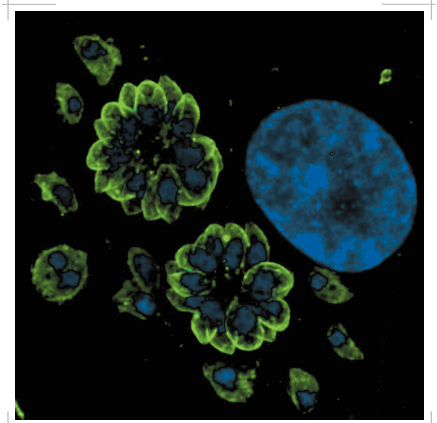
LISBETH CECILIA ROJAS BARÓN

New aspects of coccidia-triggered modulation of the host cellular cell cycle



INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
am Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

édition scientifique
VVB LAUFERSWEILER VERLAG

New aspects of coccidia-triggered modulation of the host cellular cell cycle

INAUGURAL DISSERTATION

submitted to the Faculty of Veterinary Medicine

in fulfilment of the requirements

for the PhD degree

Doctor of Philosophy

of the Faculties of Veterinary Medicine and Medicine

of the Justus Liebig University Giessen, Germany

by

Lisbeth Cecilia Rojas Barón

Biologist from Mérida, Venezuela

Gießen, 2025

1. Auflage 2025

1st Edition 2025

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, 35396 GIESSEN, GERMANY
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

Aus dem Institut für Parasitologie, Justus-Liebig-Universität Gießen

New aspects of coccidia-triggered modulation of the host cellular cell cycle

Inaugural-Dissertation zur Erlangung des Grades eines

Doctor of Philosophy (PhD)

am Fachbereich Veterinärmedizin der Justus-Liebig-Universität Gießen

eingereicht von

Lisbeth Cecilia Rojas Barón

Biologin aus Mérida, Venezuela

Gießen, 2025

From the Institute of Parasitology, Faculty of Veterinary Medicine,
Justus Liebig University of Giessen

Managing Director:	Prof. Dr. Dr. Stefan Arnhold
First reviewer/ Supervisor:	Prof. Dr. Anja Taubert
Second reviewer:	Prof. Dr. Christian Bogdan
Vice-chair/ Co-Supervisor:	Prof. Dr. Tilmann Borggrefe
Chair:	Prof. Dr. Friedemann Weber
Date of Doctoral Defense:	17.02.2025

In memory of my beloved sister, Liseth M. Rojas Barón

(29.08.1996 – 13.01.2022)

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Several data of this dissertation have already been published or presented at conferences:

Original papers

1. Velásquez, Z., **Rojas-Barón, L**., Larrazabal, C., Salierno, M., Gärtner, U., Pervizaj-Oruqaj, L., Herold, S., Hermosilla, C., Taubert, A. (2022). *Neospora caninum* infection triggers S-phase arrest and alters nuclear characteristics in primary bovine endothelial host cells. Front Cell Dev Biol 20;10:1025995.
2. **Rojas-Barón, L**., Hermosilla, C., Taubert, A., Velásquez, Z. (2024). *Toxoplasma gondii* Me49 and NED strains arrest host cell cycle progression and alter chromosome segregation in a strain-independent manner. Front Microbiol, Vol 15.
3. **Rojas-Barón, L**., Hermosilla, C., Taubert, A., Velásquez, Z. (2024). *Toxoplasma gondii* infection-induced host cellular DNA damage is strain-dependent and leads to the activation of the ATM-dependent homologous recombination pathway. Front Cell Infect Microbiol 8;14:1374659.
4. **Rojas-Barón, L**., Senk, K., Hermosilla, C., Taubert, A., Velásquez, Z. (2024). *Toxoplasma gondii* modulates the host cell cycle, chromosome segregation, and cytokinesis irrespective of cell type or species origin. Parasites & Vectors 5;17(1):180.

Conference contributions

1. **Rojas-Barón, L**., Hermosilla, C., Taubert, A., Velásquez, Z. *Toxoplasma gondii* Me49 and NED strains induce cytokinesis failure and host cell cycle dysregulation in primary endothelial host cells. Conference of the DVG specialist group “Parasitology and parasitic diseases”, May 23-25th 2022, Berlin, Germany.
2. **Rojas-Barón, L**., Conejeros I., Butterworth, S., Treeck, M., Hermosilla, C., Taubert, A., Velásquez, Z. *Toxoplasma gondii* infection-driven genome instability is haplotype- and cell type-independent. 30th Annual meeting of the German society for parasitology, March 15-17th 2023, Giessen, Germany.
3. **Rojas-Barón, L**., Hermosilla, C., Taubert, A., Velásquez, Z. *Toxoplasma gondii* infections activate the DNA damage response pathway in primary human and

bovine host cells. Conference of the DVG specialist group “Parasitologie and parasitic diseases”, May 15-17th 2023, Munich, Germany.

4. Conejeros, I., Velásquez, Z., **Rojas-Barón, L**., Espinosa, G., Hermosilla, C., Taubert, A. Role of AMPK in *Besnoitia besnoiti* and *Toxoplasma gondii* tachyzoites-induced PMN responses. European Phagocyte Workshop, March 20-23th 2024, Visegrád, Hungary.
5. **Rojas-Barón, L**., Hermosilla, C., Taubert, A., Velásquez, Z. *Neospora caninum* infections induce DNA damage in primary bovine endothelial host cells. Conference of the DVG specialist group “Parasitology and parasitic diseases”, July 08-10th 2024, Hannover, Germany.
6. Velásquez, Z., **Rojas-Barón, L**., Conejeros, I., Hermosilla, C., Taubert, A. Modulation of the host cell cycle and DNA integrity by different coccidian parasites. Conference of the DVG specialist group “Parasitology and parasitic diseases”, July 08-10th 2024, Hannover, Germany.
7. Espinosa G., Salinas-Varas C., **Rojas-Barón L**., Preußer C., Gärtner U., Conejeros I., Hermosilla C., Taubert A. *Besnoitia besnoiti* tachyzoites- and host cell-derived extracellular vesicles induce NADPH oxidase-independent NET formation. Conference of the DVG specialist group “Parasitology and parasitic diseases”, July 08-10th 2024, Hannover, Germany.
8. Espinosa G., Salinas-Varas C., **Rojas-Barón L**., Preußer C., Gärtner U., Conejeros I., Hermosilla C., Taubert A. *Besnoitia besnoiti* tachyzoites- and host cell-derived extracellular vesicles induce NADPH oxidase-independent NET formation. International Symposium Neutrophil, September 17-19th 2024, Munich, Germany.

ABBREVIATIONS

Akt/PKB: serine-threonine kinase Akt/ protein kinase B signal transduction pathway

APC: antigen-presenting cell

APC/C: anaphase-promoting complex

ATM: ataxia-telangiectasia mutated or ATM serine/threonine kinase

ATR: ataxia telangiectasia and Rad3-related proteins

BCEC: bovine colonic epithelial cells

BCS-1: cell line with epithelial morphology isolated from African green monkey kidney

Bcl2: B-cell leukemia/lymphoma 2 protein

Bcl-xL: B-cell lymphoma-extra large

BER: base excision repair

BeWo: human trophoblast cells

BRCA1/2: breast cancer gene 1/2

BrdU: bromodeoxyuridine

BSIEC: bovine small intestinal epithelial cells

BUVEC: bovine umbilical vein endothelial cells

BUBR1: budding uninhibited by benzimidazole-related 1

CD81: cluster of differentiation 81

Cdc25: cell division cycle-25

CDK: cyclin-dependent kinases

CDV: canine distemper virus

Chk1/Chk2: checkpoint kinase 1/2

c-myc: multifunctional transcription factor

DDR: DNA damage response

DNA-PKcs: DNA-dependent protein kinase catalytic sububit

DRR: direct reversal repair

DNA: deoxyribonucleic acid

DP1: heterodimeric partner DP1

E2F: transcription factor

ERK: extracellular signal-regulated kinases

***Et*ROP1:** rhoptry-derived kinase

FACS: fluorescence-activated cell sorting

Fas: tumor necrosis factor receptor superfamily member 6 (TNFRSF6) or CD95

FHs74: human small intestine epithelial cells

G0: gap 0

G1: gap 1

G2: gap 2

GABA: Gamma-aminobutiric acid

H2A.X: H2A histone family member X

hBMECs: human brain microvascular endothelial cells

HEK293T: immortalized human embryonic kidney cells 293

HFF: human foreskin fibroblast

Hepa1-6: murine hepatoma cell line from a C57L mouse

HepG2: hepatocellular carcinoma epithelial-like

HLA: human leukocyte antigen

HeLa: immortalized cancer cell line isolated from Henrietta Lacks

HR: homologous recombination

HUVEC: human umbilical vein endothelial cells

IFN-γ: interferon-gamma

IL: interleukin

K562: human lymphoma cells

Ku70: DNA repair subunit protein

LD: lethal dose

LINC: linker of nucleoskeleton and cytoskeleton complex

MAD1/MAD2: mitotic arrest deficient genes 1/2

MAD2L1/2: mitotic spindle assembly checkpoint proteins 1/2

MAPK: mitogen-activated protein kinases

Me49: *T. gondii* strain belonging to haplotype II

MMR: mismatch repair

Mre11: double DNA break repair nuclease

mRNA: messenger ribonucleic acid

MTOC: microtubules-organizing center

mTOR: mammalian target of Rapamycin

N. caninum: *Neospora caninum*

NED: *T. gondii* strain belonging to haplotype III

NER: nucleotide excision repair

NF-κB: Nuclear factor kappa B

NHDF: human dermal fibroblast

NHEJ: non-homologous end joining

NOD2: nucleotide-binding oligomerization domain containing protein 2

OCD: obsessive-compulsive disorder

p21: cyclin-dependent kinase inhibitor p21

p27: cyclin-dependent kinase inhibitor p27

p53: tumor protein p53

pBRCA: phosphorylated-breast cancer gene

PCM: pericentriolar material

PCNA: proliferation cell nuclear antigen

PI: propidium iodide

PI3-K: phosphoinositide 3-kinase

PRR: pathogen recognition receptor

PV: parasitophorous vacuole

R: restriction point

Rad50: ATP-dependent DNA-binding protein

Rb: retinoblastoma protein

RH: *T. gondii* strain belonging to haplotype I

ROP: rhoptry protein

RT-PCR: reverse transcription-polymerase chain reaction

sp: specie

S-phase: synthesis phase

T. gondii: *Toxoplasma gondii*

TEM: transmission electron microscopy

TGF-β: transforming growth factor beta

TLR: Toll-like receptor

TNF: tumoral necrosis factor

V-ATPase: vacuolar-type ATPase

Vero: tissue culture cell line from African green monkey kidney epithelial cells

Wee1: nuclear protein kinase

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1. Apicomplexan parasites

The phylum Apicomplexa consists of a large group of parasitic protists, which include more than 6,000 species with an obligate intracellular lifestyle, distributed in a wide diversity of environments, including soil, freshwater and marine habitats (del Campo *et al*., 2019; Votýpka *et al*., 2017). Many of these parasites have a significant clinical and economic relevance since they may cause severe human and veterinary diseases worldwide, thereby affecting millions of individuals each year (Rojas-Pirela *et al*., 2021; Seeber and Steinfelder, 2016). In this context, malaria (*Plasmodium* species), babesiosis (*Babesia* sp.), toxoplasmosis (*Toxoplasma gondii*), neosporosis (*Neospora* sp.) and cryptosporidiosis (*Cryptosporidium parvum*) represent important diseases caused by apicomplexan parasites (Chakraborty *et al*., 2017; Martínez-Ocampo, 2018), whose cell biology and genomes have extensively been studied (Janouškovec *et al*., 2019; Rojas-Pirela *et al*., 2021).

Apicomplexan parasites are characterized by a unique apical complex that defines the phylum name (Votýpka *et al*., 2017). These parasites are composed of a unique cytoskeleton, secretory organelles (micronemes, rhoptries and dense granules), endosymbiont-derived organelles (mitochondria and apicoplast), specific structures (acidocalcisomes and plant-like vacuoles) and universal eukaryotic organelles (nucleus, endoplasmic reticulum, Golgi apparatus and ribosomes), enclosed by a membranous pellicle forming characteristic banana-shaped stages (Dubey *et al*., 1998; Ferguson and Dubremetz, 2020; Votýpka *et al*., 2017). All these organelles and structures vary between parasite species and are involved in motility, host cell attachment, invasion and intracellular parasitophorous vacuole (PV) formation (Rojas-Pirela *et al*., 2021).

So far, the apicomplexan phylogeny has been widely discussed but not terminatory defined. Currently, the taxonomy of apicomplexan parasites includes three major parasitic classes, which include hematozoea (haemosporidians and piroplasms), coccidia (Eimeriidae and Sarcocystidae) and gregarines (eugregarines, blastogregarines and archigregarines). However, data of recent single-cell genomics and transcriptomics separated *Cryptosporidium* spp. from coccidia and gregarines and

classified them as a fourth class (Conoidasida) (Janouškovec *et al*., 2019; Mathur *et al*., 2019).

