversal of anxiety and

impairment of memory recognition of new objects (24). Swiss mice acutely infected with tachyzoites of the VEG type III *T. gondii* strain and treated with resveratrol plus TMP-SMX showed reduced number of brain cysts and amelioration of anxiety and aversive memory (30). Interestingly, in infected C57BL/6J mice, guanabenz, an anti-hypertensive drug effectively reduced hyperactivity, neuroinflammation and perivascular cuff, independently of the brain cyst load (26). Similarly, in chronically Prugniaud strain-infected BALB/cJ mice, the oral treatment with guanabenz reversed hyperactivity without a decrease in the number of brain cysts (26). Overall, improvement in behavioral and neurocognitive changes after therapy, with or without decline in brain cyst load, supports additional biological mechanisms underlying these alterations in *T. gondii* infection.

Here, we showed that in the long-term chronic *T. gondii* infection besides reducing brain cyst load, the S+P therapy also reversed meningoencephalitis and decreased inflammatory foci and hemorrhagic spots in the parenchyma, in an apparent long-lasting way. In a murine model of reactivated toxoplasmosis in an interferon regulatory factor 8-deficient mice infected with the ME-49 strain, the S+P therapy hampered the onset of toxoplasmic encephalitis and brain parasitism (66). In a murine model, the S monotherapy administered from the acute to early chronic phase partially improved microvascular damage and reduced parasite load in the brain (67). Further, monotherapy with S or P in ME-49-infected CBA/Ca mice did not reduce neuroinflammation (64). In chronically ME-49-infected Swiss Webster mice, the S monotherapy in drinking water was not effective for treating neuropathological signs, despite the reduced number of cysts (14). Therefore, our data show for the first time that combining the S+P therapy was more effective than the S or the P monotherapy to promote parasite control and reduce neuroinflammation in long-term chronic *T. gondii* infection is quite relevant.

Here, BBB integrity was impaired during the early (30 dpi) and long-term (60 and 90 dpi) chronic *T. gondii*-infection of female C57BL/6 mice, as shown previously (25). Monotherapy with S administered to Swiss Webster mice from the acute to chronic phase (from 8 to 40 dpi) partially restored BBB integrity in 27% (67). Our data show that the S+P therapy restored efficiently BBB integrity, reducing EB dye leakage into the CNS in 80% of the infected mice, reinforcing the efficacy of combined therapy. Our mouse model of early and long-term chronic infection presents a Th1 immune response profile, which is crucial to establish an efficient antiparasitic response (12). More, our chronically ME-49-infected Veh-treated infected mice exhibited high systemic levels of the inflammatory

cytokines IL-12, IL-6, TNF, IFNγ and MCP-1/CCL2, corroborating previous data (25). Crucially, brain cyst load was correlated with IFNγ, TNF and MCP-1/CCL2 serum levels. The S+P therapy resulted in reduction of inflammatory cytokine concentrations in serum, particularly decreasing the Th1 cytokines IFNγ and TNF levels. We noticed that although IFNγ and MCP-1/CCL2 levels were notably reduced, they remained elevated when compared with NI controls. In murine toxoplasmosis, one of the main functions of IFNγ is the induction of TNF, which is produced by macrophages, microglial cells and astrocytes in the CNS, playing a role in parasite control, thus enabling mice survival during the acute and chronic phases of the infection. Furthermore, TNF together with IL-6 can reduce parasite replication (12). Indeed, anti-TNF antibody aggravates toxoplasmic encephalitis in mice (68). In addition, the levels of the regulatory cytokine IL-10 increased in pre-therapy (30 dpi) and in the Veh-treated (60 dpi) mice, and the maximum values were reduced after the S+P therapy. In ME-49-infected C57BL/6 mice, short-term S monotherapy reduced IL-12, IL-10 and IFNγ levels (69). Here, we showed that 30 days after the S+P cessation of the therapy, the regulatory effect was sustained, except for IFNγ and TNF levels that remained upregulated, compared with NI controls, at this timepoint. Altogether, these data indicate that the effects of the S+P therapy, reducing neuroinflammation and systemic cytokines levels, were partially sustained in our model of chronic *T. gondii* infection (summarized in **Table 2**). Furthermore, multivariate and principal component analyses support the relationship between behavioral and neurocognitive alterations and elevated systemic cytokine levels. Thus, our results point toward systemic Th1 cytokines as key players to be further challenged as trait-related biomarker associated with efficacy of treatments for behavioral and neurocognitive changes in chronic *T. gondii* infection.

We have reported previously that in ME-49-infected female C57BL/6 mice, upregulation of intracerebral levels of TNF, MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and MCP-1/CCL2 precedes the onset and persisted after the establishment of behavioral alterations anxiety, depressive-like behavior and hyperactivity (25). In ME-49-infected male B6CBAF1/J mice, behavioral changes occur in a scenario of downregulation of physiological signaling (amine ligand-binding and serotonin receptors) and upregulation of inflammatory signaling pathways in the CNS (21). Brain inflammation may present a causal effect in the development of psychiatric disorders and cognition loss (32). In humans, peripheral immunostimulation leads to impaired spatial memory (70). Further, systemic administration of IFNα can induce

anxiety and depression in hepatitis virus-infected patients (36). Factors related to inflammation, such as peripheral pro-inflammatory cytokines, have been the subject of research as biomarkers in psychiatric disorders (33). Chronic *T. gondii* infection induces a wide variety of behavioral and neurocognitive changes, which may depend on the experimental models and protocols used for evaluation, as already mentioned (57), but having in common the presence of neuroinflammation, a feature of *T. gondii* infection (20, 21, 24-26, 28, 30, 60). Thus, it is reasonable that the behavioral and neurocognitive changes reflect the process of persistence of cysts in the brain tissue, but also in peripheral tissues, which can continuously stimulate the upregulation of systemic pro-inflammatory cytokine levels, and may increase BBB permeability and leak into the CNS, sustaining neuroinflammation, an idea supported by the profile of responses to therapy showed in the present study. Indeed, PCA supports the relation of systemic cytokine levels with behavioral and neurocognitive changes, reinforcing those distinct clusters reveal the beneficial effects of the S+P therapy. Regardless of the limitation of our results for the lack of known status of brain cytokines and other neuromediators and neurotransmitters in the S+P-treated infected mice, the favorable and, in some cases, sustained action of this therapeutic scheme on cyst control, systemic cytokine levels, and behavioral and neurocognitive changes reinforce that chronically *T. gondii*-infected patients may benefit from the combined S+P therapy and have a better quality of life.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Use Ethics Committee of IOC/Fiocruz (L014/2018).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LCB, JLV. Performed the experiments: LCB, ASP, DG, JLV. Analyzed the data: LCB, LLHV, DG, JLV. Wrote the paper: LCB, JLV. Discussed data and revised the manuscript: LCB, AAS, LLHV, JRM, NMS, JLV.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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TABLE 1. Cytokine levels in serum of *Toxoplasma gondii*-infected mice.

		Pre-therapy		**Therapy**				**Ceased Therapy**			
	NI Control	Veh		Veh		S+P		Veh		S+P	
Cytokine	Mean ± SEM	Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value
IL-6	93.87 ± 19.36	225.10 ± 41.96	**0.015**	153.00 ± 80.70	0.456	107.90 ± 26.23	0.693	97.04 ± 57.61	•0.857	90.87 ± 16.88	•0.857
IL-10	19.91 ± 1.68	26.00 ± 5.68	0.262	56.47 ± 30.20	•0.052	23.67 ± 4.18	•0.282	16.01 ± 3.22	•0.222	19.09 ± 0.10	•0.5
MCP-1/CCL2	164.5 ± 34.89	360.00 ± 33.68	**0.003**	281.9 ± 58.40	0.096	164.1 ± 34.92	0.995	319.6 ± 40.40	**0.012**	207.20 ± 54.82	0.225
IFNɣ	4.667 ± 0.69	180.20 ± 13.96	**<0.0001**	120.2 ± 27.68	**0.0005**	39.84 ± 8.51	**0.002**	110.30 ± 79.39	**0.017**	70.69 ± 20.86	**0.0008**
TNF	19.79 ± 1.74	65.74 ± 14.33	**0.003**	78.56 ± 33.08	**•0.003**	32.45 ± 4.88	**•0.040**	28.32 ± 10.59	0.177	38.04 ± 2.39	**0.001**
IL-12	35.09 ± 2.85	43.56 ± 11.24	0.413	95.83 ± 33.71	**•0.018**	55.05 ± 23.42	•0.779	18.62 ± 5.68	•0.111	43.54 ± 0.49	•0.222

•p-values calculated using the Mann-Whitney test. Statistically significant p-value < 0.05 (bold); infected group compared to NI control group.

TABLE 2. Effectiveness of sulfadiazine plus pyrimethamine therapy in long-term chronic *T. gondii* infection.

		Pre-therapy	**Therapy**		**Ceased Therapy**	
Parameter	NI Control	Veh	Veh	S+P	Veh	S+P
Weight loss	0	2	1	0	1	0
Neuromuscular function						
Muscle strength	0	1	1	0	2	2
Motor function						
Rearing	0	0	1	0	1	0
Immobility time (OFT)	0	0	0	0	1	0
Behavioral changes						
Anxiety	0	1	1	0	1	0
Depression	0	1	1	0	1	0
Hyperativity	0	0	1	0	3	1
Cognitive impairments						
Habituation memory	0	1	1	0	1	1
Aversive memory retention	0	1	1	1	0	0
Aversive memory consolidation	0	3	3	2	3	1
Brain cyst						
Number	0	3	3	2	2	1
Size	0	2	3	1	3	1
Neuropathological alterations						
Meningoencephalitis	0	2	3	1	2	1
Perivascular infiltrates	0	2	3	1	2	1
BBB integrity	0	2	3	1	2	1
Cytokine regulation						
IL-6	0	3	2	1	0	0
IL-10	0	1	2	1	0	0
MCP-1/CCL2	0	3	2	1	3	2
IFNɣ	0	3	3	1	3	2
TNF	0	3	3	2	1	2
IL-12	0	1	3	2	0	1
Total score	**0**	**35**	**41**	**17**	**32**	**17**

The changes and the effect of the therapy were rated from 0 to 4, being 0 = absent; 1= mild; 2= moderate; 3=severe; 4= aggravation.

FIGURE LEGENDS

FIGURE 1: Effect of combined sulfadiazine plus pyrimethamine therapy on weight gain and muscle strength in the long-term chronic *Toxoplasma gondii* infection. (**A**) Mice were infected with 5 cysts of the ME-49 *T. gondii* strain, and after the onset of chronic long-term infection, they were treated orally with the combination of sulfadiazine plus pyrimethamine, and evaluated immediately after finishing the treatment (therapy) and 30 days after suspension (ceased therapy). (**B**) The S+P therapy increased gain in body weight of infected mice. (**C**) The S+P therapy reversed the loss of muscle strength in infected mice, but the effect is transient. Muscle strength values are presented as gram strength (gf) / body weight (g). Each experimental group consisted of 4-6 NI mice and 8-21 mice infected with *T. gondii*. Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the Welch's test (**B**) and *t*-Student test or ordinary one-way ANOVA (**C**). **, $p<0.01$. ***, $p<0.001$, comparing mice infected with *T. gondii* and NI control mice. ##, $p<0.01$, comparing Veh-treated and S+P-treated *T. gondii*-infected mice.