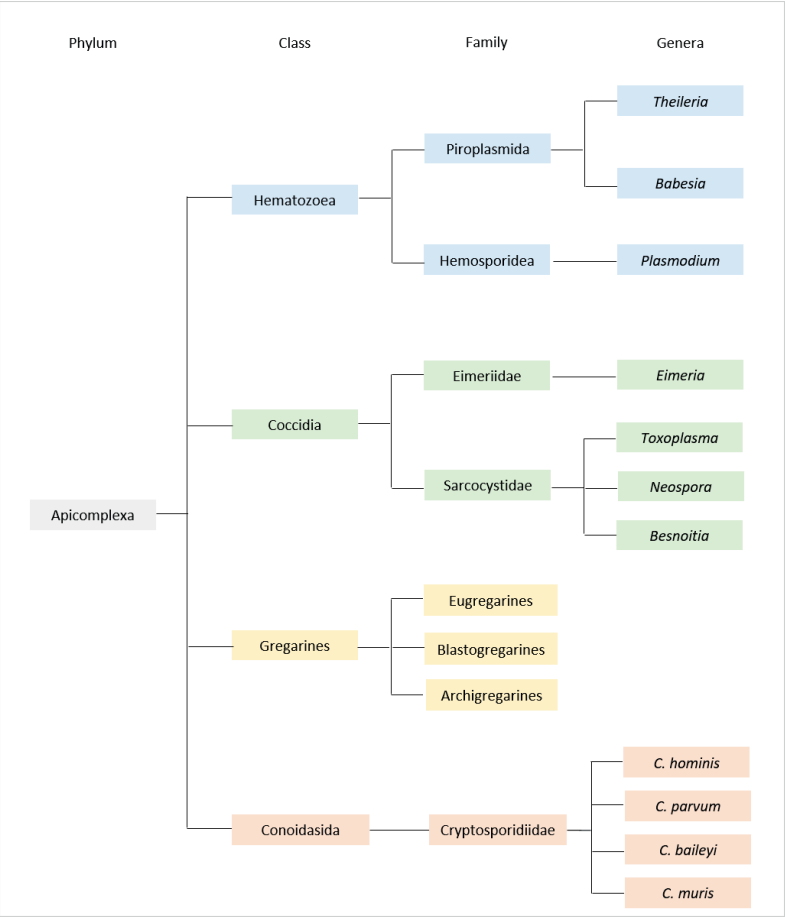


Figure 1. Taxonomic classification of the main apicomplexan species. Apicomplexan parasites are classified into four major parasitic classes: hematozoea (Piroplasmida and Hemosporidia), coccidia (Sarcocystidae and Eimeriidae), gregarines (eugregarines, blastogregarines, archigregarines) and conoidasida, which were separated according to recent genomic and transcriptomic analyses. *Toxoplasma gondii* and *Neospora caninum* are members of the Sarcocystidae family within the coccidian class. [Adapted and modified from Janouskovec *et al*. (2019) and Mathur *et al*. (2019)].

The hematozoean class includes parasites of the apicomplexan clade with relevance for human and veterinary medicine like *Plasmodium*, *Babesia* or *Theileria,* while the gregarine class bears some potential as biological control agents for several pest organisms (insects) (Rueckert and Devetak, 2017). Additionally, coccidian parasites

comprise two important parasite families, Eimeriidae and Sarcocystidae, the latter of which includes *Toxoplasma gondii*, an important zoonotic pathogen. Both families show distinct biological differences but also share common characteristics, such as asexual merogonic proliferation cycles occurring in intermediate and/or definitive hosts, followed by a sexual gametogonic proliferation that exclusively takes place in definitive hosts (Daugschies and Najdrowski, 2005; Lindsay and Dubey, 2020; Martorelli Di Genova and Knoll, 2020; Votýpka *et al*., 2017). Moreover, the conoidasida class includes several *Cryptosporidium* subspecies causing cryptosporidiosis in a broad range of host species.

Given that toxoplasmosis is a highly important zoonosis, the current study focused on molecular mechanisms involved in *T. gondii* infection-driven host cell cycle dysregulation. For comparative reasons, related effects of the closely related parasite *Neospora caninum* were also included in this work.

1.2. *Toxoplasma gondii*

T. gondii, the etiological agent of toxoplasmosis, is an obligate intracellular coccidian parasite that was first described in 1908 in North Africa and Brazil (Ferguson, 2009; Nicolle *et al*., 1909). The species designation originated from the name of the North African rodent *Ctenodactylus gondii* from which the parasite was isolated. *Toxoplasma* is considered as a protozoan pathogen of major medical and veterinary relevance, based on the wide range of hosts (Gubbels *et al*., 2008; Shapiro *et al*., 2019; White and Suvorova, 2018) and its highly sophisticated ability to modulate a broad range of host cell functions to guarantee its intracellular development and replication (Fernández-Escobar *et al*., 2022). Consequently, its life cycle represents a complex transmission process, affecting not only public health, but also livestock industry and wildlife animals (Calero-Bernal *et al*., 2022).

1.2.1. Life cycle and transmission pathways

T. gondii infects a broad range of hosts including terrestrial and aquatic mammals and birds as intermediate hosts where - by definition - exclusively asexual development occurs. In contrast, sexual stages exclusively develop in members of the Felidae family, including domestic cats, thereby acting as definitive hosts (Attias *et al*., 2020; S. Al-Malki, 2021) Intermediate hosts like humans can be infected by several transmission routes, such as (*i*) ingestion of water, vegetables and fruits contaminated

with sporulated oocysts, (*ii*) intake of raw or undercooked meat, such as lamb or pork, containing viable tissue cysts (Dubey, 2009a; Hill and Dubey, 2002; S. Al-Malki, 2021), (*iii*) congenital transmission via the placenta of the mother or *iv*) by transplantation of organs containing cysts or tachyzoites (Figure 2). In contrast, definitive hosts (felines) are typically infected by carnivorism, thereby engorging tissue cysts. Of note, felines act in both categories, i. e. as definitive and intermediate hosts and can therefore also be infected by the oral uptake of sporulated oocysts. Alike, intermediate hosts from wild habitats, such as skunks, racoons, foxes or bears, are also infected by ingestion of sporulated oocysts or prey animals containing cysts (Hill and Dubey, 2002; Lindsay *et al*., 2004). In case of humans, the ingestion of non-pasteurized milk from sheep, goats and cows or milk products also represents a potential source of transmission due to the resistance of tachyzoites to high temperatures and proteolytic enzymes, although not common (Riemann *et al*., 1975; Sacks *et al*., 1982; Stelzer *et al*., 2019; Tenter *et al*., 2000).

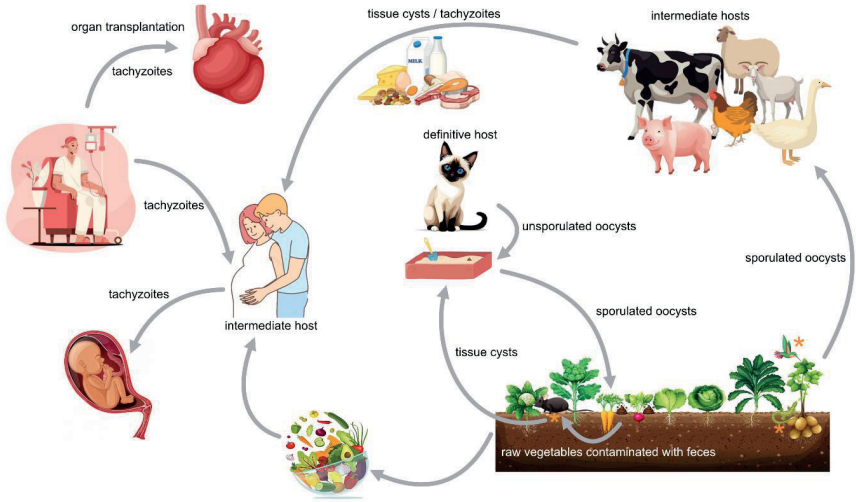


Figure 2. Transmission pathways of *T. gondii*. Sexual stages of *T. gondii* only develop in felid definitive hosts, which shed unsporulated oocysts in the feces. These stages are highly resistant to environmental conditions and develop into sporulated oocysts. When released into the environment through feces, oocysts contaminate vegetables and food crops, which are consumed by both humans and animals, such as cattle, sheep, poultry and pigs (intermediate hosts). The ingestion of contaminated water, vegetables and fruits is one of the most common route of transmission, in addition to the intake of un/undercooked meat and other products from farm animals (water- and food-borne toxoplasmosis). Moreover, pasteurized milk or milk products from infected animals are also considered as a potential source of transmission, although not common. In case of primary infected pregnant women, tachyzoite stages can be transmitted congenitally from the mother via the placenta to the fetus. Other routes of horizontal transmission include the transfer of tachyzoites via blood transfusion or transplantation of organs with cysts or tachyzoites. Typical intermediate hosts like mice, rats, lizards or birds (orange asterisks) are also involved in the life cycle of *T. gondii*, rendering it highly successful and widespread.

Considering its development, *T. gondii* infection follows the typical phases of coccidian parasites, including three infectious stages: (*i*) tachyzoites, a rapidly multiplying form characteristically present in acute infections, (*ii*) bradyzoites, a slowly replicating form which develops within tissue cysts typically found in chronic infections, and (*iii*) sporozoites, which are formed during sporogony within oocyst stages, being produced by definitive hosts and released into the environment via felid feces (Attias *et al*., 2020; Blader *et al*., 2015; Dubey *et al*., 1998). The latter stages result from sexual development (gamogony) in the gut of felines and are ingested by intermediate hosts, where sporozoites are released, invade host cells and convert into tachyzoites, which then multiply by endodyogenies (merogonies, asexual development) in any nucleated cell type during the acute phase of infection (Dubey *et al*., 1998; Hofer, 2019). Following continuous cycles of merogonies, tachyzoites spread within the host body. Thereafter, and most likely triggered by adverse host immune responses, tachyzoites convert into bradyzoites within tissue cysts during the chronic phase of infection. Tissue cysts are predominantly found in the central nervous system (brain) and muscle tissue but may occur in any organ, where they can reside for the total life span of the host (Black and Boothroyd, 2000). Cysts stages are then taken-up by ingestion of infected tissues. During their passage through the digestive tract, bradyzoites are released from cysts and infect epithelial cells of the intestine, where they convert again into tachyzoite stages and multiply for dissemination throughout the body (Frénal *et al*., 2017; Kato, 2018; Opitz and Soldati, 2002).

1.2.2. Population structure of *T. gondii*

T. gondii is a widely spread parasite and approximately one third of the global human population is assumed to have been infected by this protozoa (Calero-Bernal *et al*., 2022). In the domestic cycle of *T. gondii*, a highly clonal population structure was described with three main widespread genotypes (genotypes I-III), varying substantially in their pathogenicity (Calero-Bernal *et al*., 2022; Sibley and Ajioka, 2008). Thus, *T. gondii* possesses a significant genetic diversity, responsible for variations in clinical manifestation (Fernández-Escobar *et al*., 2021). Originally, these clonal types were described by pathogenicity and lethality in laboratory mice. In the murine host system, *T. gondii* type I strains (haplotype I) were classified as highly pathogenic with 100% of cumulative mortality (LD_{100} = 1), while type II strains (haplotype II) were considered as intermediate pathogenic (30% of cumulative mortality, $LD_{50} \geq 10^3$) and type III strains (haplotype III) as non-pathogenic (mortality

index < 30%, LD_{50} > 10^5) (Dardé *et al*., 2014; Sibley and Boothroyd, 1992; Su *et al*., 2002).

In Europe and North America, *T. gondii* genotype II is most common, showing a rather low pathogenicity for humans. In contrast, in South America, a high genetic variety of *T. gondii* strains (genotypes I-III) was stated, leading to the rise of atypical and recombinant genotypes with a high human pathogenicity. Likewise, most of the South American divergent strains (haplogroups 4-10) have been characterized as highly pathogenic in mice (Grigg and Suzuki, 2003; Khan *et al*., 2007). Such atypical and recombinant strains can also rarely be found in Europe or in the United States (Ajzenberg *et al*., 2009).

Referring to human disease, type II haplotypes predominate in congenital toxoplasmosis in Europe, while haplotype I is more frequently present in atypical and recombinant genotypes, being linked to severe forms of toxoplasmosis. However, both, haplotype I and II have been associated with severe cases of acquired toxoplasmosis in immunocompetent patients and with ocular toxoplasmosis (Carme *et al*., 2002; Grigg *et al*., 2001).

1.2.3. Clinical manifestations in toxoplasmosis

Overall, several factors are involved in clinical manifestation of the disease. Thus, strain pathogenicity on one hand and individual immune response on the other hand critically influence the outcome of infection. These depend on factors like haplotype, route of infection, life stage of *T. gondii*, number of passages of the parasite in mice or cell culture, and genetic background of the host (Nayeri *et al*., 2024).

In general, *T. gondii* infections in immunocompetent hosts present as benign self-limiting disease and are therefore mostly asymptomatic (Robben *et al*., 2004). In contrast, in chronically immunocompromised individuals (e. g. HIV patients), *T. gondii* infection may induce an exacerbated immune response, inducing tissue damage with severe and eventual fatal inflammation and necrosis (Chen *et al*., 2024; Niedelman *et al*., 2013; Robben *et al*., 2004; Sana *et al*., 2022; Teimouri *et al*., 2022). Due to its preference for neural cells, *Toxoplasma* infection may also cause neurological manifestations or acute brain damage like toxoplasmic encephalitis (Alvarado-Esquivel *et al*., 2017; Flegr, 2015; Schlüter and Barragan, 2019).

In pregnant women experiencing primary *T. gondii* infections, tachyzoite stages may cross the placenta, and pose severe damage to the foetus, such as long-term disabling sequelae, stillbirths or foetal death (Montoya and Liesenfeld, 2004). The severity of congenital toxoplasmosis depends on the number of parasites crossing the placenta, the immunological maturity of the fetus and the gestational stage (Rojas-Pirela *et al*., 2021).

A highly prevalent clinical manifestation, especially in South America, is ocular toxoplasmosis, which presents as single or multiple areas of necrosis in the retina that may result in blindness. Choroid scars usually result from inflammatory responses with edema and leukocyte infiltrates (Kalogeropoulos *et al*., 2022).

Since recent decades, there is also increasing evidence on statistical correlations between latent *T. gondii* infections and neurological or mental disorders like schizophrenia, bipolar or obsessive-compulsive disorder (OCD), depression, epilepsy, anxiety, personality changes and behavioral disorders, amongst others (Bartolomé del Pino and Zanón-Moreno, 2024). Of note, these correlative analyses in the human system have to be regarded with care since they are mainly of statistical character, often lacking experimental reassurance. However, the initial acute stage of *T. gondii* infection is effectively controlled in healthy hosts, forcing the parasite into a chronic intracellular tissue cyst development that takes place in a variety of tissues, including skeletal muscles, heart and, importantly, the brain, which houses the majority of tissue cysts in both murine and human models (Fabiani *et al*., 2015; Konradt *et al*., 2016; Mangot, 2016). Chronic *T. gondii* infections induce alterations in rodent behavior, giving hints that *T. gondii* infection may also cause equivalent states in humans (Virus *et al*., 2021). The mechanisms behind these effects await elucidation, but it has been hypothesized that the distribution and/or activity of the parasite in distinct brain regions may contribute to altered behavioral responses (Berenreiterová *et al*., 2011; Vyas and Sapolsky, 2010; Webster and McConkey, 2010). These regions include the amygdala (fear responses) and the *nucleus accumbens* (responsible for brain stimulation reward), which seem infected at higher tissue cyst densities (Gulinello *et al*., 2010; House *et al*., 2011; Ikemoto, 2010), besides other areas like the cerebellum, the *pontine nuclei*, the *caudate putamen*, the accessory olfactory bulb and virtually all compact masses of myelinated axons, suggesting that *T. gondii* may indeed exert considerable effects on proper central nervous system operativeness (Berenreiterová

et al., 2011; Virus *et al*., 2021). However, this tissue cyst distribution mainly occurs in rodent models, but - on a stochastic level - also seems apparent in humans (House *et al*., 2011). Referring to parasite-driven neurotransmitter alterations, *T. gondii* chronic infection was reported to raise whole brain dopamine levels up to 15% in mice (Kamerkar and Davis, 2012). Of note, tyrosine hydroxylase genes of the parasite were found associated with neurological signs since their products interfere with dopamine synaptic transmission and, consequently, may mediate neurological changes (McFarland *et al*., 2018). However, recent gene deletion studies gave evidence that tyrosine hydroxylase is not essential for neurobehavioral changes, which rather seem to be fostered by neuroinflammation (Fuks *et al*., 2012). Besides tyrosine hydroxylase-mediated effects, a plethora of other mechanisms are discussed, either based on parasite-driven immune responses or on secreted parasite kinase activities, which affect host cell signaling leading to dysregulated functions of neurotransmitters like GABA, serotonin, noradrenalin, nitric oxide, kynurenic acid, glutamate, or proinflammatory cytokine production (Bottari *et al*., 2015; Dincel and Atmaca, 2015; Fuks *et al*., 2012; Gatkowska *et al*., 2013; Kannan *et al*., 2016; Müller and Schwarz, 2007; Ngô *et al*., 2017; Tonin *et al*., 2014). Interestingly, *T. gondii* infection-driven host cell alteration shares transcriptional pathways with other CNS disease states (i. e. NF-κB, TGFβ, NOD2, TLR, HLA) and modulates immune processes during chronic infections, thereby causing changes in neuronal connectivity, synaptic plasticity and altered brain functions (Alvarado-Esquivel *et al*., 2017; Ngô *et al*., 2017; Virus *et al*., 2021), all of them potentially linked to the development of neurological and psychiatric disorders.

Besides humans, *T. gondii* also plays a major role in veterinary medicine leading to considerable economic losses, worldwide. In this context, primary *T. gondii* infections can result in reproductive failure, embryonic death, resorption, mummification, abortion, stillbirths and neonatal death, especially in small ruminants, (mainly sheep) (Dubey, 2009b; Holec-Gąsior and Sołowińska, 2023; Stelzer *et al*., 2019). The severity of ovine infections depends on the stage of gestation at which the ewe becomes primary infected; i. e. the earlier in gestation, the more severe the consequences (Dubey, 2009c; Innes, 2010). Hence, in the early phase of pregnancy, toxoplasmosis may result in foetal death with subsequent resorption or mummification while infections at later gestation phases may cause abortion, stillbirth or perinatal death of the new-bornes (Dubey, 2009c). Even though toxoplasmosis is an important parasitic infection,

the precise number of losses in lambs is difficult to estimate since the disease does not cause clinical manifestations in the ewes. *T. gondii* DNA was detected in up to 25% of aborted fetuses with pathological findings in placentas, muscle samples, brains, abomasi, livers and spleens (Dubey, 2009c; Owen *et al*., 1998). Overall, *T. gondii* DNA was detected at higher quantities in muscle and brain samples of the fetuses (Masala *et al*., 2007, 2003). Alike sheep, *T. gondii* infections are also highly prevalent in goats worldwide, with serious implications in reproductive disorders (de Oliveira *et al*., 2022; Dubey, 2009c), including abortions as the most predominant consequence. In this context, *T. gondii* induces tissue lesions in infected animals, fostering necrosis and inflammation in the central nervous system, liver and lungs of aborted fetuses (de Barros *et al*., 2022; de Oliveira *et al*., 2022). Moreover, necrosis and calcification of cotyledonary tissues was observed in the placenta of infected goats (Unzaga *et al*., 2014). Moreover, in acute infections, thrombosis and infarct areas have been found in the caruncles (Castaño *et al*., 2014), all of them contributing to abortive processes.

In dogs and cats, toxoplasmosis mainly includes general clinical signs, such as fever, anorexia or dyspnea. In most cases, *T. gondii* infections are associated with low morbidity and mortality in both dogs and cats. Feline toxoplasmosis can aggravate in transplacentally infected kitten, resulting in hepatitis, cholangiohepatitis, pneumonia and encephalitis being accompanied with ascites, lethargy and dyspnea (Dubey and Carpenter, 1993). Of note, hepatic failure and hyperplastic cholangitis was also described in adults besides other signs like intestinal enteritis, inflammatory intestinal disease, thickening of the gastric wall and lymphadenopathy (De Tommasi *et al*., 2014). Hence, feline toxoplasmosis may rarely become fatal in cats showing severe respiratory or neurological signs (Brennan *et al*., 2016; Cohen *et al*., 2016; De Tommasi *et al*., 2014; Dubey and Carpenter, 1993; McConnell *et al*., 2007; Peterson *et al*., 1991). Dogs rarely suffer from toxoplasmosis as a primary disease. In most cases, the disease is linked to immunosuppression or a lack of vaccination against canine distemper virus (CDV). In clinical cases, signs may include neurological disorders like seizures, cranial nerves deficits, tremors, ataxia, paresis and paralysis, muscle wasting and stiffness (Patitucci *et al*., 1997). However, pneumonia seems one of the main signs of toxoplasmosis and an acute respiratory distress syndrome and septic shock may occur (Brownlee and Sellon, 2001; Evans *et al*., 2017). Ocular diseases are also described for both cats and dogs including necrotizing conjunctivitis, uveitis, endophthalmitis and chorioretinitis (Dubey and Carpenter, 1993; Swinger *et al*.,

2009; Wolfer and Grahn, 1996). Moreover, cutaneous manifestations have been documented, characterized by erythematous epidermal nodules, pyogranulomatosus necrotizing dermatitis with multifocal vasculitis and vascular thrombosis (Bernsteen *et al*., 1999; Hoffmann *et al*., 2012; Oliveira *et al*., 2017).

1.3. The cell cycle in mammalian cells

Given that this work focuses on *T. gondii*-driven modulation of the host cell cycle, the general principles of cell cycling will here be reviewed. Cell division is a highly complex and tightly regulated biological process, which follows the aim to divide the mother cell and to correctly duplicate chromosomal DNA to deliver the cellular information of the mother cell to two genetically identical daughter cells (Satyanarayana and Kaldis, 2009). This process includes two major phases, interphase and mitosis.

1.3.1. Interphase

The interphase includes the sequential events of gap 1 (G1)-, synthesis (S)- and gap 2 (G2)-phases (Alberts *et al*., 2007). During G1-phase, the cell grows significantly, pivotal organelles are copied, and molecular building blocks are generated to be used in later steps (Bertoli *et al*., 2013). During S-phase, the cell synthesizes a complete copy of the genomic DNA and duplicates the centrosome, a microtubule-organizing structure, which assists in separating chromosomes during mitosis (Kõivomägi *et al*., 2011; Westendorp *et al*., 2012) During G2-phase, the cell continues growing, produces proteins and organelles and starts to reorganize its content in preparation for mitosis (Alberts *et al*., 2007; Kousholt *et al*., 2012).

It is important to highlight that G1- and G2-phases not only reflect cell growth and building block synthesis. These phases also include the control of internal and external environmental parameters to ensure suitable cell cycling conditions and to guarantee the fulfillment of all molecular requirements before progressing to S-phase and mitosis (Wang, 2021). In this respect, G1-phase is especially important since its duration can greatly vary depending on external conditions or on extracellular signals coming from other cells. For example, if extracellular conditions are unfavorable, cells delay their progression through G1-phase and enter a specialized resting phase, called G0-phase, in which they can remain for days, weeks or even years before returning to a proliferative state (Cheung and Rando, 2013; Sajiki *et al*., 2018). Two different G0-state types are described and are categorized as quiescence and senescence. Whilst

quiescence refers to a reversible G0-state, senescence mirrors an irreversible status of cells in response to severe DNA damage, which would lead to nonviable progeny formation. Overall, G0-states represent strategies alternative to cellular suicide, e. g. driven by apoptosis (Anvarbatcha *et al*., 2023; Burton and Krizhanovsky, 2014; Campisi and d'Adda di Fagagna, 2007; Fukada *et al*., 2007; Rodier and Campisi, 2011).

1.3.2. Mitosis

Mitosis also represents a highly regulated process and includes several consecutive phases: prophase, prometaphase, metaphase, anaphase and telophase (Alberts *et al*., 2007). Mitosis comprises two main events: nuclear division, in which the chromosomes are segregated, and cytokinesis, in which the cytoplasm is divided, finally resulting in two identical daughter cells (McIntosh, 2016) (Figure 3).

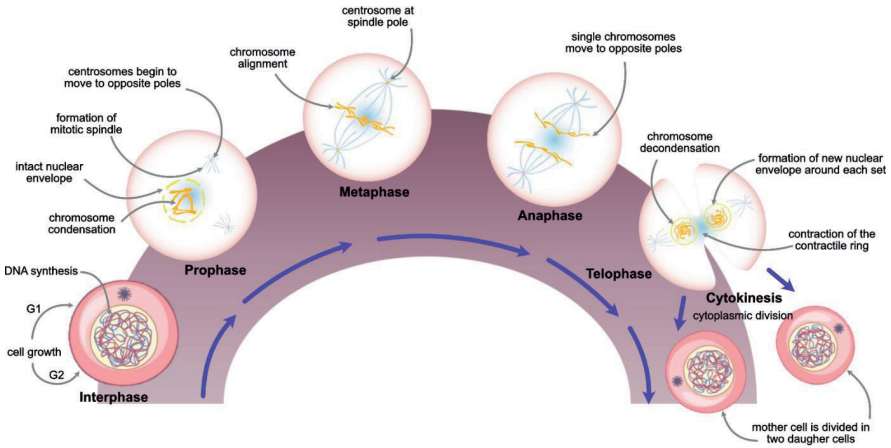


Figure 3. Principle steps of mitosis. Mitosis includes chromosome condensation in prophase, chromosome alignment in metaphase, separation of sister chromatids to opposite poles in anaphase, and mitotic spindle rupture and formation of two new nuclei (one for each set of chromosomes) in telophase, finally leading to cytoplasmic division during cytokinesis.

In prophase, chromosomes condense and the mitotic spindle begins to form, originating from centrosomes, which start to move towards opposite cell poles. As consequence, the nuclear envelope starts to break down (Ong and Torres, 2019; Yim and Erikson, 2011). In prometaphase, the nuclear envelope is fully disassembled and microtubules from the spindle apparatus attach to the kinetochores, which are protein

structures in the centromeres of the chromosomes (Alberts *et al*., 2007; Feitoza *et al*., 2017). Metaphase is then characterized by a chromosome alignment along the metaphase plate, an imaginary plane equidistant to the two spindles poles (Guo *et al*., 2013; Orr and Maiato, 2019). Then, each sister chromatid is attached to spindle fibers from opposite poles. During anaphase, cohesin proteins binding the sister chromatids together are cleaved, allowing the sister chromatids to separate. Then, the single chromatids are pulled towards opposite poles of the cell via the spindle fibers. In telophase, the two sets of daughter chromosomes arrive at the poles of the spindle and decondense (Chu *et al*., 2022). A new nuclear envelope reassembles around each set of chromosomes, resulting in two separate nuclei within the mother cell, marking the end of mitosis (Yim and Erikson, 2011). Ultimately, during cytokinesis, the cytoplasm is divided into two portions by a contractile ring of actin and myosin filaments forming a cleavage furrow and finally dividing the mother cell into two identical daughter cells, each with one nucleus (Figure 3).