FIGURE 2: Sulfadiazine plus pyrimethamine strategy hampers locomotor and exploratory alterations in the long-term chronic *T. gondii* infection. (**A**) In long-term chronic infection the number of rearings decreased, and reversed with the S+P therapy. (**B**) The S+P therapy prevented the onset of locomotor alteration in long-term chronic *T. gondii*-infected mice. Each experimental group consisted of 4-6 NI mice and 7-14 mice infected with *T. gondii.* Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the *t*-Student test (**A**) and *t*-Student test or ordinary one-way ANOVA (**B**). *, $p<0.05$. ***, $p<0.001$, comparing mice infected with *T. gondii* and NI control mice. #, $p<0.05$, ###, $p<0.001$ comparing Veh-treated and S+P-treated *T. gondii*-infected mice.

FIGURE 3: Sulfadiazine plus pyrimethamine therapy reverses behavioral changes present in the early and long-term chronic *T. gondii* infection. Chronically infected mice showed (**A**) anxiety, (**B**) depressive-like behavior, and (**C**) hyperactivity. The S+P therapy reversed these behavioral changes permanently (**A-B**) or transiently (**C**). Each experimental group consisted of 4-6 NI mice and 8-15 mice infected with *T. gondii.* Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the *t*-Student test (**A**) and *t*-Student test or ordinary one-way ANOVA (**B-C**). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$,

comparing mice infected with *T. gondii* and NI control mice. #, p<0.05, ##, p<0.01 ###, p<0.001 comparing the Veh-treated and the S+P-treated *T. gondii*-infected mice.

FIGURE 4: Sulfadiazine plus pyrimethamine therapy selectively reverses impairments of memory present in the early and long-term chronic *T. gondii* infection. (**A**) The effect of the S+P therapy in reversing the habituation memory alteration is transitory. (**B**) Chronically *T. gondii*-infected mice showed transient aversive memory retention impairment, or were unresponsive to the S+P therapy, and (**C**) aversive memory consolidation alterations showed partial improvement after the S+P therapy. Each experimental group consisted of 4-6 NI mice and 7-14 mice infected with *T. gondii*. Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the *t*-Student test or ordinary one-way ANOVA (A), the Mann-Whitney test (**B**) and the *t*-Student or Mann-Whitney test (**C**) . *, p<0.05, **, p<0.01, ***, p<0.001, comparing mice infected with *T. gondii* and NI control mice. ##, p<0.01 ###, p<0.001 comparing Veh-treated and S+P-treated *T. gondii*-infected mice.

FIGURE 5: Sulfadiazine plus pyrimethamine therapy impacts the number and size of parasite cysts and histological changes in the CNS in the early and long-term chronic *T. gondii* infection. (**A**) Infected C57BL/6 mice showed gradual control of brain cysts, which was improved by the S+P therapy. (**B**) The presence of anxiety, depressive-like behavior and habituation memory impairments was positively correlated with cyst numbers. (**C**) The S+P therapy reduced the size of brain cysts, (**D**) reversed meningoencephalitis and reduced hemorrhagic foci in chronically infected mice. Bar = 100 and 300 µm. Each experimental group consisted of 8-12 mice infected with *T. gondii*. Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the ordinary one-way ANOVA (**A**) and Pearson's correlation coefficient (**B**). ##, p<0.01 ###, p<0.001 comparing the pre-thetrapy group whit the others group, $, p<0.01, $$$, p<0.001 comparing the Veh-treated with others, and &&, p<0.01, comparing the Veh-treated whit the S+P-treated.

FIGURE 6: Sulfadiazine plus pyrimethamine therapy partially restores the integrity of the blood-brain barrier and reduces parcially the cerebral edema in the early and long-term chronic *T. gondii* infection of C57BL/6 mice. (**A**) Representative brain images show EB extravasation macroscopically visible, and the S+P therapy reversed this effect. (**B**) EB

concentrations increased in the brain tissues of infected mice were decreased by the S+P therapy. (**C**) The increase in the relative brain weight of infected mice, an indicative of cerebral edema, was partially reversed by the S+P therapy. Each experimental group consisted of 4-6 NI mice and 8-13 mice infected with *T. gondii.* Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the Mann-Whitey test (**B**) *t*-Student test (**C**). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, comparing mice infected with *T. gondii* and NI control mice, $^{\#\#}$, $p<0.01$ $^{\#\#\#}$, $p<0.001$ comparing the Veh-treated and the S+P-treated *T. gondii*-infected mice.

FIGURE 7: Sulfadiazine plus pyrimethamine therapy reduces the increased inflammatory cytokine serum levels in the early and long-term chronic *T. gondii* infection**.** (**A**) The images show representative data plots of the FACS analysis of CBA of NI control, infected and therapy groups. (**B**) IL-6, IL-10, MCP-1/CCL2, IFNγ, TNF, IL-12 of the Veh-treated infected mice were upregulated compared with NI controls, and the S+P therapy affected the levels of all cytokines. Each experimental group consisted of 2-3 NI mice and 2-8 mice infected with *T. gondii.* Each circle represents an individual mouse. Data are expressed as means ± SEM and were analyzed using the Mann-Whitey test (**B**) and *t*-Student test (**C**). *, $p<0.05$, **, $p<0.01$, comparing mice infected with *T. gondii* and NI control mice, #, $p<0.05$ ##, $p<0.01$.

FIGURE 8: Relationship between brain cyst load, cytokines and behavioral and neurocognitive changes. (**A**) Significant correlation is represented by a circle, the color indicates the strength of this correlation. The "x" indicates the absence of a significant correlation between the variables. (**B-D**) The four first principal components explain 73.5% of the variance, and the 2D projection of (**B**) CP1-CP2, (**C**) CP1-CP3 and (**D**) CP1-CP4 indicated the association between brain cyst load, serum cytokine levels and behavioral and neurocognitive abnormalities. (**E**) The 3D projections, with the three first principal showed that the NI control and the S+P-treated clusters showed a similar behavior, and demonstrated the differences between the Veh-treated and NI control cluster and the S+P-treated and the Veh-treated cluster.

LEGENDS OF SUPPLEMENTARY FIGURES

FIGURE S1: Flow chart showing the experimental protocol with the experimental *n* in 2 (pre-therapy and therapy group) or 1 (ceased therapy group) independent experiments and survival.