1.3.3. Mechanisms of cell cycle control

The cell cycle represent a series of highly regulated steps that govern cell proliferation. Key molecules of cell division control are the cyclins and their binding partners, the cyclin-dependent kinases (CDKs), a family of serine/threonine kinases (Suryadinata *et al*., 2010). Cyclins and CDKs control cell cycle progression by phosphorylation of regulatory proteins. Hence, both CDKs and cyclins form part of cell cycle checkpoints, which represent surveillance mechanisms monitoring the order, integrity and fidelity of major cell cycle events to guarantee correct phase progression (Barnum and O'Connell, 2014; Morgan, 1996; Pietenpol and Stewart, 2002). The transition from one to another cell cycle phase is mainly regulated by the formation of cyclin-CDK complexes (Satyanarayana and Kaldis, 2009). The different members of the cyclin family are expressed in a stimulus-dependent manner and thereafter are rapidly degraded leading to a cell cycle phase-specific, periodic cyclin presence or absence (see Figure 4). Hence, Cyclin D starts to accumulate in early G1-phase, cyclin E in late G1-early S- and cyclin A during S-phase with a gradual decrease in G2-phase (Coverley *et al*., 2002; Gu *et al*., 1992) (Figure 4). Progression to mitosis depends on the accumulation of cyclin B, which is then degraded with cell cycle finalization

(Vorlaufer and Peters, 1998). In contrast, CDKs are constitutively expressed, but are activated via their binding to cyclins in cyclin complexes (Figure 4).

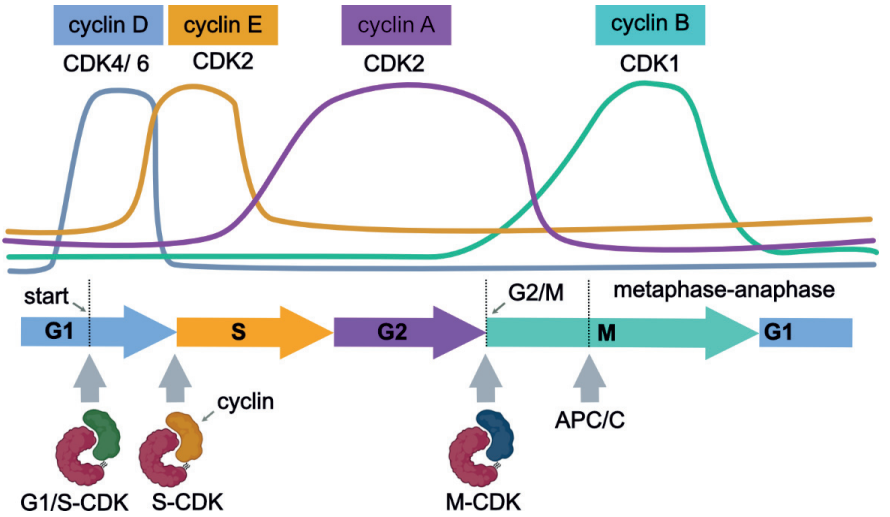


Figure 4. Cyclins expression and formation of cyclin-CDK complexes during cell cycling. Cyclins experience synthesis and degradation in each cell cycle phase due to cyclic changes in phosphorylation. The activity of CDKs depends on the presence of corresponding cyclins. The expression of cyclin D is enhanced in early G1-phase, declining in late G1-phase. A progressive accumulation of cyclin E appears in late G1-phase with a prominent peak in the S-phase and degradation before early G2-phase. Then, cyclin A accumulates in S-phase with degradation in early M-phase, which is partially mediated by the APC/C protein. The onset of M phase is mediated by cyclin B accumulation. Cyclin B degradation coincides with the end of cell division.

In G1/S-transition phase, a pivotal checkpoint - also known as restriction (R) point - ensures that all cellular requirements are properly fulfilled for adequate DNA synthesis (Figure 5). Therefore, the cell is monitored for adequate size, energy reserves and absence of DNA damage (Bertoli *et al.*, 2013). Key molecules of the G1/S-checkpoint are the retinoblastoma protein (Rb) and other transcription factors, such as E2F and p53, which are central tumour supressors regulating cell division by promoting cell cycle arrest to repair DNA damage or to induce apoptosis in case of irreversible damage (Engeland, 2022a; Ozaki and Nakagawara, 2011). In that case, p21 is transcriptionally activated by p53 with concomitant activation of p27 (cell cycle inhibitors), inhibiting the kinase activity of CDK2 and CDK1 and leading to growth arrest at specific stages in the cell cycle. Taken together, these elements constitute the p53-p21-Rb-signaling pathway (Abbas and Dutta, 2009; Abbastabar *et al.*, 2018). After progression through S- and G2-phases, the G2/M checkpoint ensures that the cell

fulfills all criteria to enter mitosis (Figure 5). At foremost, the cell is controlled for completeness and accuracy of DNA replication in addition to the absence of any DNA damage (Sale *et al*., 2012; Tang *et al*., 2024). The most important factors of this checkpoint are p53, ATM/ATR kinases and Chk1/Chk2 (Stark and Taylor, 2004) (Figure 5).

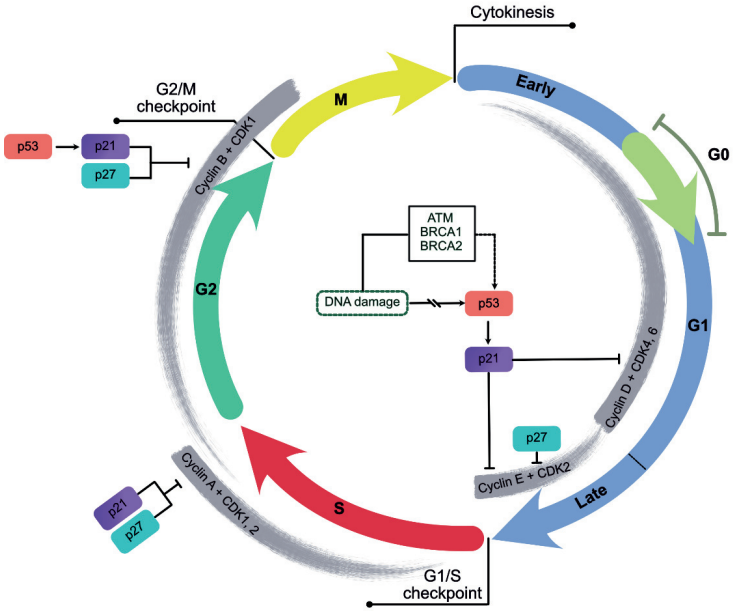


Figure 5. Main proteins and transcription factors of cell cycle checkpoints. Cyclins and CDKs promote cell cycle progression while CDK inhibitors block the transition between phases. The balance between both determines cell proliferation. In early G1-phase, the presence of growth factors is required for progression. The DNA integrity is monitored before DNA replication (S-phase) via the G1/S checkpoint. P53 is a fundamental tumour suppressor, which regulates cell division via cell cycle arrest to repair DNA damage or to induce apoptosis. CDK inhibitors like p21 and p27 block cell cycle progression by inhibiting cyclins A/B and CDK1/2, which are pivotal for G1/S and G2/M checkpoints. The G2/M checkpoint controls DNA integrity before procession to mitosis.

Besides the highly important G1/S- and G2/M-checkpoints, a further relevant checkpoint exists in the metaphase of mitosis, which controls both, the proper attachment of the chromosomes to the spindle apparatus and the correct alignment of chromosomes in the metaphase plate before the cell progresses into anaphase. Key elements of this checkpoint are MAD1 and MAD2 (mitotic arrest deficient genes), BUBR1 (budding uninhibited by benzimidazole-related 1), APC/C (Anaphase promoting complex/cyclosome), securin and separase (Bertoli *et al*., 2013; Lu *et al*.,

2014; Luo and Tong, 2021a; Raaijmakers *et al*., 2018). MAD1 recruits the anaphase inhibitor MAD2 to accumulate at kinetochores and generates a wait signal, thereby preventing the cell from progressing to anaphase, until spindle microtubules have correctly been aligned with the kinetochores on each chromosome. Here, BUBR1 is directly involved, i. e. if any chromatids remain unattached, BUBR1 expression prevents cell division (Matsuura *et al*., 2006; Sironi *et al*., 2002; Suijkerbuijk *et al*., 2010). Then, APC/C controls sister chromatid segregation and the exit from mitosis by catalyzing ubiquitylation of cyclins and the other cell cycle regulatory proteins under regulation of separase-securin complex (Barford, 2011; Hellmuth *et al*., 2020; Luo and Tong, 2021b).

1.3.4. DNA damage repair pathways

Given that DNA damage signifies a highly severe insult for a cell which could be abortive for daughter cells, different cellular pathways of DNA repair exist to maintain genetic stability and integrity, once mammalian cells experience endogenous (e. g. cellular metabolic processes including reactive oxygen species production, oxidation, alkylation or hydrolysis) or exogenous (e. g. environmental factor like UV light, ionizing radiation or chemotherapy agents) DNA-damaging events (Tubbs and Nussenzweig, 2017). If damaged DNA is not properly repaired, this can lead to genomic instability, apoptosis or senescence, predisposing cells to immunodeficiency, neurological disorders and cancer (Hakem, 2008; Subba Rao, 2007; Thoms *et al*., 2007). To prevent this scenario, cells own a series of mechanisms, summarized as DNA damage response (DDR), which include both DNA-damage checkpoints and repair pathways (Li *et al*., 2021). However, the mode of DDR depends on the type of DNA damage occurring in the cell, i. e. different repair systems respond to DNA single- or double-strand lesions (Giglia-Mari *et al*., 2011) (Figure 6). In this context, the major pathways are (*i*) direct reversal repair (DRR), which mainly repairs the lesion induced by alkylating agents; here *de novo* DNA synthesis is not required to correct errors (Gutierrez and O'Connor, 2021), (*ii*) base excision repair (BER), which corrects small base lesions mainly caused by oxidation; here a DNA glycosylase removes the damaged base and fills the gaps by short- or long-patch repair (Krokan and Bjørås, 2013), (*iii*) nucleotide excision repair (NER), which primarily removes helix-distorting DNA lesions (Li *et al*., 2021), (*iv*) mismatch repair (MMR), that recognizes and repairs erroneous insertions, deletions and mis-incorporation of bases arising during DNA

replication and recombination (Li, 2008) and, (*v*) recombinational repair, which includes the homologous recombination (HR) and non-homologous end joining (NHEJ) repair pathway, operating at double-strand DNA breaks (Hakem, 2008; Li *et al*., 2021). Double-strand DNA breaks are considered as the most severe and lethal DNA damage type that cells can experience, ultimately leading to cell death or serious genetic mutations, if left unrepaired. Interestingly, the recombinational repair operates cell cycle-dependent. Thus, the HR pathway is activated during S- and G2-phase, whilst the NHEJ repair pathway in principle is activated at any cell cycle phase but seems more important in G1-phase (Li and Heyer, 2008; Watanabe and Lieber, 2022) (Figure 6). Both, the HR and NHEJ pathways are considered as the two most important pathways for the repair of double-strand DNA breaks, fostering a complex process which requires the activation of a cascade of proteins (Lieber, 2010) (Figure 6). In this sense, the HR pathway branches into ATM and ATR pathways, two repair routes which act according to the type of damage, i. e. either double-strand DNA breaks (ATM) or stalled replication forks (ATR) (Awasthi *et al*., 2015; Goodarzi *et al*., 2003; Hakem, 2008; Maréchal and Zou, 2013) (Figure 6). Within these pathways, ATM, ATR and DNA-PKcs phosphorylate several target molecules, which contribute to the overall DNA damage response (Figure 6). As an immediate event occurring after double-strand DNA breaks, phosphorylation of histone H2A.X resulting in γH2A.X takes place, which is the first step in recruiting DNA repair proteins, acting as substrates and mediators of damage responses, including BRCA1, Chk1 and Chk2 (Kuo and Yang, 2008). When activated, ATM, ATR, Chk1 and Chk2 promote the phosphorylation and subsequent activation of p53, a key player in the DNA damage checkpoint (Su, 2006) (see also Figure 5). When p53 transactivates p21, inhibition of CDK2 and CDK4 takes places (= G1/S-promoting cyclin-dependent kinases), leading to a sustained G1 arrest to prevent damaged DNA replication (Hakem, 2008). The ATM and ATR pathways are

essential for the G1/S, S-phase and G2/M DNA damage checkpoints and are critically involved in the maintenance of genomic integrity.