Figure 1.TIF

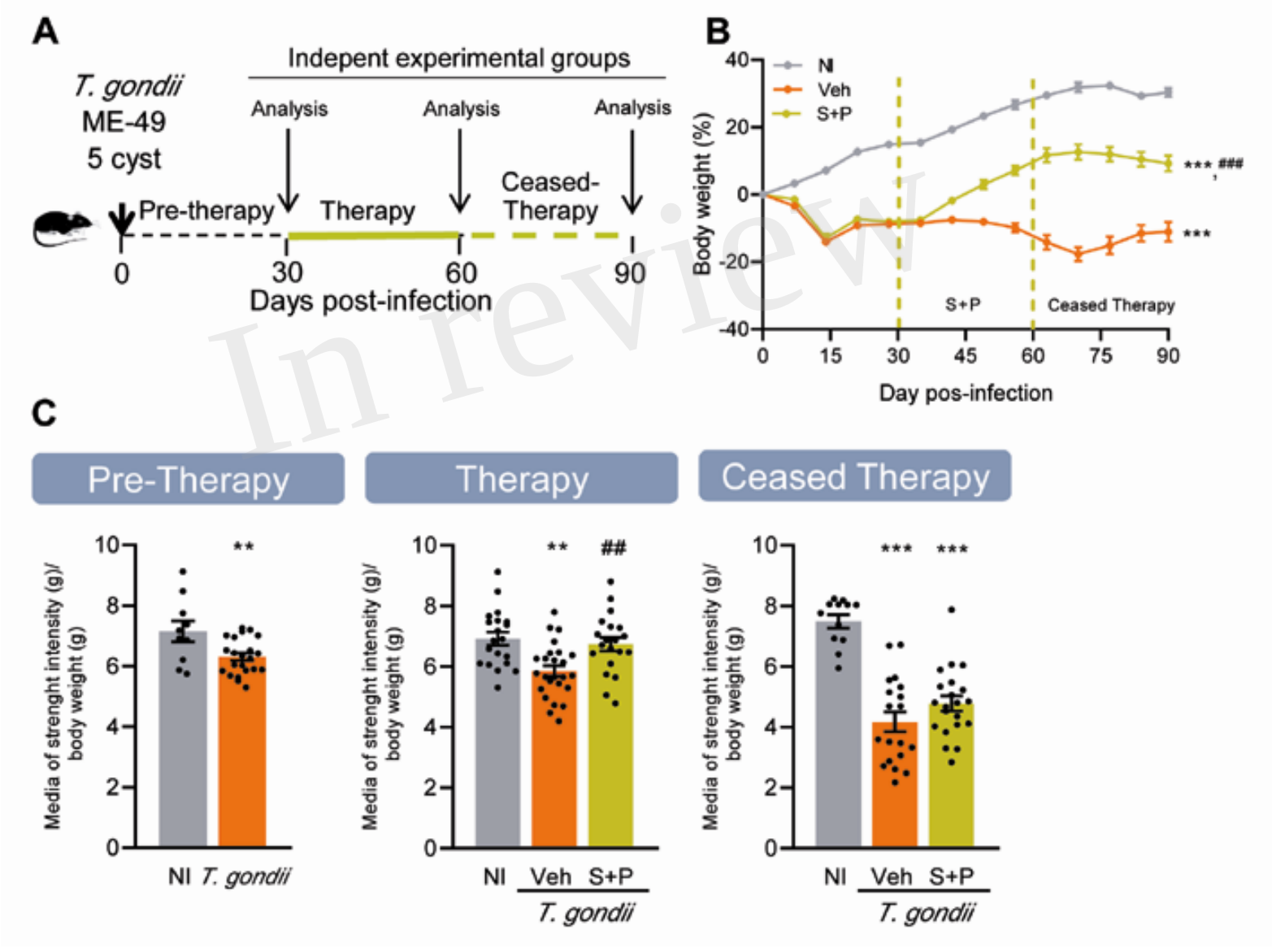


Figure 2.TIF

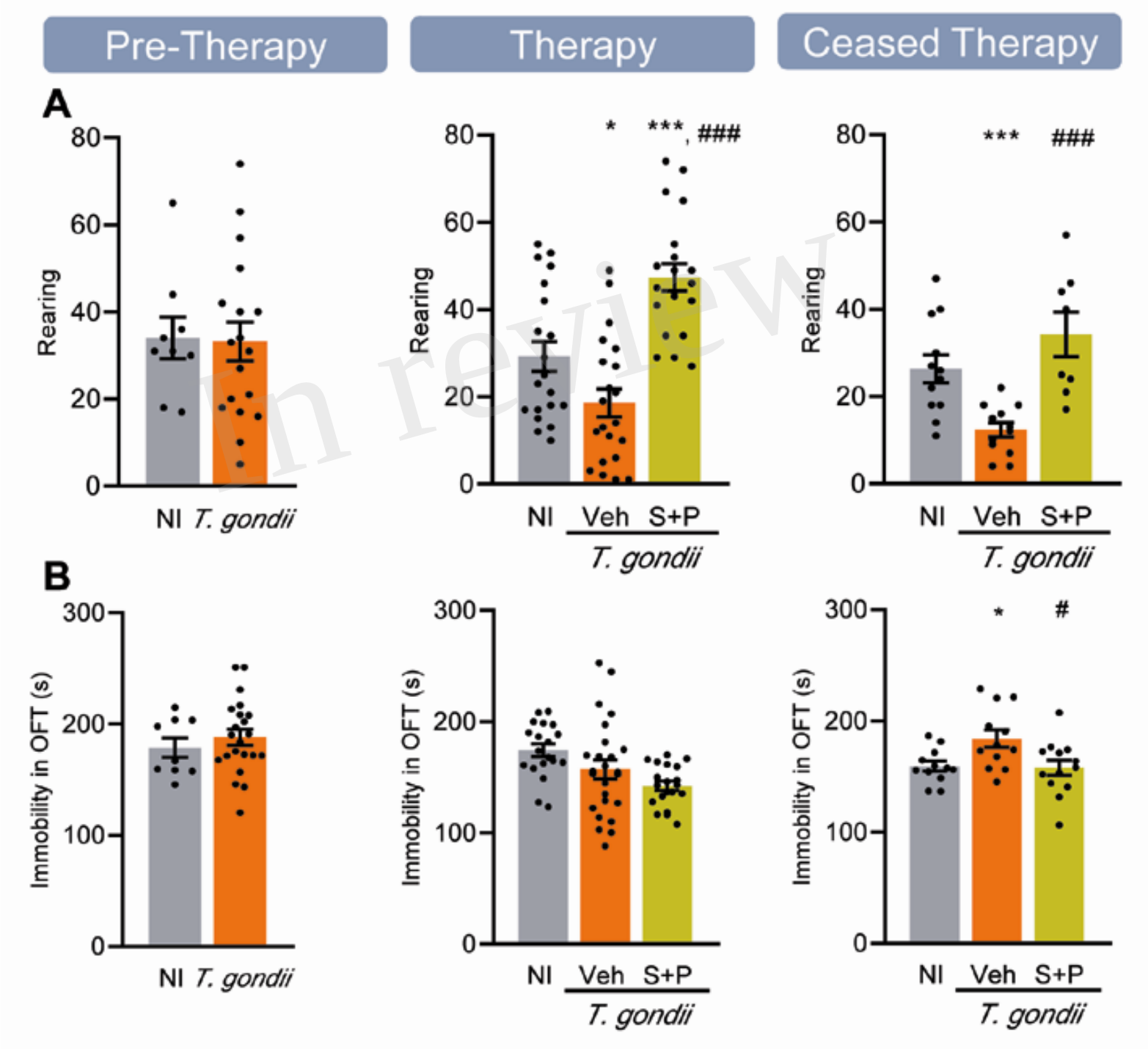


Figure 3.TIF

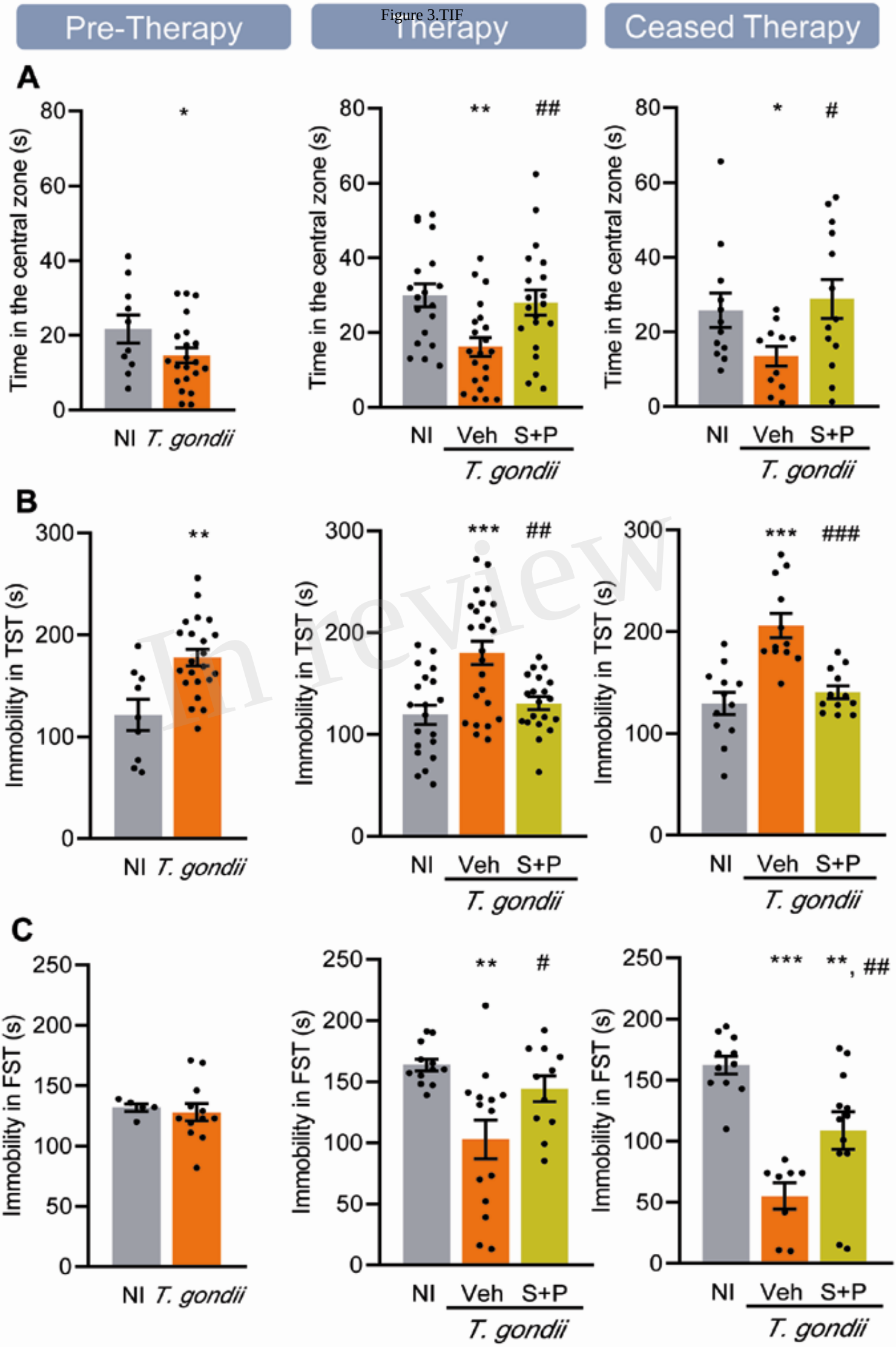


Figure 4.TIF

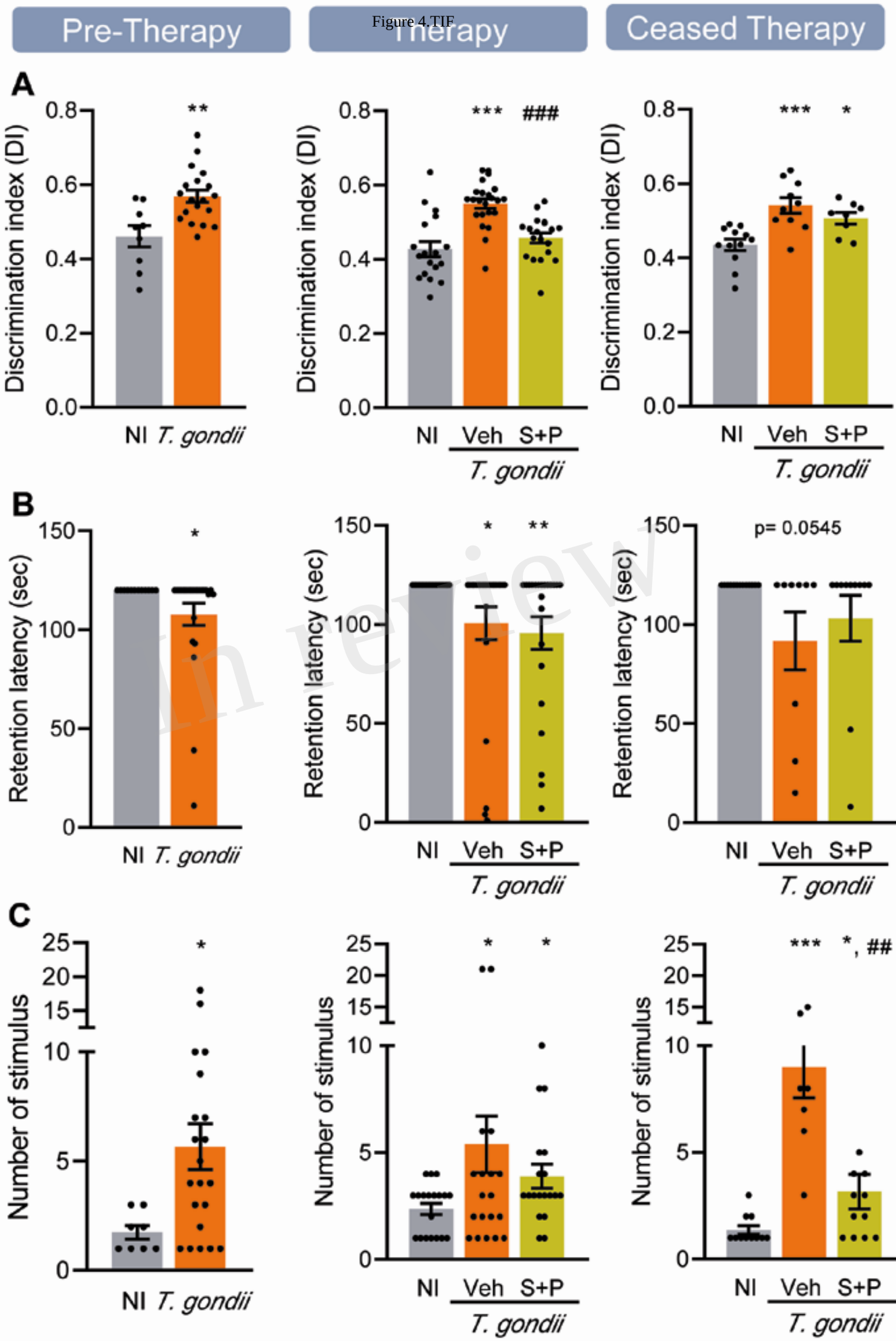
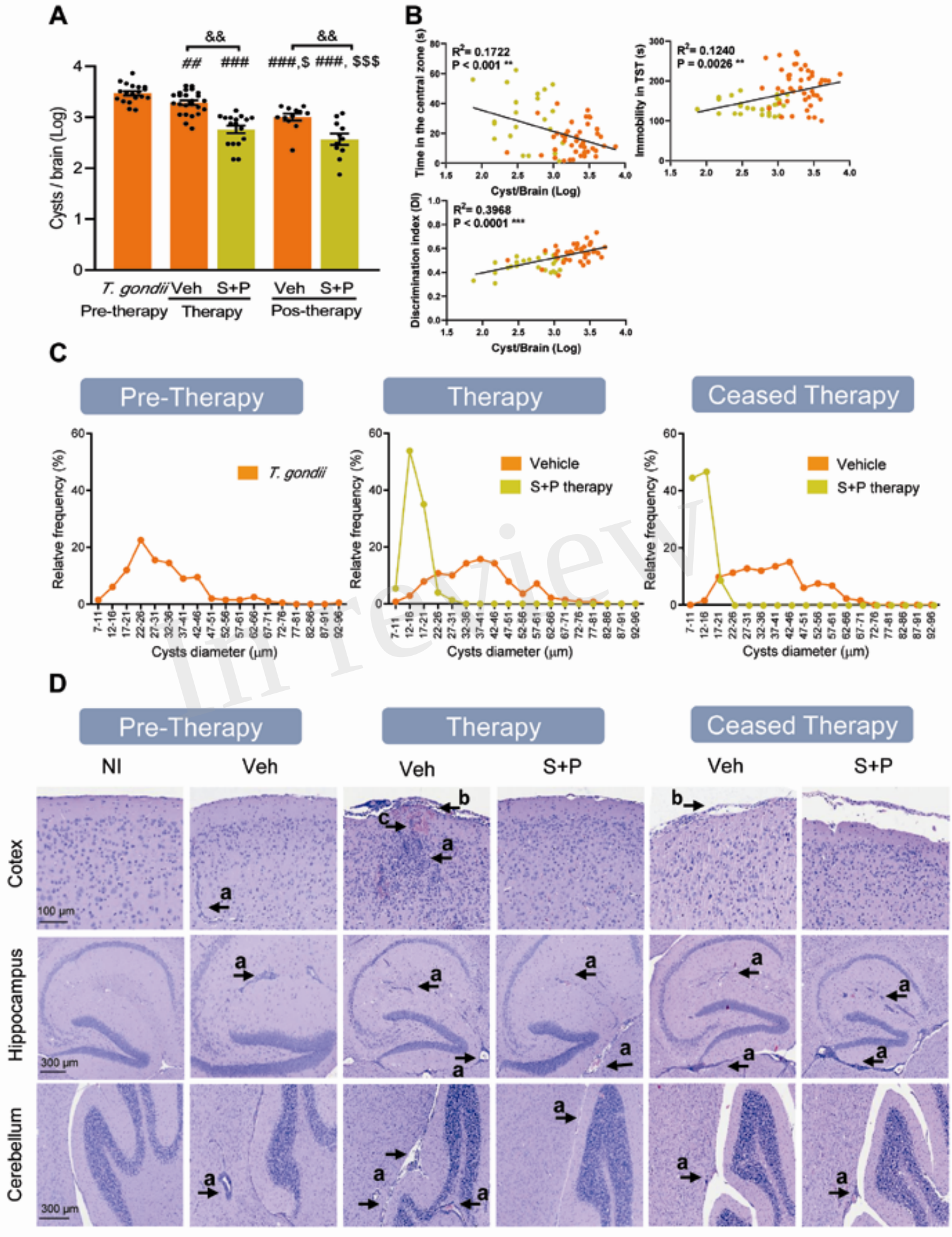


Figure 5.TIF



a- Meningoencephalitis
b- Perivascular inflammatory cuffs
c- Hemorrhagic spots

Figure 6.TIF

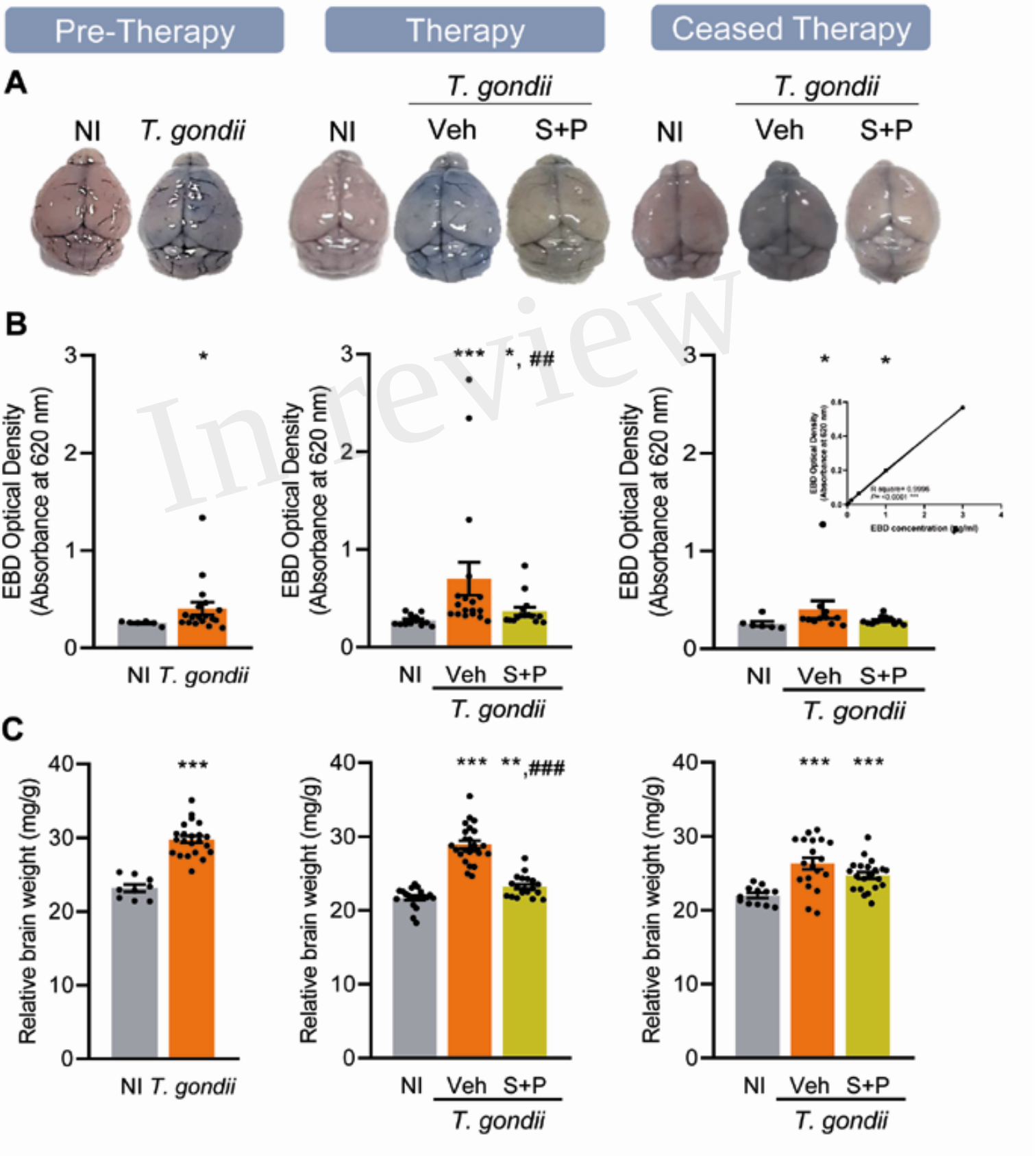


Figure 7.TIF

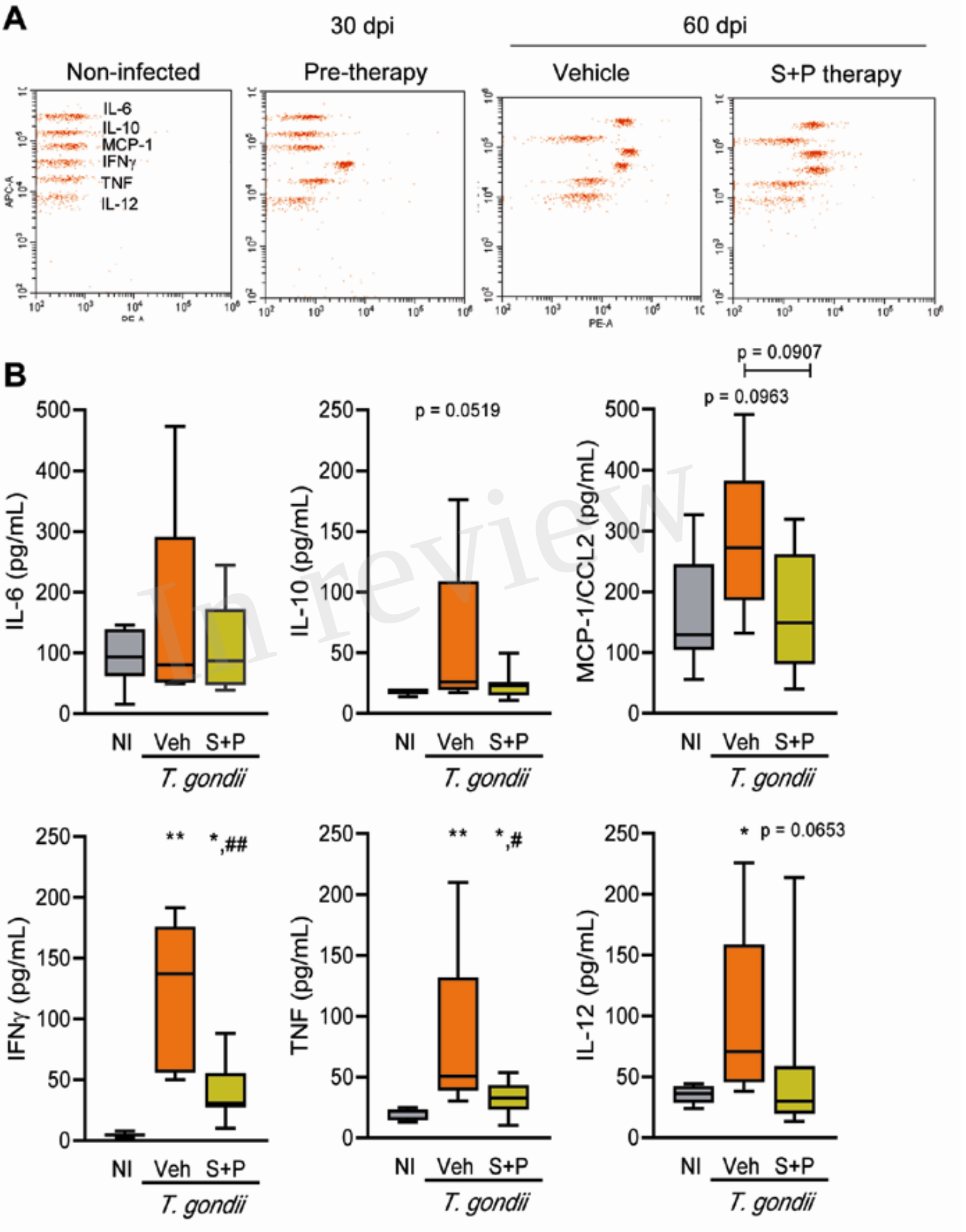
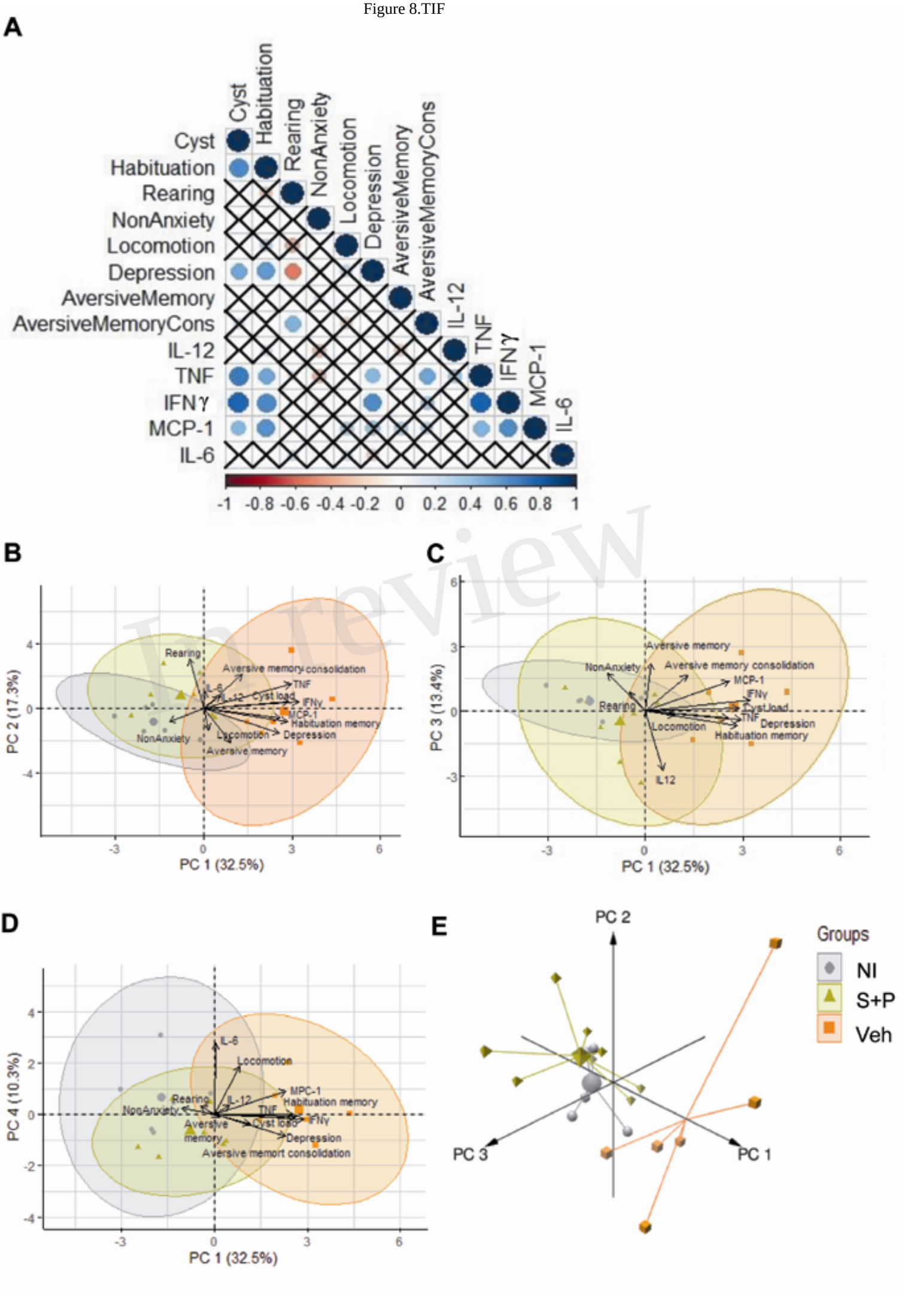