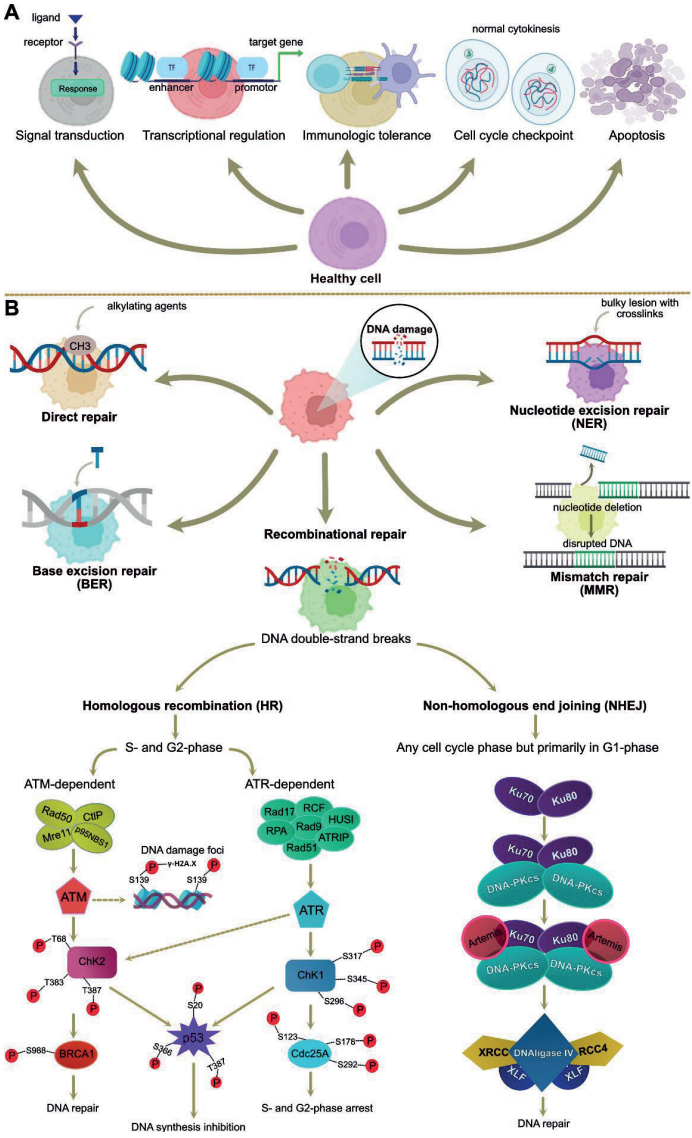


Figure 6. DNA repair pathways in cells. (A) Under physiological conditions, cells efficiently control DNA synthesis and replication by diverse mechanisms, which include adequate signal transduction, transcriptional regulation, immunologic tolerance, regulation via cell cycle checkpoints and, if necessary, apoptosis induction. **(B)** In case of cellular DNA lesions, DNA damage responses (DDR) are elicited to protect the cells from eventually arising detrimental defects. Here, DNA single-strand damages are

corrected by (*i*) direct reversal repair (DRR) (mainly active when lesions were induced by alkylating agents), (*ii*) base excision repair (BER) that corrects small base lesion, (*iii*) nucleotide excision repair (NER) removing helix-distorting DNA lesions and, (*iv*) mismatch repair (MMR), which corrects erroneous insertions or deletions. DNA double-strand breaks are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ) repair pathways. Both pathways are cyclin-dependent. Hence, the HR pathway operates when DNA damage occurs in S- or G2-phase, whilst the NHEJ pathway can be activated at any cell cycle phase, but mainly operates in G1-phase. The HR pathway branches into ATM and ATR pathways, which both involve a cascade of molecular reactions finally resulting in DNA repair and cell cycle arrest, respectively.

1.4. Modulation of the host cell cycle by intracellular parasites

In line to other obligate intracellular parasites, *T. gondii* extensively manipulates a broad range of pivotal biological processes in its host cell to guarantee intracellular replication and survival. In former studies, *T. gondii* indeed revealed as a master of host cell modulation since it significantly inhibits host cellular defense mechanisms (Chen *et al*., 2024; Hunter and Sibley, 2012; Zhu *et al*., 2019), exploits the host cellular metabolism (Ewald *et al*., 2024; Shunmugam *et al*., 2022), changes host cellular skeleton and autophagy (Besteiro, 2019; Cheng *et al*., 2022; Walker *et al*., 2008; Wang *et al*., 2009; White *et al*., 2024), controls apoptosis (Besteiro, n.d.; DU *et al*., 2022; Su *et al*., 2023) and also affects host cell cycle (Brunet *et al*., 2008; Kim *et al*., 2016; Molestina *et al*., 2008; Pierre-Louis *et al*., 2022; Sabou *et al*., 2020), amongst other cell functions. Referring to the latter, progression through cell cycle phases creates a high demand of nutrients, metabolites, aminoacids, proteins and sugars to provide sufficient energy and building blocks for DNA synthesis and cellular development. Thus, the cellular metabolism continuously changes, depending on the specific requirements in each cell cycle phase. Given that intracellular parasite proliferation also signifies a highly energy- and building block-demanding process, parasite-driven modulation of essential biological host mechanisms like the cell cycle represents a fundamental strategy to ensure successful parasite development and proliferation. Hence, it represents a common target of intracellular parasite-driven manipulation.

1.4.1. Host cell cycle manipulation by apicomplexa other than *T. gondii*

Apicomplexan parasites are obligate intracellular protozoan widely recognized as efficient modulators of a broad range of host cellular functions, such as the cell cycle. Studies on different *Plasmodium* species demonstrated that erythrocyte infections - even though this cell type does not own a nucleus and consequently cannot divide - trigger changes in cell signaling pathways that mimic certain aspects of cell cycle progression, thereby aiding in efficient parasite replication (Hanson *et al*., 2015). Thus, *Plasmodium falciparum*-infected erythrocytes exhibit changes in cycle progression

leading to abnormal mitotic events (Hanson *et al*., 2015). In contrast, *P. falciparum*-infected hepatocytes were found arrested in G2/M-phase to create a cellular environment that facilitates parasite development and replication (Hanson *et al*., 2015). At transcriptional and post-transcriptional level, *P. berghei* infections manipulate the G2/M checkpoint by repressing cyclin A and B in Hepa1-6 cells as well as the APC complex in anaphase (Albuquerque *et al*., 2009; Wang *et al*., 1999). However, other findings come from HepG2-CD81 cells showing that *P. yoelii* infections maintain the proliferative state of host cells with activated retinoblastoma protein (Rb) and low p53 abundance (Kaushansky *et al*., 2013), thereby controlling G1- to S-phase transition. In contrast, *P. berghei*-infected HepG2 cells showed an S-phase stasis after sporozoite invasion (Hanson *et al*., 2015). Moreover, a substantial proportion of binucleated cells (31%) was here stated, indicating cytokinesis failure, which was mainly found in cells containing large schizonts (Hanson *et al*., 2015).

Another apicomplexan master modulator of host cell cycle progression is *Theileria*, which extensively drives host cell transformation, finally resulting in continuous clonal cell expansion (Chakraborty *et al*., 2017). Of note, at each host cell division cycle, parasite stages are transferred to each daughter cell, thereby leading to an exponential spread of the pathogen. Indeed, *Theileria* spp. transform their host cells (mainly lymphocytes) into a cancerous phenotype by modulating host cell kinases and transcription factors like CDK2, CDK4, CDK6, cyclin D, NF-kB, PI3-K, p53 (Dobbelaere *et al*., 2000). Here, the constitutive activation of cyclin D seems pivotal for oncogenic host cell transformation (Marshall, 1999). Both NF-kB and PI3-K regulate cyclin D at the transcriptional level. The translation of cyclin D mRNA is regulated via the mTOR pathway, whilst its stability is enhanced via Akt/PKB-dependent inactivation of glycogen synthase kinase 3β, which induces phosphorylation, ubiquitination and degradation of cyclin D (Galley *et al*., 1997). The main target of cyclin D/CDKs is the tumor suppressor molecule Rb, which maintains E2F transcription factors in their inactive forms (Engeland, 2022b). Cyclin D-dependent kinases initiate the phosphorylation of Rb, thereby disrupting its association with E2F. This results in E2F-dependent transcription of an array of genes necessary for DNA synthesis, amongst them are c-myc and cyclin E (Dobbelaere *et al*., 1999; Dobbelaere and Heussler, 1999). Cyclin E-dependent CDK2 then induces the phosphorylation and degradation of p21 and p27, which maintain cyclin E- and cyclin A-dependent kinases in an inactive form (Engeland, 2022b). From this moment onwards, the cells are irreversibly

committed to continue S-phase and to proceed to complete the cell cycle. Thus, with NF-kB, PI3-K and potentially AP-1 inducing constitutive transcription of the cyclin D gene and PI3-K-dependent pathways ensuring cyclin D stability and translation, constitutively high levels of cyclin D stimulate continuous cell cycle progression in *Theileria*-infected cells (Dobbelaere *et al*., 2000).

Eimeria tenella, one of the most pathogenic etiological agents of avian coccidiosis, was also demonstrated to trigger host cell cycle dysregulation (Diallo *et al*., 2019). Here, the overexpression of *Et*ROP1, a rhoptry-derived kinase, induced a G0/G1 arrest and a reduction of cells in S-phase in transfected HEK293T cells. Additionally, a stimulation of p21 gene transcription and protein expression was stated, findings which were consistent with a previously observed host cell cycle arrest via activation of the p53/p21 pathway (Diallo *et al*., 2019).

In the bovine system, *E. bovis* infection induced an arrest at G1/S transition in primary bovine endothelial host cells with concomitant upregulation of cyclin E1 and downregulation of cyclin B1 at the late phase of macromeront formation, which was additionally characterized by premature senescence (Velásquez *et al*., 2021b). Moreover, *E. bovis* infection triggered a phenotypic change in host cell nuclei being characterized by nucleolar fusion and heterochromatin-enriched peripheries (Velásquez *et al*., 2021b).

1.4.2. *T. gondii*-driven cell cycle modulation

T. gondii was shown to profoundly reprogram the gene expression of infected cells, thereby also affecting transcription factors that regulate host cell proliferation and cell cycling (Khelifa *et al*., 2021; Li *et al*., 2023; Menard *et al*., 2021; Molestina and Sinai, 2005; Sun *et al*., 2021). Interestingly, the cell cycle division autoantigen-1, a host cell-derived molecule with inhibitory functions on cell cycle, slows parasite growth when being overexpressed and induces the expression of bradyzoite-specific proteins (Behnke *et al*., 2008; Radke *et al*., 2005; Sokol-Borrelli *et al*., 2023). These findings provided evidence that changes in host cell transcription, particularly in profiles affecting the cell cycle machinery, own the capacity to affect parasite development. Other hints on *T. gondii*-driven cell cycle interference are (*i*) an increase in transcripts associated with metabolic processes in the host cell including glycolysis and gluconeogenesis, which is consistent with typical events occurring in S-phase, such as

synthesis of DNA, nucleotides, aminosugars, amongst others (Blader and Saeij, 2009), (*ii*) relocation of host cellular microtubules around the PV which may alter cell cycle progression to mitosis (Coppens *et al*., 2006a; Coppens and Romano, 2018), and (*iii*) an infection-driven downregulation of mitotic proteins, such as cyclin B1 (Nelson *et al*., 2008).

First evidence on *T. gondii*-mediated interference with the host cell cycling machinery came from past observations on host cell invasion as the first step of infection. Thus, the infective capacity of *T. gondii* increased when synchronized host cells proceeded from G1- or S-phase and decreases when cells entered G2/M transition-phase (Dvorak and Crane, 1981; Grimwood *et al*., 1996). In this respect, reports on *T. gondii*-driven cell cycle perturbation included somewhat varying results in different cells types but primarily documented an arrest in early cell cycle phases. Thus, infected human epithelial cells promoted a G1/S transition followed by G2-phase arrest and cyclin B1 downregulation, the latter of which is probably the major cause of arrest (Brunet *et al*., 2008). However, *T. gondii*-infected human foreskin fibroblast (HFF) were arrested in S-phase (Lavine and Arrizabalaga, 2009; Molestina *et al*., 2008; Pierre-Louis *et al*., 2022), accompanied by a delayed or lacking increase of cyclin A2 and cyclin B1 in combination with an early elevation of cyclin E1 and an oscillatory profile of ERK (extracellular signal-regulated kinases) activation. These findings suggest a missing exit from S-phase and failure to progress toward mitosis in *T. gondii* infection (Molestina *et al*., 2008). This kind of cell cycle perturbation depended on parasite viability and proliferation since replication-defective tachyzoites barely influenced the host cell cycle (Molestina *et al*., 2008). Referring to parasite-derived molecules being involved in cyclin regulation, recent findings demonstrated that a tachyzoite-derived effector protein, HCE1 (host cell cyclin E1), bound to host cellular E2F/DP1 heterodimers and triggered robust host cyclin E upregulation (Panas *et al*., 2019). *T. gondii* infections also fostered non-infected bystander cells to enter S-phase, thereby indicating that soluble factors contributed to cell cycle modulation (Lavine and Arrizabalaga, 2009). In line, extracellular vesicles from *T. gondii*-infected L6 rat myoblast cells induced both, a decreased proliferation and S- or G2/M-phase stasis (Kim *et al*., 2016). Other reports suggested that driving host cells into S-phase was beneficial for the parasite since *T. gondii* preferentially invaded cells in S-phase (Grimwood *et al*., 1996). In contrast, human trophoblast and dermal fibroblast cells infected with *T. gondii* were arrested in G2-phase whilst a G2/M-based cell cycle arrest

was driven in primary bovine endothelial cells (Brunet *et al*., 2008; Velásquez *et al*., 2019). G2-phase arrest was accompanied by a cyclin B1 downregulation in trophoblasts, whilst cyclin B1 levels remained unaffected in endothelial host cells (Brunet *et al*., 2008; Velásquez *et al*., 2019). Of note, cyclin B1 gene promotor silencing was mediated by *T. gondii*-secreted ROP16, targeting the host cellular transcription factor UHRF1. This event triggered host cell cycle arrest by inducing a series of epigenetic reactions, such as deacetylation and histone H3 methylation around the cyclin B1 promotor (Sabou *et al*., 2020).

Referring to later stages of cell division, studies on *T. gondii*-infected murine RAW264.7 cells showed an enhanced proportion of polyploid cells (8n), thereby reflecting a successful DNA replication but a failure of cytokinesis (Franco *et al*., 2016). Cytokinesis failure will result in the presence of binucleated host cells, as it was also observed in *T. gondii*-infected primary endothelial cells (Velásquez *et al*., 2019). In this cell type, *T. gondii* infections not only perturbed early phases of the host cell cycle but also affected mitotic events by inducing chromosome segregation errors, mitotic spindle alteration and blockage of cytokinesis progression, being accompanied by a downregulation of Aurora B kinase expression (Velásquez *et al*., 2019). In general, chromosome segregation failures and cytokinesis impairment are linked to chromosomal instability (Holland and Cleveland, 2009), a phenomenon which may mirror DNA damage, resulting from DNA strand breaks and cellular stress during DNA replication (Wilhelm *et al*., 2020). In fact, recent data confirmed that *T. gondii* RH strain-infected tumour cells experienced parasite-driven DNA double-strand breaks and responded by activating the homologous recombination repair pathway (Zhuang *et al*., 2020).

1.5. *Neospora caninum*

Neospora caninum is an obligate intracellular parasite (Apicomplexa: Sarcocystidae), closely related to *T. gondii*. Since its discovery in dogs with encephalomyelitis and myositis (Dubey, 1999; Hemphill and Gottstein, 2000), *N. caninum* has been recognized as an important infectious agent causing major reproductive failures in cattle with concomitant economic losses worldwide (Dubey, 1999; Hemphill and Gottstein, 2000; Innes *et al*., 2005).

1.5.1. Life cycle, transmission routes and pathology

N. caninum has a heteroxenous life cycle comprising two modes of reproduction: asexual reproduction in intermediate hosts and sexual reproduction in definitive canid hosts including dogs, coyotes, gray wolves and dingoes (Gondim *et al*., 2004; King *et al*., 2011; McAllister *et al*., 1998) (Figure 7). The precise details on *N. caninum* merogony and gamogony *in vivo* are still unknown. The only known endogenous sexual stage of *N. caninum* is the unsporulated diploid oocyst, which is shed into the environment. Unsporulated oocysts undergo sporogony in the environment to form eight haploid sporozoites being contained in two sporocysts (Dubey *et al*., 2007). Of note, oocysts stages proved resistant to freezing and drying. After ingestion of sporulated oocysts by intermediate hosts, sporozoites are released, infect host cells and convert into rapidly proliferating stages, known as tachyzoites, which disseminate in the body host. Tachyzoites invade nucleated host cells, form a parasitophorous vacuole (PV) and replicate asexually by repeated endodyogenies. After several rounds of endodyogeny, parasites egress to re-infect new host cells (Ojo *et al*., 2014), subsequently fostering innate and adaptative immune responses. Most likely due to host immune reactions and other environmental factors, tachyzoites convert in the chronic phase into a slowly growing, semi-dormant stage known as bradyzoites, which are enclosed in tissue cysts. Tissue cysts can persist within host cells for a long time and bradyzoites can re-convert into tachyzoites to generate active infection, particularly in immunocompromised hosts (Dubey, 1999; McAllister et al., 1998).

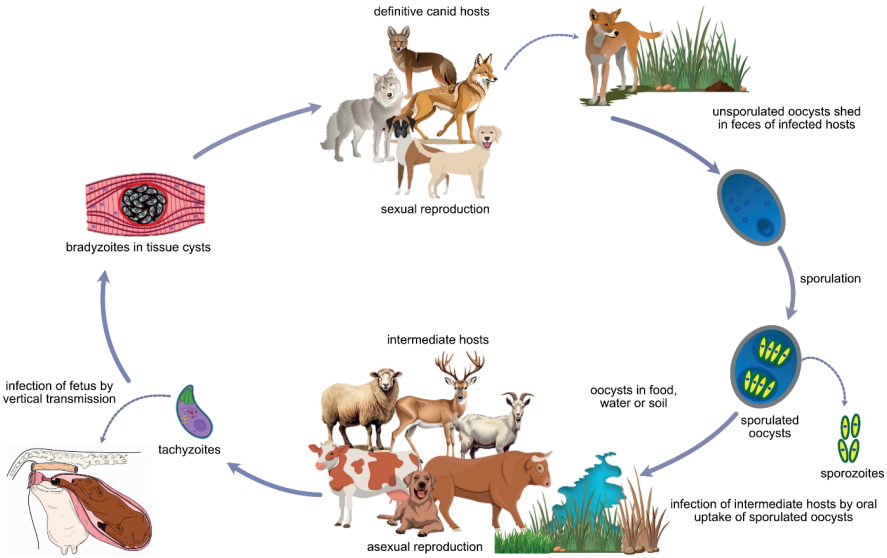


Figure 7. The heteroxenous life cycle of *N. caninum*. The sexual reproduction exclusively occurs in definitive canid hosts, including dingoes, coyotes, grey wolves, domestic and wild dogs. In the gut, unsporulated oocysts are formed, which are shed via the feces into the environment. Unsporulated oocysts undergo sporogony, thereby two sporocysts, each containing four sporozoites, are formed. After ingestion of sporulated oocysts by intermediate hosts, sporozoites are released from oocysts, invade host cells and convert into tachyzoites, which replicate asexually by endodyogenies, and egress from host cells to infect new cells. In chronic infections, tachyzoites convert into bradyzoites within tissue cysts, persisting for a long period and generating latent infections, particularly in immunocompromised hosts. Cattle can become infected either horizontally via oocyst ingestion or vertically by tachyzoite transfer from the cow to the fetus, with the latter infection route by far representing the predominant one.

Despite having a rather wide host range and a heteroxenous life cycle, *N. caninum* mainly infects cattle and dogs as its primary intermediate and definitive hosts, respectively (Dubey, 1999). Cattle can become infected either horizontally by ingestion of sporulated oocysts, or vertically, through transplacental transmission of tachyzoite stages (Figure 7). Vertical transmission is considered as the predominant and most efficient route of *Neospora* transmission in cattle herds and it can either result from primary infection of a pregnant heifer, or from latent infections reactivated during pregnancy (Bergeron *et al*., 2000; Pereyra *et al*., 2019). Fetuses infected with *Neospora* can be aborted at any gestational age, be resorbed, mummified, autolyzed, or born with persistent infection. In contrast to *T. gondii*, *N. caninum* infection can be vertically transmitted in several consecutive pregnancies, thereby leading to a high prevalence in cattle herds being associated with a significantly increased risk of abortion. Transplacental transmission has also been documented in other intermediate

hosts, including sheep, goats, deer, and dogs; moreover, venereal transmission by semen and transmission by milk was reported (Donahoe *et al*., 2015; Ortega-Mora *et al*., 2003; van Velsen, 2021; Villa *et al*., 2024). Dogs are generally infected orally via ingestion of tissue cysts. Given the close association of dogs and cattle in herds (Dubey *et al*., 2007; McAllister *et al*., 1998) and the likelihood of vertical transmission through generations of cattle (Barber and Trees, 1998; Thurmond and Hietala, 1996), the probability of transmission is relatively high, since *N. caninum* can be maintained and propagated between definitive and intermediate hosts for a long time within the same herd. This special transmission cycle between cattle and dogs through asexual and sexual reproduction is suggested to shape the population structure of *N. caninum* (Khan *et al*., 2020).

The pathogenesis of neosporosis involves complex interactions between the parasite and the host immune system. After ingestion of oocysts or cysts, *N. caninum* sporozoites or bradyzoites, respectively, infect intestinal epithelial cells, convert to tachyzoite stages and disseminate via bloodstream and lymphatic system. Tachyzoite stages invade different cell types like macrophages, endothelial cells and neurons during acute phase of infection. They rapidly multiply within host cells, causing cell lysis and tissue necrosis. This parasitic stage is highly immunogenic and elicits a strong inflammatory response (Buxton *et al*., 2002). Infected animals usually generate a strong T cell-mediated immune response with concomitant IFN-γ production, which activates macrophages to kill tachyzoite stages (Fereig and Nishikawa, 2020). In the chronic phase, tachyzoites transform into bradyzoites in cysts, being primarily formed in the central nervous system and muscles. Tissue cysts may persist for the lifetime of the host and are hardly recognized by the host immune response (Innes, 2007).

The pathology of *N. caninum* infections varies in relation to the stage of infection, the gestational stage and the host species. In pregnant cattle, infection can cause, fetal resorption, abortion or birth of weak calves due to placentitis and fetal infection (Silva and Machado, 2016). Young dogs may rarely develop a polyradiculoneuritis-myositis-syndrome, characterized by neurological symptoms, such as ataxia, paralysis and muscle atrophy, resulting from inflammation and tissue damage in the central nervous system (Knowler and Wheeler, 1995; Kul *et al*., 2015; Saey *et al*., 2010; Sánchez-Sánchez *et al*., 2018).

1.5.2. *N. caninum*-driven host cell modulation

The aim of this project was to compare mechanisms of host cell cycle modulation driven by *T. gondii* and *N. caninum* as closely related parasites. So far, data on *N. caninum*-driven host cell cycle perturbation is rather scarce. Undoubtedly, as a typical apicomplexan parasite, *N. caninum* modulates different host cellular functions to ensure its survival and proliferation. Thus, proteomic and transcriptomic analyses revealed a multitude of functional categories to be modulated by *N. caninum* infection in bovine trophoblasts including protein synthesis/turnover, metabolism, mitochondrial function, stress response and host cell cycle (Horcajo *et al*., 2016; Regidor-Cerrillo *et al*., 2020). In detail, *N. caninum* activates NF-kB, phosphoinositidae 3-kinase (PI3K)/Akt- and MAPK-related signaling pathways, thereby affecting gene expression and inducing the synthesis of chemokines and adhesion molecules being involved in immune responses and cell survival, e. g. by inhibiting apoptosis via upregulating anti-apoptotic proteins and downregulating pro-apoptotic factors in bovine endothelial cells (Taubert *et al*., 2006). Moreover, in infected bovine macrophages and trophoblasts, *N. caninum* infections trigger the upregulation of pro-inflammatory genes like TNF-α, IL-8, TLR-2, IFN-γ and downregulation of IL-6 and TGF-β (García-Sánchez *et al*., 2020; Jiménez-Pelayo *et al*., 2019). Interestingly, *N. caninum* invades the host cell avoiding the recognition by APC and PRRs for CD8 T cells and resulting in parasitophorous vacuole formation to reside inside the host cells (Jordan and Hunter, 2010). Once the parasitophorous vacuole is established, the production of host cellular IFNγ is blocked (Correia *et al*., 2015), thereby abrogating the predominant host protective mechanism conferred by CD8 T cells. Additionally, *N. caninum* infections induce an increased production of IL-10, a cytokine known for its anti-inflammatory properties and concomitant inhibition of pro-inflammatory cytokines (TNF). Thus, cellular and humoral immune responses support the maintenance of infection (Correia *et al*., 2015; Innes *et al*., 2002).

Concerning host metabolism, *N. caninum* scavenges cholesterol and sphingolipids from host cell organelles, specifically from Golgi vesicles, thereby downregulating fatty acid synthesis and tryptophan degradation (García-Sánchez *et al*., 2020). Moreover, *N. caninum* recruits host mitochondria to the PV and retains them close to the parasitophorous vacuole membrane for energy acquisition (Nolan *et al*., 2015).

Furthermore, to ensure its intracellular development, *N. caninum* represses lysosome-mediated degradation processes. In detail, when phagolysosome activity was studied, a downregulation of V-ATPase genes was observed, demonstrating a lysosome pathway inhibition. The expression of lysosomal-associated membrane proteins, transport proteins and the activation of lysosomal enzymes, together with peptides and enzymes with microbicidal properties was found overall dysregulated in *N. caninum*-infected host cells (Nalpas *et al*., 2015; Uribe-Querol and Rosales, 2017). As expected, MAPK signaling pathways controlling cell proliferation, differentiation and stress responses were also altered in infection models with concomitant activation of the PI3K/Akt pathway to generate an appropriate environment for replication (García-Sánchez *et al*., 2020; Remmerie and Scott, 2018).

In line to other apicomplexan parasites, *N. caninum* infection hampers host cell apoptosis by downregulating the release of cytochrome c from mitochondria (García-Sánchez *et al*., 2020), thereby avoiding the activation of caspases, which are essential for the apoptosis process. On molecular level, an increased expression of anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, impeding the stabilization of mitochondrial membranes and the release of apoptotic factors was detected (Nishikawa *et al*., 2001). These alterations interfere with death receptor pathways (especially Fas and TNF receptors) and their ligands (Green, 2022), finally leading to inhibition of caspase 8 activity, a key initiator caspase in the extrinsic pathway of apoptosis (Fritsch *et al*., 2019; Qian *et al*., 2022).

Importantly, besides few hints on *N. caninum*-driven cell cycle modulation deduced from proteomic analyses of infected host cells, some findings come from Elsheikha *et al*. (2020), showing that *N. caninum* infection induced a cell cycle arrest in S- and G2-phases in human brain microvascular endothelial cells, thereby blocking host cellular DNA replication and mitosis. Furthermore, in contrast to *T. gondii*, *N. caninum* infections failed to induce cyclin E upregulation in human cells (Panas *et al*., 2019). However, detailed analyses on effects and mechanisms of *N. caninum*-driven host cell dysregulation await further investigation.

2. ORIGINAL PUBLICATIONS

2.1. *TOXOPLASMA GONDII* MODULATES THE HOST CELL CYCLE, CHROMOSOME SEGREGATION, AND CYTOKINESIS IRRESPECTIVE OF CELL TYPE OR SPECIES ORIGIN

Rojas-Barón L., Senk K., Hermosilla C., Taubert A., Velásquez Z. (2024).

Parasit Vectors. 2024 Apr 5;17(1):180. doi: 10.1186/s13071-024-06244-2. PMID: 38581071; PMCID: PMC10996137.