A
30 dpi
60 dpi
Non-infected
Pre-therapy
Vehicle
S+P therapy
IL-6
IL-10
MCP-1
IFNγ
TNF
IL-12
APC-A
PE-A
B
IL-6 (pg/mL)
IL-10 (pg/mL)
MCP-1/CCL2 (pg/mL)
IFNγ (pg/mL)
TNF (pg/mL)
IL-12 (pg/mL)
p = 0.0907
p = 0.0963
p = 0.0519
p = 0.0653
** *,##
** *,#
* p = 0.0653
NI Veh S+P
T. gondii

Figure 8.TIF



4. DISCUSSÃO

Em nossos estudos publicados ou submetido que compõem esta tese, além da contribuição com dados inéditos, tentamos trazer discussão sobre o tema integrando nossos dados e dados da literatura. Notamos que a área de pesquisa em modelos de infecção pelo *T. gondii* e estudo de alterações comportamentais e neurocognitivas é muito ativa, com publicações relevantes e contribuições sobre mecanismos biológicos. Assim, tentamos contribuir com o impacto da terapia etiológica na fase crônica da infecção nas alterações observadas e trabalhar os dados com análise integrada com o perfil inflamatório sistêmico. Por outro lado, o estudo na área de infecção pelo *T. cruzi* e comprometimento do SNC e alterações comportamentais e neurocognitivas é muito menos ativa, tendo o nosso grupo dado contribuições na descrição e estudos de processos biológicos associados a alterações comportamentais. Assim, em nosso estudo buscamos contribuir com a descrição da presença de alterações neurocognitivas e o impacto do tratamento etiológico em fase crônica, através de um modelo murino, trazendo informações básicas sobre possíveis processos biológicos,

Assim, questionamos se a colonização do SNC pelos parasitos *T. cruzi* e *T. gondii*, está associada à presença de alterações comportamentais e neurocognitivas durante a infecção crônica em modelos experimentais em camundongos e a relação destas alterações com os processos biológicos gerados como resposta à infecção parasitária, como a (i) neuroinflamação, (ii) ruptura da integridade da BHE e (iii) aumento dos níveis sistêmicos de citocinas. Para explorar esta questão, utilizamos camundongos C57BL/6 (H-2^b) fêmeas, uma vez que estudos anteriores publicados (141, 142) e nossos estudos preliminares mostravam que a infecção de animais desta linhagem e deste gênero permitia sobrevivência e desenvolvimento de fase crônica, de modo que nossas questões poderiam ser desafiadas. Camundongos C57BL/6 (H-2^b) fêmeas infectadas por *T. cruzi* e *T. gondii*, parasitos que persistem no SNC na fase crônica de infecção, mostram resultados da relação parasito/hospedeiro bem diversos. Por um lado, camundongos fêmeas da linhagem C57BL/6 são resistentes à neuroinflamação durante a infeção experimental pelo *T. cruzi*, seja na fase aguda, seja na fase crônica, que ocorre com persistência do parasitismo no SNC (33), o que foi corroborado em trabalho anterior de nosso grupo (35) e aqui no **Artigo 1** (143). Por outro lado, nesta mesma linhagem de camundongos, a infecção por *T. gondii* resulta em neuroinflamação sustentada,

ou seja, que se mantém ao longo da fase crônica, com presença de cistos do parasito no SNC (38-40), também mostrado aqui no Artigo 2 (69). Adicionalmente, nos Artigos 1 (143) e 3 (submetido) desafiamos o papel da persistência do parasito na fase crônica da infecção nas alterações comportamentais e neurocognitivas, por meio da terapia etiológica de primeira escolha estabelecida para cada patógeno (41, 42).

Inicialmente, demostramos que a infecção crônica por *T. cruzi* e *T. gondii* em camundongos C57BL/6 (H-2^b) fêmeas, reproduzem alterações comportamentais e neurocognitivas reportadas em portadores crônicos da DC (19, 20, 26-28, 72), e em pacientes soropositivos para *T. gondii* (30, 106).

No **Artigo 1**, mostramos que as alterações mnemônicas, pela primeira vez descritas em modelo crônico da DC, ocorrem na ausência de inflamação no tecido nervoso. Como dito, ausência de neuroinflamação neste modelo foi inicialmente descrita pelo nosso grupo (33), o que corrobora dados descritos em portadores da DC (36). Mostramos também que os camundongos C57BL/6 (H-2^b) fêmeas infectadas por *T. cruzi* tem, na ausência de neuroinflamação, atrofia cerebral, que anteriormente foi descrita em portadores crônicos da DC (66). Atrofia cerebral tem sido descrita em situações de esclerose múltipla (144), DA e demência (145), associada a alterações e declínio neurocognitivo. Níveis aumentados de citocinas pró-inflamatórias sistêmicas (TNF e IL-6), também têm sido associadas à atrofia cerebral em pacientes sem acidente vascular cerebral, demência e ataque isquêmico transitório (146). Além dos dados do **Artigo 1**, trazemos um dado que mostra que animais cronicamente infectados pelo *T. cruzi,* 120 dpi, apresentaram níveis aumentados de TNF e IFNγ, quando comparadas com níveis séricos dos animais controles não infectados pareados por idade (**Anexo 1**). Assim, parte do objetivo 5 foi desafiado através da avaliação dos níveis séricos de citocinas pró-inflamatórias nos animais cronicamente infectados (120 dpi). Níveis séricos elevados destas citocinas estão relacionados à gravidade da DC em portadores, particularmente, na CCC, estando relacionados ao perfil inflamatório sitêmico (147-150). Nossos dados anteriores mostraram o aumento de níveis séricos de TNF e da expressão desta citocina no tecido cardíaco de camundongos C57BL/6 cronicamente infectados (35, 142). TNF tem sido relacionado à indução de alterações neurocognitivas em situações de neuroinflamação crônica não infecciosa em modelos experimentais (151), e em humanos (152). Na infecção crônica pelo *T. cruzi*, com ausência de expressão detectável de TNF no SNC, a administração de

anticorpo bloqueador anti-TNF (infliximab) ou de pentoxifilina, um modulador da expressão do receptor 1 de TNF (TNFR1), resultou na melhora do comportamento semelhante à depressão, sugerindo que poderia haver participação de citocinas sistêmicas nas alterações comportamentais (35). Do mesmo modo, a administração do análogo da talidomida, 3,6'-ditiothalidomida (DT), um inibidor da síntese de TNF, reverteu déficits cognitivos em ratos F344 com neuroinflamação crônica induzida pela infusão de lipopolisacarídeos bacteriano (151). Em pacientes com DA ou demência, a inibição de TNF com administração de etanercepte, uma proteína de fusão dimérica recombinante que se liga ao TNF e bloqueia sua interação com receptores de TNF da superfície celular, possibilitou a melhora da fluência verbal, aprendizagem e memória (152). Também, em modelo murino e primata não humano de DA induzida por administração de oligômeros de β-amilóide, foi demonstrada a participação de TNF na alteração mnemônica e relacionando a fatores patogênicos comuns a diabetes (153). Em conjunto, estes dados sugerem que TNF é um mediador crítico nas alterações comportamentais e neurocognitivas. Ademais, TNF estaria envolvido em mecanismos relacionados à disfunção neuronal induzida por neuroinflamação (151), mas também parece ocorrer na ausência desta (35, 143) (**Artigo1**), o que precisa ser explorado.

Aqui, no **Anexo 1**, mostramos que IFNγ também está elevado no soro dos animais C57BL/6 (H-2^b) usados em nossos experimentos, o que corrobora dado anterior neste modelo (142). Uma das limitações de nosso estudo foi a não exploração da participação das citocinas nas indução e/ou progressão das alterações comportamentais e neurocognitivas. Estudo anterior mostrou que a deleção de IFNγ evitou a perda da memória epacial de curto prazo em um modelo murino deficiente em IFNγ em fundo genético C57BL/6J expostos a estresse crônico induzidos por diversas condições adversas ou “regime de estressores”, como troca de gaiolas com exposição a odores aversivos ou de individuos desconhecidos, exposição social, restrição de movimentos, gaiolas sujas, entre outros, por 6 semanas, alguns minutos por dia (154). Por outro lado, citocinas também poderiam ser produzidas no SNC por células da glia, como astrócitos (155). Abordagens *in vitro* mostraram que tanto IFNγ quanto TNF favorecem a entrada e o crescimento do *T. cruzi* em astrócitos murinos e humanos, que seriam possíveis fontes de parasito na reativação da infecção em situação de imunossupressão (156, 157), e em pessoas imunocompetentes e infectadas estas citocinas produzidas *in situ* poderiam, *per se* ou associadas à infiltração a partir do soro no SNC, contribuir para as

alterações comportamentais e neurocognitvas. Estas questões precisam ser exploradas.

Com nosso trabalho, no estudo da infecção pelo *T. cruzi* no modelo C57BL/6 (H-2^b), a neuroinflamação é ausente e podemos considerar que citocinas sistêmicas poderiam estar “inundando” o SNC por áreas desprovidas de BHE, como os orgãos circunventriculares (158), que possuem vasos fenestrados. Alternativamente, este processo pode se dar diretamente através do transporte ativo, quando há ruptura da BHE, embora até hoje não tenha sido descrita a quebra da BHE na infecção em modelos experimentais de infecção pelo *T. cruzi*. A avaliação da BHE durante a infecção aguda por *T. cruzi* (13 dpi), em ratos Holtzman fêmeas infectadas com as cepas Y, PNM ou CL-Brener, mostrou uma leve penetração do azul de Evans no cérebro, com maior intensidade na matéria cinzenta que na matéria branca. Contudo. a interpretação deste dado é dficultada pela falta de registro do resultado do azul de Evans no grupo controle e ausência de determinação da absorbância (159). Por outro lado, mostramos a existencia de raras células mononucleraes inflamatórias infiltrando o plexo coróide nos animais cronicamente infectados. O plexo coróide é uma barreira especializada, que constitui a barreira sangue-líquido cefalorraquidiano e, assim como a BHE, contribue com a homeostase do SNC (160). Assim, seria conveninte pesquisar o envolvimento da BHE na patogenia e alterações comportamentais e neurocognitivas da na fase crônica da infecção experimental pelo *T. cruzi*. Dados preliminares de nosso grupo, entretanto, não demonstraram extravasamento detectável de azul de Evans no SNC na infecção crônica (Silva AA, não publicado), o que não exclui o extravasamento de moléculas no SNC e/ou a ativação de celulas endoteliais ou gliais por mocélulas de citocinas circulantes, que poderiam amplificar a produção de citocinas no SNC. De modo importante, mostramos que as alterações mnemônicas nos animais cronicamente infectados pelo *T. cruzi* estavam relacionadas ao aumento da peroxidação lipídica, que reflete presença de estresse oxidativo associado à prejuízo da memória em distúrbios neurodegenerativos (161). De modo relevante, a terapia com Bz, que reverteu ou evitou a instalação das alterações de memória, também reduziu o estresse oxidativo no SNC. Se foi um efeito direto ou indireto, via controle do parasito, pela terapia com Bz é algo a ser esclarecido.

A persistência do *T. cruzi* no SNC foi demonstrada em camundongos C3H/He cronicamente infectados pela cepa Colombiana, que em condição de imunossupressão, após 160 dpi, desenvolvem reativação da infecção pelo parasito,

na ausência de parasitemia e restrita ao SNC, indicando a persistência do parasito durante a fase crônica da DC experimental (33), o que foi corroborado no modelo C57BL/6 (157). A administração de Bz na fase aguda da infecção experimental pelo *T. cruzi* em modelo murino inibiu a instauração de comportamento depressivo na fase crônica da infecção (35), mostrando que fase aguda menos grave (redução de parasitemia) pode contribuir para fase crônica menos grave. Aqui nós desafiamos o efeito de Bz em alterações mnemônicas e demonstramos que a forma nervosa da DC crônica experimental é independente do processo de neuroinflamação, com persistência do parasito, que poderia estar "minando" o SNC, seja diretamente, seja induzindo a expressão de citocinas, que também contribuiriam para o estresse oxidativo, e outros neuromediadores, que em conjunto poderiam contribuir para as alterações comportamentais ou neurocognitivas ao longo prazo. Como citado no nosso **Artigo 1**, este estudo apresentou limitações referentes a ausência de dados sobre níveis moleculares da ação do Bz no SNC, lacuna que precisa ser preenchida. Mas com este artigo, sugerimos que Bz poderia ser usado em portadores da DC, seja para controle da cardiomiopatia, questão em debate, mas também para melhoria da qualidade da saúde mental dos pacientes.

No **Artigo 2** mostramos a existência de alterações comportamentais (depressão, ansiedade e hiperatividade), de forma cinética, durante a infecção crônica, precoce e de longo termo, pelo *T. gondii,* corroborando dados existentes na literatura em outros modelos de estudo da relação/parasito hospedeiro (117, 133, 162, 163). Considerando que a localização dos cistos poderia ser um possível mecanismo na indução de alterações comportamentais (34, 121), procuramos esclarecer a existência de um possível tropismo de cistos por regiões especificas do SNC, além de avaliar o tamanho dos cistos. O isocórtex, tálamo e mesencéfalo foram mais colonizados na infecção crônica precoce e de longo termo. Nossos dados corroboraram dados recentemente publicados sobre maior colonização de áreas especificas (39, 164). Embora inicialmente tenhamos sido tentados a afirmar a existência de tropismo por isocórtex, tálamo e mesencéfalo no nosso modelo de infecção de camundongos C57BL/6 (H-2^b) com a cepa ME-49 do *T. gondii* não foi possível estabelecer uma relação concreta estrutura cerebral/presença de cisto, já que estes foram observados em todas as áreas do encéfalo, sem significância estatística que indicasse uma estrutura diferencialmente parasitada. No entanto, sugerimos a ampliação de estudos nesta área, modelos experimentais padronizados; em inóculo, linhagem de camundongos e cepas idênticas, que

permitam uma maior reprodutibilidade e análise de dados originados em laboratórios diferentes, contribuindo para esclarecer se há região de localização preferencial de cistos no SNC, o que poderia comprometer determinada ação/função. Certamente, estes parâmetros devem ser levados em consideração diante do atual conhecimento sobre o potencial de plasticidade sinápticas, que contraria a existência de uma relação entre localização preferencial dos cistos e comprometimento cognitivo, e da baixa (ou inexistente) relação entre a estrutura cerebral parasitada e a (dis)função comportamental/neurocognitiva.

Neste modelo, o perfil inflamatório sistêmico e intracerebral também foi explorado. Demonstramos a persistência de inflamação no SNC, através de análises histolólogicas, correlacionadas com estudos moleculares da expressão de RNAm de citocinas TNF e IFNγ e CC-quimiocinas (MCP1/CCL2, MIP1α/CCL3, MIP-1β/CCL4) intracerebrais, e perfil inflamatório sistêmico, revelado por aumento dos de níveis de citocinas séricas (TNF e IFNγ, MCP1/CCL2). A demonstração de neuroinflamação está em acordo com o que foi observado em outros modelos murinos experimentais de infecção pelo *T. gondii* (38-40, 133). Assim, comprovamos que o modelo de infecção crônica de camundongos C57BL/6 (H-2^b) pela cepa ME-49 gera uma resposta inflamatória do tipo Th1, que, envolvendo perfil de citocinas e CC-quimiocinas, atuam de modo determinante no controle do parasitismo e redução do número de cistos no SNC (165). Estes dados também estão de acordo com o perfil de expressão gênica de IFNγ, IL-12, TNF, IL-27 e Alox5ap relacionada à inflamação detectado no modelo de infecção de camundongos B6CBAF1/J com a cepa ME-49 (39). A persistência dos cistos cerebrais do *T. gondii* em nosso modelo pode estimular a produção de citocinas e CC-quimiocinas intracerebrais, contribuindo para o recrutamento de células inflamatórias. Por outro lado, a BHE neste modelo, mostrou-se comprometida, como demonstrado pelo extravasamento de azul de Evans, o que pode facilitar a entrada de mediadores e células inflamatórios ao tecido cerebral (166), agindo, junto à circulação sistêmicas e produção intracerebral de citocinas pró-inflamatórias, como mantenedor da neuroinflamação. Alterações locomotora e neurocognitiva estão associadas ao aumento da permeabilidade da BHE e neuroinflamação em ratos Wistar com sepse polimicrobiana, induzida por ligação e perfusão cecal (167). Assim, na toxoplasmose crônica experimental, o meio inflamatório sistêmico e cerebral pode contribuir para a instauração das alterações comportamentais que detectamos.

Nossos dados nos levaram a considerar a persistência dos cistos como um fator crucial que poderia contribuir, direta ou indiretamente, para a ativação do sistema imune de forma sistêmica e localizada no SNC, assim como alterar outros fatores e vias não explorados em nosso trabalho (neuromediadores/ neurotransmissores) e, assim, alterar o comportamento do animal na fase crônica da infecção. Deste modo, decidimos intervir com o tratamento etiológico nesta fase da infecção.

No **Artigo 3**, reproduzimos alterações comportamentais (ansiedade, depressão e hiperatividade) anteriormente descritas no modelo de infecção crônica precoce e de longo termo ou tardia e avaliamos alterações neurocognitivas (memória de habituação, memória aversiva e consolidação da memória aversiva), presentes no modelo. Desafiamos o efeito da resposta imune intrínseca que resulta no controle do número e tamanho de cistos intracerebrais (107) e a associação entre o tratamento etiológico S+P (41) no controle do parasito. Analisamos as alterações inflamatórias locais e sistêmicas, assim como as alterações comportamentais e neurocognitivas.

Uma vez mais, as alterações comportamentais e neurocognitivas aqui avaliadas corroboraram dados da literatura descritos de forma independente e usando modelos distintos e análises em tempos diversos após a infecção (34, 39, 69, 114, 117-120, 133, 163, 168-170). Assim, as alterações comportamentais e neurocognitivas aqui avaliadas são conservadas na infecção pelo *T. gondii*.

Como descrito no **Artigo 2** e expandidas no **Artigo 3**, as alterações comportamentais e neurocognitivas no nosso modelo foram relacionadas à persistência de cistos cerebrais, neuroinflamação, ruptura de BHE e manutenção do perfil inflamatório sistêmico. Ainda que seja observada redução da carga de cistos no SNC, a medida em que a infecção evolui da fase crônica inicial ou precoce para a fase crônica tardia, o que pode ser associado à resposta imune intrínseca (107), esta não foi suficiente para impactar de forma relevante as alterações comportamentais e neurocognitivas. Demonstramos pela primeira vez que a terapia combinada S+P apresenta efeito benéfico seletivo (total, parcial ou transitório) nas alterações comportamentais e neurocognitivas. Verificamos a redução da carga de cistos cerebrais, da neuroinflamação, restauração parcial da integridade da BHE e diminuição dos níveis sistêmicos de citocinas inflamatórias, que, como já discutido acima, podem estar envolvidos na indução de alterações comportamentais e neurocognitivas. Ademais, observamos correlação positiva entre os níveis séricos de

IFNγ, TNF e MCP-1/CCL2, carga de cistos cerebrais e alterações comportamentais e neurocognitivas. Curiosamente, a diminuição de níveis de citocinas pro-inflamatórias e anti-inflamatórias no SNC, foram associadas ao comportamento parecido com a ansiedade em camundongos BALB/c infectados pelo protozoário *Leishmania amazonenses* (171). No entanto, nossos dados estão em concordância com dados anteriormente publicados em camundongos B6CBAF1/J cronicamente infectados com a cepa ME-49, onde níveis de expressão gênica e concentração de citocinas pró-inflamatórias plasmáticas e a carga de cistos estavam correlacionados com a gravidade da alteração no comportamento de esquiva de predadores (39). Associação entre carga parasitária no SNC e alterações da estrutura do risco (34), aversão ao odor de predadores e ansiedade (172) também têm sido reportadas.