Own part in the publication:

Project planning:	50%, together with co-authors and supervisors
Experimentation:	50%, together with co-authors
Data evaluation:	50%, together with co-authors
Manuscript writing:	60%, together with co-authors

Parasites & Vectors

RESEARCH

Open Access

Toxoplasma gondii modulates the host cell cycle, chromosome segregation, and cytokinesis irrespective of cell type or species origin

Lisbeth Rojas-Baron[1], Kira Senk[1], Carlos Hermosilla[1], Anja Taubert[1] and Zahady D. Velásquez[1*]

Abstract

Background *Toxoplasma gondii* is an apicomplexan intracellular obligate parasite and the etiological agent of toxoplasmosis in humans, domestic animals and wildlife, causing miscarriages and negatively impacting offspring. During its intracellular development, it relies on nutrients from the host cell, controlling several pathways and the cytoskeleton. *T. gondii* has been proven to control the host cell cycle, mitosis and cytokinesis, depending on the time of infection and the origin of the host cell. However, no data from parallel infection studies have been collected. Given that *T. gondii* can infect virtually any nucleated cell, including those of humans and animals, understanding the mechanism by which it infects or develops inside the host cell is essential for disease prevention. Therefore, we aimed here to reveal whether this modulation is dependent on a specific cell type or host cell species.

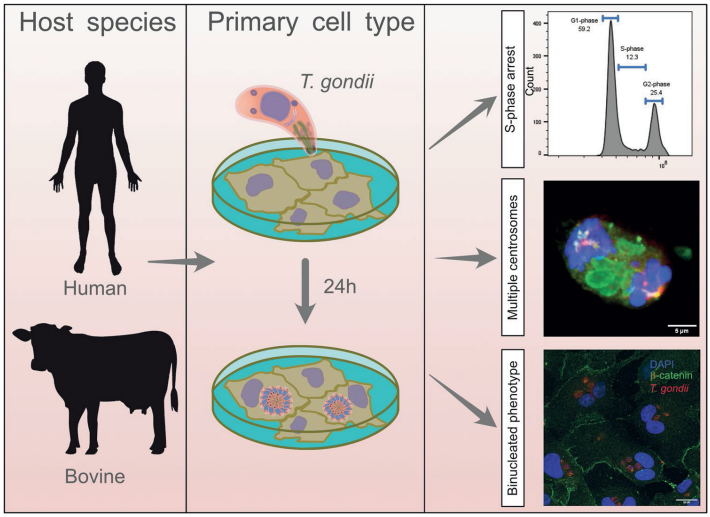
Methods We used only primary cells from humans and bovines at a maximum of four passages to ensure that all cells were counted with appropriate cell cycle checkpoint control. The cell cycle progression was analysed using fluorescence-activated cell sorting (FACS)-based DNA quantification, and its regulation was followed by the quantification of cyclin B1 (mitosis checkpoint protein). The results demonstrated that all studied host cells except bovine colonic epithelial cells (BCEC) were arrested in the S-phase, and none of them were affected in cyclin B1 expression. Additionally, we used an immunofluorescence assay to track mitosis and cytokinesis in uninfected and *T. gondii*-infected cells.

Results The results demonstrated that all studied host cell except bovine colonic epithelial cells (BCEC) were arrested in the S-phase, and none of them were affected in cyclin B1 expression. Our findings showed that the analysed cells developed chromosome segregation problems and failed to complete cytokinesis. Also, the number of centrosomes per mitotic pole was increased after infection in all cell types. Therefore, our data suggest that *T. gondii* modulates the host cell cycle, chromosome segregation and cytokinesis during infection or development regardless of the host cell origin or type.

Keywords Cell cycle, *Toxoplasma gondii*, Binucleated cells, Human and bovine cells

*Correspondence:
Zahady D. Velásquez
zahady.velasquez@vetmed.uni-giessen.de
Full list of author information is available at the end of the article

Graphical Abstract



Background

The obligate intracellular parasite *Toxoplasma gondii* is a globally spread zoonotic protozoan that causes severe health problems in both humans and animals. Prenatal infections can cause abortion or harm the progeny's welfare. Acute infections can be life-threatening in immunocompromised individuals, and several studies have postulated a correlation between latent *T. gondii* infections and neurological/psychiatric disorders in humans [1, 2].

Intracellular protozoa are well known for modulating their host cells to ensure efficient intracellular development and proliferation. As such, they have been shown to modulate a variety of host cellular functional categories, such as apoptosis, autophagy, cytoskeleton, metabolism, immunological responses and cell cycle [3–8]. Some parasite-triggered cell cycle disruption is both parasite species- and host cell type-specific. *Toxoplasma gondii*, *Leishmania* spp., *Trypanosoma cruzi* and *Encephalitozoon* spp. induce cell cycle arrest and dampen host cell proliferation, whilst *Theileria* stimulates host cell division and proliferation [9–14]. *Leishmania amazonensis* interferes early in the cell cycle with the G0/G1 phase, and *T. cruzi* triggers host cell progression to the S-phase, besides blocking host cell mitosis and impairing cytokinesis after nuclear replication [10, 12]. The host cell type seems to be important in *Plasmodium* species; for example, *P. falciparum* infections in HepG2 cells were found to affect mitosis and lead to binucleated phenotype formation without cell division [15]. However, cell cycle-dependent reactions played no role in *P. berghei*- or *P. yoelii*-infected mouse models either in vivo or in vitro. *Plasmodium falciparum*-infected primary hepatocytes provide evidence that primary cell types differ considerably from permanent cell lines in their reactions. To date, there is no information whether the control

Table 1 Primary cells used in the current study

Name of primary cell	Company	Cat. number	Lot number (donor)
HUVEC-p single donor	PromoCell	C-12250	449Z018.1
HUVEC-p single donor	PromoCell	C-12250	466Z026
HUVEC-p single donor	PromoCell	C-12250	467Z015
HUVEC-p single donor	PromoCell	C-12250	478Z023
HUVEC-p single donor	PromoCell	C-12250	486Z004
HUVEC-p single donor	PromoCell	C-12200	469Z003.1
HUVEC-p single donor	PromoCell	C-12200	473Z022.1
HFF-1	ATCC	SCRC-1041	
FHs74	ATCC	CCL-241	

that *T. gondii* exerts on the host cell cycle is restricted to specific cell types or to only some host cell species. However, it has been shown that *T. gondii* infections shift G0/G1 cells through the S-phase in human foreskin fibroblasts (HFF) [4], but arrest cells in the G2-phase in a human trophoblast cell line and human dermal fibroblasts [3], or even both in the L6 rat myoblast cell line [16]. The G2-phase arrest in human dermal fibroblasts or a human trophoblast cell line was linked to decreased cyclin B1 abundance without alterations in other G2/M checkpoint-related proteins [3]. *T. gondii* arrest of HFF during the S-phase or G2/M was accompanied by a delayed or absent increase in cyclin A and cyclin B1, thereby indicating a missing exit from the S-phase and failure to progress [4]. *T. gondii* infections increased the proportion of polyploid cells (8n) in the murine RAW264.7 cell line, most likely reflecting DNA replication without subsequent cytokinesis [17]. All of the above suggests that *T. gondii* altered the host cell cycle based on the origin of the host or the cell type infected. However, no experiment has been carried out in parallel using different cell types and species in order to establish comparative analyses. Therefore, the current study sought to determine whether *T. gondii* regulates the host cell cycle progression based on the host cell species or type. We used commercially available primary human cells and two primary bovine cell lines in-house-isolated, controlling the passage number to maintain the original phenotype. Our findings showed that cell cycle arrest occurred in almost all primary cells excluding bovine colonic epithelial cells (BCEC). Nonetheless, *T. gondii*-induced chromosome mis-segregation and cytokinesis failure were detected in all cell types studied, suggesting that *T. gondii* modulates these two mechanisms independently of host cell origin.

Methods

Primary human and bovine host cells and parasite maintenance

Primary human umbilical vein endothelial cells (HUVEC, six donors in total, PromoCell), HFF ($n=3$, PromoCell) and human small intestine epithelial cells (FHs74, American Type Culture Collection [ATCC]) were cultured at 37 °C and 5% CO_2 following the supplier's protocols [(media: HUVEC: Endothelial Cell Growth Medium, PromoCell; HFF: Dulbecco's modified Eagle medium [DMEM]-GlutaMAX, Gibco; FHs74: HybriCare, ATCC; BCEC: RPMI1640, Sigma; bovine small intestinal epithelial cells [BSIEC]: DMSM/F12, Gibco); see primary cell information in Table 1]. Each experiment was performed at a maximum of four passages after isolation to enable the best comparison. All cell lines were seeded at the same time and infected with the same batch of tachyzoites. *T. gondii* RH tachyzoites were maintained by serial passages in primary HFF cells (maximum passage: 10). Therefore, free-released *T. gondii* tachyzoites were harvested from HFF supernatants, pelleted (400×*g*, 12 min), counted and suspended in the corresponding medium for each host cell type. Infections were performed in subconfluent cell layers. All experiments were performed at a multiplicity of infection (MOI) of 1:1 (cells: parasites).

Flow cytometry-based analysis of cell cycle phases

Cellular DNA content was measured using FxCycle PI (propidium iodide)/RNAse staining solution (Invitrogen, F10797) according to the manufacturer's instructions. Cyclin B1 quantification was performed in cells fixed with BD fixation/permeabilization solution (BD, 554714, Becton, Dickinson and Company, Heidelberg, Germany) and stained with cyclin B1-AF647 (Cell Signaling Technology, 4118). The samples were analysed using a BD Accuri C6 Plus flow cytometer (Becton–Dickinson)

Table 2 Primary and secondary antibodies used in the western blot (WB) and immunofluorescence assays

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies immunofluorescence assay				
B-catenin	Abcam	Ab32572	Rabbit	1:200
Toxoplasma gondii	Thermo Fisher	PA1-7256	Goat	1:100
Primary antibodies FACS				
Cyclin B1-Alexa Fluor 647	Cell Signaling Technology	ab32053	Rabbit	1:50
Secondary antibodies				
Antigen/conjugate	Company	Cat. number	Host/target	Dilution
Goat anti-mouse IgG peroxidase conjugated				
Alexa Fluor 488	Thermo Fisher	A11001	Goat/mouse	1:500
Alexa Fluor 594	Thermo Fisher	A21468	Chicken/rabbit	1:500
Alexa Fluor 647	Thermo Fisher	A21235	Goat/mouse	1:500

applying 535/5 nm excitation and emission collected in a 617/20 band-pass. Cells were gated according to their size and granularity; only morphologically intact host cells were included in the analysis. All analyses were performed in FlowJo v.10 software.

Immunofluorescence assays

The method was performed as described by Velásquez et al. [18]. Briefly, uninfected and infected cells were fixed with paraformaldehyde (PFA, 4%, 15 min, room temperature [RT]), washed with phosphate-buffered saline (PBS) and incubated in blocking/permeabilization solution (PBS with 3% bovine serum albumin [BSA], 0.1% Triton X-100; 1 h, RT). Thereafter, samples were incubated in primary antibodies (Table 2) and diluted in blocking/permeabilization solution (overnight at 4 °C in a humidified chamber). After three washes in PBS, the samples were incubated in secondary antibody solutions (Table 2; 30 min at RT and complete darkness). Cell nuclei were labelled using a DAPI-supplemented mounting medium (Fluoromount-G, Thermo Fisher).

Image acquisition and reconstruction

Image acquisition and reconstruction were carried out according to the description published by Velásquez et al. [18]. A ReScan confocal microscope (RCM 1.1 Visible, Confocal.nl) equipped with a fixed 50 µm pinhole size and combined with a Nikon Ti2-A inverted microscope was used to acquire fluorescence and confocal images. The RCM unit was connected to a Toptica CLE laser with the following excitations: 405/488/561/640 nm. Images were acquired using a scientific CMOS [complementary metal–oxide–semiconductor] (sCMOS) camera (pco. edge, PCO) with a CFI Plan Apochromat 60× lambda-immersion oil objective (NA 1.4/0.13; Nikon). The system was operated using NIS-Elements software (version 5.11). Images were acquired via z-stack optical series with a step size of 0.1 micron depth to cover all structures of interest within the analysed host cells. Z-series were displayed as maximum z-projections. Identical brightness and contrast conditions were applied for each data set within one experiment using Fiji software [19].

Statistical analysis

All data are expressed as mean ± standard deviation (SD) from three independent experiments. When two groups were compared, a Mann–Whitney test was performed. When three or more experimental groups were compared, a Kruskal–Wallis one-way analysis of variance was applied. Significance was defined as $P \leq 0.05$. All graphs and statistical analyses were performed using GraphPad Prism 9 software.

Results

Toxoplasma gondii has been described to modulate the host cell cycle between the G1 and G2/M phase depending on the experimental model used [3, 4, 14, 20]. Given that these experiments were performed under different experimental conditions and cell types, we wanted to know whether *T. gondii* uses this mechanism as an infection strategy or whether it is dependent on the host type or species. As host cells, we tested three human primary cells, HUVEC, FHs74 and HFF, and two primary bovine cells, BSIEC and BCEC. To avoid artefacts due to the immortalization of the cell or tumour phenotype, we worked only with primary cells, at a limit of four passages after isolation. The host cells were infected at sub-confluence, and at 24 h post-infection (p.i.) they were analysed for DNA content using fluorescence-activated cell sorting (FACS) flow cytometry. The gating process started by selecting the cell population according to their shape and granularity (Fig. 1). Cells were then plotted as the number of cells versus PI signal. The first peak represents the cells in the G1-phase, the second peak represents those cells in the G2/M-phase, and the S-phase corresponds to the cells located between the two peaks. Cells in each cell cycle phase are shown as a proportion of the total number of cells counted. DNA quantification results showed that HUVEC, HFF and FHs74 from humans and BSIEC were arrested in the S-phase after infection (Fig. 1), whereas *T. gondii* infection did not affect cell cycle progression in BCEC (Fig. 1). It should be noted that HUVEC and HFF cells showed a reduction in the number of cells in the G1-phase, and HFF also showed a reduced percentage of cells in the G2-phase (Fig. 1).