De forma altamente relevante, nosso estudo evidenciou a diferenciação de três “clusters” ou grupos distintos, nos quais os animais infectados que receberam terapia S+P, se distinguem dos infectados que receberam veículo e, mais importante, tiveram um comportamento global similar aos animais controles não infectados. Demonstramos ainda o efeito benéfico do tratamento S+P como adjuvante na resposta imune intrínseca no controle de cistos no SNC, de modo a impactar nas alterações comportamentais e neurocognitivas.

Em conjunto, nossos dados demonstram a presença de alterações comportamentais e neurocognitivas reportadas em humanos durante a infecção crônica da DC e da toxoplasmose, em modelos experimentais murinos e possíveis benefícios da adição da terapia etiológica na fase crônica destas doenças na qualidade de vida dos pacientes. Indubitavelmente, modelos experimentais constituem uma ferramenta valiosa no entendimento das doenças humanas. Porém, oferecem um potencial translacional limitado em relação à doença humana, principalmente devido a diferenças evolutivas entre estes e roedores, incluindo diferenças na composição e complexidade dos tecidos (173). O fato da arquitetura básica e celular do cérebro de mamíferos ser bem conservada, permite a correspondência de tipos homólogos e previsões de propriedades de tipos de células humanas. Apesar desta similaridade, há diferenças na composição celular do córtex humano, quando comparado ao córtex cerebral do camundongo, o córtex humano é mais de 1.000 vezes maior em área e em número de neurônios. Além de grandres diferenças entre os tipos de células homólogas, alterações marcantes nas proporções, distribuições laminares, expressão gênica e morfologia dos cérebros de

ambas as espécies (174). Pelo que é necessario cautela para tecer qualquer extrapolação de dados.

Há cada vez mais evidências de que as citocinas e outros elementos inflamatórios do sistema imunológico contribuem de maneira importante para os transtornos mentais (13, 15, 35, 175, 176). A inflamação periférica pode estimular a manutenção de um meio inflamatório intracerebral, pela ativação de células residentes no SNC através do endotélio vascular, ou mesmo através do transporte ativo ao SNC pelas regiões circunventriculares e da BHE (158, 160). Demonstramos que alterações comportamentais e neurocognitivas estão presentes em camundongos cronicamente infectados pelo *T. cruzi*, independente de neuroinflamação, e paralelo à neuroinflamação sustentada no modelo crônico de infecção pelo *T. gondii*. Porém ambos os modelos têm em comum a persistência do parasito no SNC, assim como inflamação sistêmica persistente, que podem contribuir para instauração de alterações comportamentais e neurocognitivas. Ademais, demonstramos que as terapias etiológicas, seja da DC (com Bz), ou da toxoplasmose (com S+P), têm potencial de melhora na evolução clínica e, consequentemente, na qualidade de vida dos pacientes afetados, embora as reações adversas ainda sejam uma grande desvantagem. Como já mencionado, no caso da terapia para DC, baseado em estudos anteriores em CCC (91, 92), usamos o equvalente a ¼ da dose de Bz recomendada para humanos, o que poderia reduzir os efeitos colaterais e contribuir para maior aceitação da terapia em pacientes. Tal estudo ainda não foi feito em relação à redução das doses de S+P, mantendo eficácia, o que precisaria ser explorado.

Curiosamente, O SARS-CoV-2, induz uma forte resposta inflamatória sistêmica, considerada como uma tempestade de citocinas, com liberação descontrolada de citocinas pró-inflamatórias (177) e, de modo importante, mesmo na ausência de colonização do SNC pelo virus, há desenvolvimento de sintomas neurólogicos, com alterações inflamatórias no cérebro e no plexo coróide (178). Como aqui proposto na DC e na toxoplasmose experimental, acredita-se que essa liberação de citocinas pró-inflamatórias induz alterações comportamentais em pacientes sobreviventes, como depressão, ansiedade, transtorno de adaptação e estresse pós-traumático (179). Este pode então ser um modelo valioso para o estudo de alterações comportamentais induzidas pela inflamação periférica sem a invasão do SNC.

A propósito da menção do SARS-CoV-2 e a pandemia desencadeada, queremos mencionar que este projeto se viu limitado pelas medidas restritivas que se fizeram necessárias para salvaguardar a vida e a integridade da população, incluindo nós e nossos colaboradores. Assim não foi possivel abordar a participação das citocinas na indução e/ou progressão das alterações comportamentais e neurocognitivas, com estudos de bloqueio de atividades ou atuando em seus receptores, bem como usando animais geneticamente modificados. Também fomos limitados em elucidar outros mecanismos neurobiológicos e os mecanismos moleculares que possam sustentar a persistência destes parasitos no SNC e as bases moleculares que contribuem para as alterações descritas acima. Ainda com as limitações enfrentadas, nosso trabalho conseguiu oferecer uma possibilidade de melhoria na qualidade de vida em pacientes portadores da DC, mostrando que a terapia com Bz oferece benefícios, mesmo na fase crônica, diminuindo a parasitemia, o parasitismo, e inclusive abolindo os poucos infiltrados inflamatórios e o estresse oxidativo no SNC. Assim, apoiamos a indicação do uso de Bz na fase crônica dos pacientes portadores da DC, ressaltando que nosso estudo utilizou ¼ da dose recomendada do Bz. Ademais, esperamos que os estudos que avaliam a redução da dose do mesmo em humanos, como o estudo BENEDITA (83), venham permitir maior adesão e tolerância ao tratamento que promete melhorar a saúde mental e, por consequência, a qualidade de vida dos portadores crônicos da DC. Por outro lado, também conseguimos demonstrar a associação existente entre a carga de *T. gondii* no SNC*,* citocinas pró-inflamatórias circulantes e intracerebrais, ruptura da BHE e as alterações comportamentais, mostrando ainda o efeito benéfico na imunidade intrínseca protetora contra cistos teciduais, com a ação adjuvante da terapia S+P. De fato, esta estratégia terapêutica, embora tenha reações adversas, é amplamente aceita na prática clínica em pacientes com reativação da doença e encefalite toxoplásmica, mas também indicamos o seu uso em pacientes soropositivos com infecção latente que não apresentam este tipo de quadros graves da doença, mas que manifestam alterações neurocognitivas e comportamentais, oferecendo também melhora na qualidade de vida das pessoas afetadas.

5. CONCLUSÕES

Camundongos cronicamente infectados com cepa Colombiana de *T. cruzi* apresentam alterações neurocognitivas da memória de longo prazo, reproduzindo aspectos de alterações comportamentais descritas nos portadores crônicos da DC. Com ausência de neuroinflamação, mas com presença estresse oxidativo, atrofia cerebral e inflamação sistêmica, além de persistência do parasito no SNC. A terapia com Bz tem potencial benéfico na interrupção da progressão e da instauração destas alterações, além de impactar a parasitemia, o parasitismo e diminuir o estresse oxidativo no SNC. De forma similar, camundongos cronicamente infectados pela cepa ME-49 de *T. gondii* apresentam alterações comportamentais e neurocognitivas descritas nos pacientes com infecções latentes, que cursam concomitantemente com neuroinflamação, quebra da BHE, inflamação sistêmica e persistência do parasito no SNC. A terapia etiológica, S+P, reverte parcial, total ou de forma transitória estas alterações e ainda impacta em diferentes medidas os parâmetros biológicos já mencionados.

O perfil elevado de citocinas sistêmicas e persistência do parasito no SNC mostrou-se como um fator em comum entre os dois modelos experimentais aqui avaliados, reforçando a ideia de que as citocinas e outros elementos inflamatórios do sistema imunológico contribuem para o desenvolvimento de doença ou transtornos mentais.

6. PERSPECTIVAS DO PROJETO

Esta pesquisa foi idealizada com a ambição de, além dos objetivos aqui apresentados, identificar mecanismos moleculares que favorecem a persistência de parasitos e a influência destes nas alterações comportamentais. Ademais, procurávamos elucidar mecanismos moleculares pelos quais as citocinas IFNγ e TNF poderiam favorecer a persistência de *T. cruzi* e do *T. gondii* no SNC. Mas, diante do cenário da atual pandemia por COVID-19, nos vimos forçados a modificar estes objetivos. Assim, como perspectivas pretendemos abordar estes mecanismos, além de avaliar a participação das citocinas Th1 periféricas e intracerebrais na indução e progressão das alterações comportamentais e neurocognitivas em ambos os modelos.

Adicionalmente, considerando que o mecanismo pelo qual outros *Trypanosomas,* como o *T. brucei,* coloniza o SNC já foi elucidado, pretendemos abordar também esta questão, pensando na existência da quebra da BHE como possível mecanismo de invasão, sustentado na ausência de dados nesta questão e a manutenção de níveis aumentados de citocinas periféricas aqui relatadas.

Claramente, deste projeto nasceram muitas perguntas. Ficamos profundamente curiosos pelo fato de as alterações comportamentais na toxoplasmose responderem de forma distinta durante o tratamento com S+P. Acreditamos que este fato esteja relacionado com localizações específicas de cistos persistentes após a terapia, como proposto anteriormente (34), à neurogênese dos comportamentos avaliados e, inclusive, a limitações quanto à adequação e sensibilidade dos testes comportamentais utilizados. Assim, faz-se necessário abordar estas questões para um melhor entendimento das patologias e do benefício da terapia.

Por último, observamos que a persistência dos cistos cerebrais de *T. gondii,* neuroinflamação, inflamação sistêmica e ruptura da BHE esteve presente durante todo o curso da doença, associados com a apresentação de alterações comportamentais e neurocognitivas observadas. No entanto, não exploramos se estas alterações estariam presentes se os animais fossem tratados na fase aguda da infecção, se isto permitiria modular a quebra da BHE, a carga de cistos no SNC e, especialmente, a presença da neuroinflamação, que poderiam ser outros desdobramentos a serem considerados.

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8. ANEXOS

8.1. Anexo 1

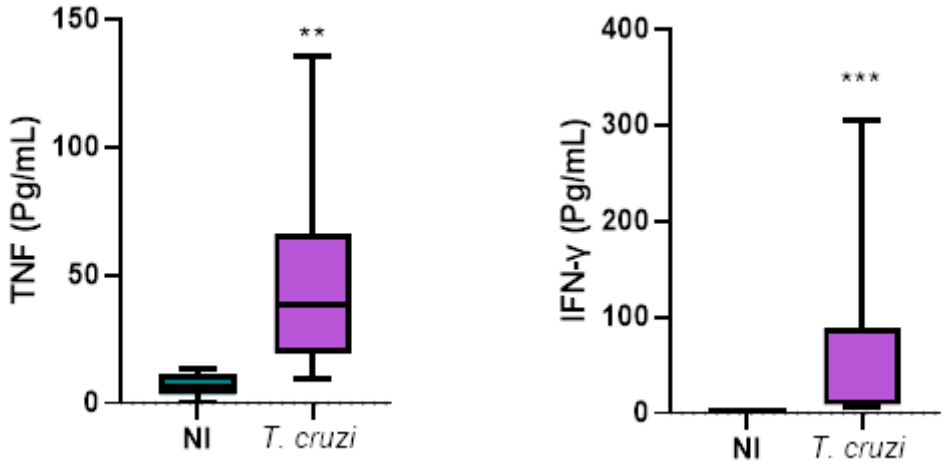

Figura 1. Níveis séricos de citocinas pró-inflamatórias em camundongos C57BL/6 cronicamente infectados pelo *T. cruzi*. TNF e IFNɣ são significativamente aumentados em infecção experimental crônica de DC, quando comparados com animais NI. Método: BD - CBA Mouse Inflammation Kit, Catálogo 552364, Lote 0031162

8.2. Anexo 2

Produtividade

Disciplinas cursadas e aprovadas:

- ✓ Imunologia geral
- ✓ Aplicações da PCR em tempo real no diagnóstico, expressão gênica e genotipagem em doenças infecciosas e parasitárias
- ✓ Parasitologia I
- ✓ Parasitologia II
- ✓ Procedimentos de Biossegurança em laboratórios de Pesquisa Biomédica
- ✓ Centro de Estudos
- ✓ Apreciação de defesa de Tese.
- ✓ Seminários discente do programa de Biologia Parasitária
- ✓ Vacinas- estado da arte, reflexões e desafios
- ✓ Tópicos de neurociências: pavimentando o caminho e novas conexões

Produção Intelectual

Artigos

Gibaldi, Daniel; Vilar-Pereira, Glaucia; Pereira, Isabela Resende ; Silva, Andrea Alice; Castaño, Leda; Ramos, Isalira Peroba; Mata dos Santos, Hílton Antônio; Gazzinelli, Ricardo; Lannes-Vieira, Joseli. CCL3/Macrophage Inflammatory Protein-1α Is Dually Involved in Parasite Persistence and Induction of a TNF and IFNγ-Enriched Inflammatory Milieu in *Trypanosoma cruzi*-Induced Chronic Cardiomyopathy. Frontiers in Immunology, v. 11, p. 2020.00306, 2020.

Vilar-Pereira, Glaucia; Castaño Barrios, Leda; Silva, Andrea Alice; Martins Batista, Angelica; Resende Pereira, Isabela; Cruz Moreira, Otacílio; Britto, Constança; Mata Dos Santos, Hílton Antônio; Lannes-Vieira, Joseli. Memory impairment in chronic experimental Chagas disease: Benznidazole therapy reversed cognitive deficit in association with reduction of parasite load and oxidative stress in the nervous tissue. PLoS One, v. 16, p. e0244710, 2021.

Castaño Barrios, Leda; Da Silva Pinheiro, Ana Paula; Gibaldi, Daniel; Silva, Andrea Alice; Machado Rodrigues e Silva, Patrícia; Roffê, Ester; da Costa Santiago, Helton; Tostes Gazzinelli, Ricardo; Mineo, José Roberto; Silva, Neide Maria; Lannes-Vieira, Joseli. Behavioral alterations in long-term *Toxoplasma gondii* infection of C57BL/6 mice are associated with neuroinflammation and disruption of the blood brain barrier. PLoS One, v. 16, p. e0258199, 2021.

Castaño Barrios, Leda; Silva, Andrea Alice; Hernandez, Lina; Da Silva Pinheiro, Ana Paula; Gibaldi, Daniel; Mineo, José Roberto; Silva, Neide Maria; Lannes-Vieira, Joseli. Sulfadiazine plus pyrimethamine therapy reversed multiple behavioral and neurocognitive changes in long-term toxoplasmosis by reducing brain cyst load and inflammation-related alterations. Submetido (Front. Immunol).

Palestras:

- ✓ *Toxoplasma gondii* e alterações no comportamento em um modelo murino, UFF, 2021

Resumos em congressos:

a. Poster

- ✓ Tratamento combinado de sulfadiazina e pirimetamina reduz número de cistos no cérebro de animais cronicamente infectados por *Toxoplasma gondii*". 54º Congresso da Sociedade Brasileira de Medicina Tropical, 2018.
- ✓ Infecção pelo *Toxoplasma gondii* induz alterações da memória aversiva e de reconhecimento. 55º Congresso da Sociedade Brasileira de Medicina Tropical 2019, o XXVI Congresso Brasileiro de Parasitologia e a 34a Reunião de Pesquisa Aplicada em Doença de Chagas e 22ª Reunião de Pesquisa Aplicada em Leishmanioses, o CHAGASLEISH, 2019.
- ✓ Anxiety and depressive-like behavior in long-term *Toxoplasma gondii* infection of C57BL/6 mice are associated with disruption of the blood brain barrier and increase in situ and systemic cytokine expression. XXXVI Reunião Anual da Sociedade Brasileira de Protozoologia/XXXVII Reunião Anual sobre Pesquisa Básica em Doença de Chagas, 2021.

- ✓ Hyperactivity in a murine model of long-lasting chronic *Toxoplasma gondii* is refractary to sulfadiazine and pyrimethamine therapy. XXXVI Reunião Anual da Sociedade Brasileira de Protozoologia/XXXVII Reunião Anual sobre Pesquisa Básica em Doença de Chagas, 2021.

b. Apresentação oral

- ✓ Sulfadiazina e pirimetamina não revertem a hiperatividade na infecção crônica de *Toxoplasma gondii*. 56º MEDTROP – Congresso da Sociedade Brasileira de Medicina Tropical - MedTrop Play, 2021.

- ✓ Ansiedade e comportamento depressivo na infecção crônica pelo *Toxoplasma gondii* em um modelo murino. 56º MEDTROP – Congresso da Sociedade Brasileira de Medicina Tropical - MedTrop Play, 2021.

Atividades de extensão:

- ✓ Fiocruz *pra* você. Fiocruz 2017.
- ✓ Semana Nacional da Ciência e Tecnologia. Fiocruz, 2017.
- ✓ Fiocruz vai aí *pra* você - Ciência na Estrada. São Gonçalo, 2017.
- ✓ Domingo com Ciência na Quinta de Boa Vista. 2019.
- ✓ Expresso Chagas XXI, 15 dias – diversas cidades do Estado de Minas Gerais. 2019.
- ✓ Ciência, Arte e Cultura na Saúde. Rio de Janeiro,2019.

Congressos, simpósios e cursos:

- ✓ V Ciclo Carlos Chagas de Palestras, Fiocruz, 2017
- ✓ VII Ciclo Carlos Chagas de Palestras, Fiocruz, 2019
- ✓ Curso de atualização em Neuroinfecção. INI – Fiocruz, 2019.
- ✓ Curso de Manejo e Ciências de Animais de Laboratório – CEUA-IOC, 2019.
- ✓ IV Seminário MEI - Metodologias de Ensino Inovadoras - Pós-graduação em Docência do IFMG, 2020
- ✓ I SINTOX - Simpósio Internacional de Toxoplasmose- UFRRJ, 2020
- ✓ "I Simpósio Online de Imunologia: Entendendo a Imunidade: o conhecimento ao alcance de todos “- SBI, 2020
- ✓ Curso de Neurofisiología - Centro educacional sete de Setembro, 2020

- ✓ Curso de Parasitologia Clínica - Liga Acadêmica de Doenças Parasitárias e Zoonoses da Universidade Federal de Mato Grosso (Ladop), 2020
- ✓ Curso ENSINO REMOTO - Caminhos e Conexões- FIOCRUZ, 2021
- ✓ Ciclo de Palestras Carlos Chagas – Fiocruz, 2021

Atividades extracurriculares:

- ✓ Aula prática de testes de comportamento e análise de dados na II Edição da Disciplina Ciências através de modelos experimentais- IOC, 2018.
- ✓ Aula prática de testes de comportamento e análise de dados na III Edição da Disciplina Ciências através de modelos experimentais – IOC, 2019.

Colaborações:

- ✓ Dr Edécio Cunha-Neto e Dr. Ramendra Pandey. Novo medicamento tripanossomicida – projeto FAPESP.
- ✓ Dra. Joseli Lannes-Vieira. Novas estratégias para cardiopatia chagásica crônica, FAPERJ e CNE/FAPERJ.
- ✓ Dra. Joseli Lannes-Vieira Enzima conversora de angiotensina/receptor AT1 como alvos da ação reguladora de benznidazol, FAPERJ.

8.3 Anexo 3

Licenças da Comissão de Ética no Uso de Animais (CEUA)

Ministério da Saúde
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/IOC

IOC
Instituto Oswaldo Cruz

LICENÇA ADITIVA

L-014/2018- A1

A Comissão CEUA/IOC, em atenção à solicitação da pesquisadora **JOSELI LANNES-VIEIRA,** responsável pela licença (L-014/2018), do protocolo (CEUA/IOC-002/2018), intitulado "**Mecanismos moleculares que favorecem a persistência do T. gondii no sistema nervoso central e sua relação com as alterações da memória.**", que atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL), autoriza o presente aditivo. A referida licença aditiva não exime a observância das Leis e demais exigências legais na vasta legislação.

Esta licença aditiva tem validade até 28/02/2022 e altera a composição dos membros da equipe:

- Exclusão de Cássia da Silva Santos da Rocha e Leonardo Alexandre de Souza Ruivo

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 18 de dezembro de 2018.

Cecília J. G. de Almeida

Cecília Jacques de Almeida
Coordenadora da CEUA/Instituto Oswaldo Cruz
Fundação Oswaldo Cruz

Cecília Jacques G. de Almeida
Coordenadora da CEUA - IOC
IOC - FIOCRUZ
Mat. SIAPE: 1535407-0

LICENÇA ADITIVA

L-006/2018- A1

A Comissão CEUA/IOC, em atenção à solicitação da pesquisadora **JOSELI LANNES-VIEIRA,** responsável pela licença (L-006/2018), do protocolo (CEUA/IOC-001/2018), intitulado "**Mecanismos moleculares que contribuem para a cardiomiopatia crônica e alterações comportamentais na infecção experimental pelo *Trypanosoma cruzi*: busca de perspectivas terapêuticas**", que atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL), autoriza o presente aditivo. A referida licença aditiva não exime a observância das Leis e demais exigências legais na vasta legislação.

Esta licença aditiva tem validade até 10/04/2022 e altera a composição dos membros da equipe:

- Exclusão de Cássia da Silva Santos da Rocha e Leonardo Alexandre de Souza Ruivo

- Inclusão de Hílton Antônio Mata dos Santos, Rubem Figueiredo Sadok Menna-Barreto, Yasmin Pedra Resende da Silva e Priscila Cupertino da Silva.

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 14 de dezembro de 2018.

Cecília J. G. de Almeida

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