Cell cycle arrest in the S-phase could be explained by a blockage entry into the G2/M-phase. Therefore, we analysed the expression of one of the main regulators of the G2-to-M-phase transition, cyclin B1. Cyclin B1 accumulates throughout the S-phase, with a peak at the onset of the M-phase [21]. After that, cyclin B1 needs to be degraded to allow cells to enter mitosis. Thus, we quantified the cyclin B1 protein 24 h p.i., using FACS-based quantification. The cells were gated according to their shape and granularity and then plotted as the total number of counted cells and the cyclin B1 intensity (Fig. 2). The results showed that none of the cells modulated cyclin B1 expression after *T. gondii* infection (Fig. 2), suggesting that *T. gondii* does not arrest the host cell cycle in the S-phase due to modulation of the mitosis checkpoint protein cyclin B1.

It has been previously demonstrated that *T. gondii* infection affects mitosis progression itself, and thus we performed an immunofluorescence assay in cells infected for 24 h with *T. gondii* tachyzoites. Firstly, we quantified the percentages of cells in mitosis in the non-infected and

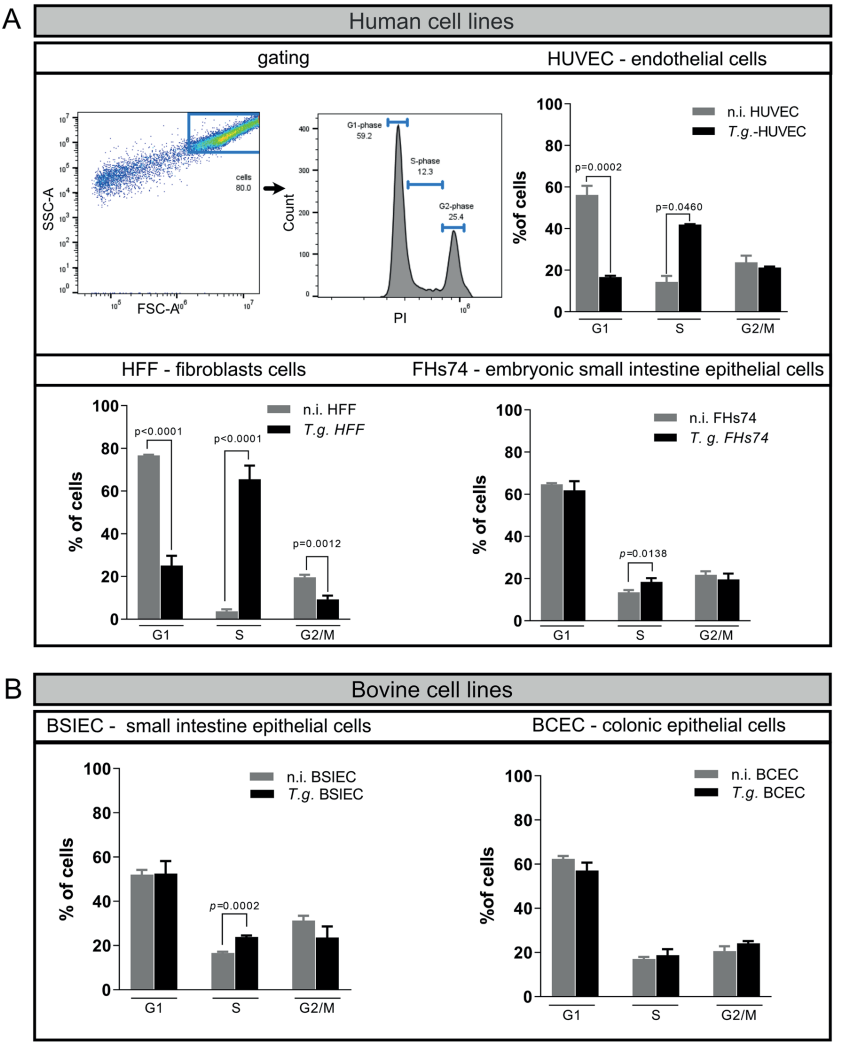


Fig. 1 *Toxoplasma gondii* arrests primary human and bovine cells in the S-phase 24 h p.i. Three isolates of HUVEC, HFF, FHs74, BSIEC and BCEC were infected with *T. gondii* tachyzoites, and the samples were collected 24 h p.i. Fixed samples were stained with propidium iodide (PI) and analysed using FACS. Gating was performed by first selecting the cell population according to the shape and granularity. Then, the cell cycle phases were analysed using a histogram of the number of cells versus the PI signal. Cells in the first peak correspond to those in the G1-phase, the second peak to cells in the G2/M-phase, and cells in between the two peaks were cells in the S-phase. The results showed that all human cells were arrested in the S-phase (**A**), whilst only one bovine cell showed no effect on cell cycle progression before *T. gondii* infection (**B**). Graph bars represent the median ± SD of three biological replicates

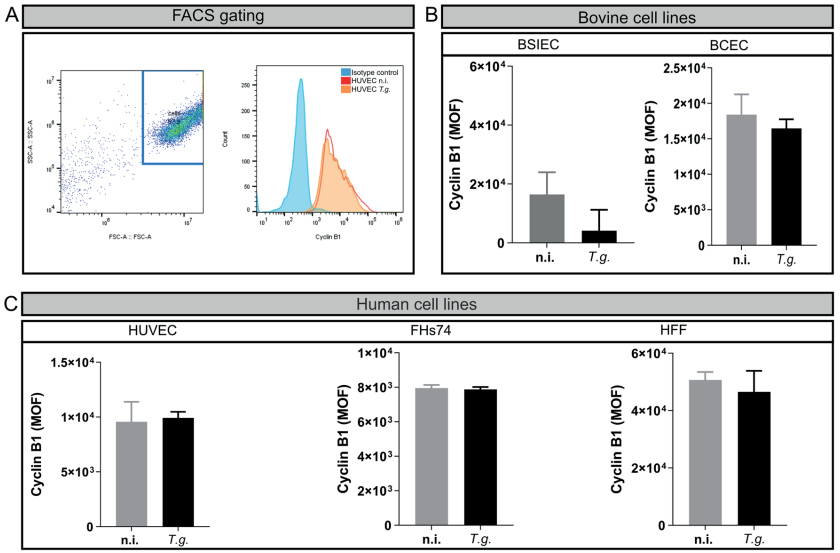


Fig. 2 Cell cycle arrest in *T. gondii*-infected cells is independent of the mitosis checkpoint protein control cyclin B1. Three donors of HUVEC, HFF, FHs74, BSIEC and BCEC were infected with *T. gondii* tachyzoites and fixed 24 h p.i. Cells were stained against cyclin B1 and an isotype control and analysed using FACS. Gating was done first by choosing the cell population and then selecting the same number of cells in each sample (**A**). The cyclin B1 signal was analysed as the mean of fluorescence (MOF) and plotted for non-infected and *T. gondii*-infected cells. The results showed that cyclin B1 was not affected after infection in any of the cell types studied (**B**, **C**). Graph bars represent the median ± SD of three biological replicates

infected monolayers (total number of cells: FHs74:1169; BSIEC:1248; BCEC:1531; HFF:6280; HUVEC:4287), with no significant differences in the mitotic index between the bovine cell lines BCEC and BSIEC and the human cell line HUVEC (Fig. 3A, B). Mitosis was evaluated from prophase (chromosome condensation) to telophase (chromosomes sets were pulled to opposite poles of the cell). On the contrary, human cell lines FHs74 and HFF increased the percentage of mitotic cells in the *T. gondii*-infected monolayer (Fig. 3B). Secondly, we quantified the aberrant mitotic cells in all human and bovine cell lines. Fixed cells were stained for chromosomes (DAPI, blue), centrosome (γ-tubulin, red) and *T. gondii* tachyzoites (green; Fig. 3C). Aberrant mitoses were detected in all phases of mitosis, as shown in Fig. 3C. We observed prometaphases or metaphases with more than two centrosome poles (Fig. 3C**,** white arrows). Similar results were observed in telophase, suggesting that *T. gondii* affects the centrosome number and therefore the chromosome segregation throughout mitosis, independently of the cell origin or type. In order to determine whether this effect was significant after infection, we quantified the percentage of aberrant mitosis in non-infected and infected monolayers. We defined aberrant mitosis as those mitotic spindle problems in chromosome segregation, mislocated centrioles or an increased number of centrosomes. The results showed that only the bovine cell line BCEC exhibited a significant increase in the number of aberrant mitoses after infection (Fig. 3C). Human endothelial cells and fibroblasts displayed an increased percentage of aberrant mitosis, while epithelial cell mitosis was not affected by infection (Fig. 3C).

Finally, we wanted to know whether the cytokinesis failure observed after *T. gondii* infection was cell-type or cell-origin-specific. Cytokinesis failure means a failure in cytosol division at the end of mitosis; therefore, we quantified the total number of cells displaying more than one nucleus per cell (binucleated cells; Fig. 4). Human and bovine cells were fixed 24 h p.i. and stained against *T. gondii* and DAPI, and the total number of binucleated cells was counted (Fig. 4). The results showed that all cells tested had increased percentages of binucleated cells, independently of the origin or cell type (Fig. 4). We consistently observed parasite-driven

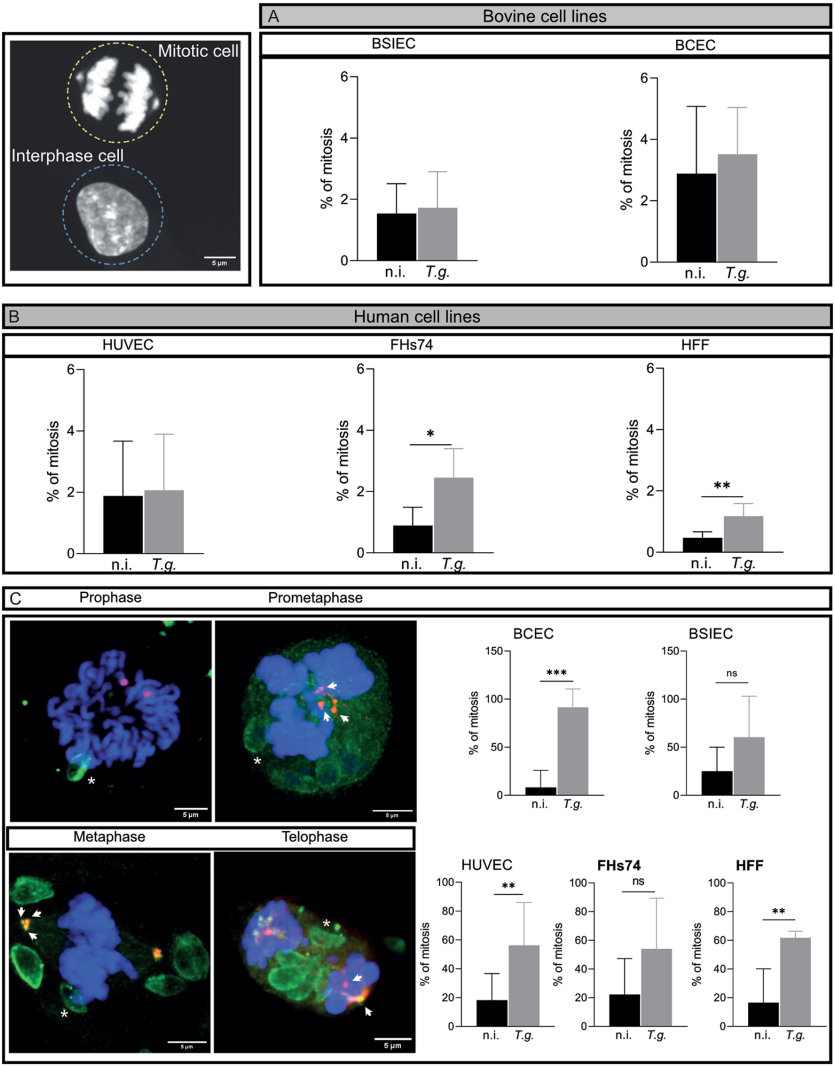


Fig. 3 *Toxoplasma gondii* infection affects the mitosis rate and the chromosome segregation at the mitosis phase. HUVEC, HFF, FHs74, BSIEC and BCEC were infected with *T. gondii* tachyzoites, and were fixed 24 h p.i. with PFA and stained against DAPI (chromosome marker, blue), γ-tubulin (centrosome marker, magenta) and *T. gondii* (green, asterisks). The mitosis index was counted as the total number of cells facing mitosis (see scheme) related to the total number of cells in the field of view. This percentage was calculated for the bovine (**A**) and human cell lines (**B**). The results showed that no bovine cell line studied here modified its mitosis rate after infection with *T. gondii*. However, FHs74 and HFF showed an increased proportion of mitotic cells. **C** The mitosis phases were followed by chromosome segregation, and the results showed that all cell lines developed chromosome segregation errors mainly due to an abnormal number of centrosomes (white arrows). Thereafter, the proportion of aberrant mitosis was determined in both bovine and human cells by counting the total number of aberrant mitoses relative to the total mitotic cells (normal and aberrant). The scale bar represents 5 µm

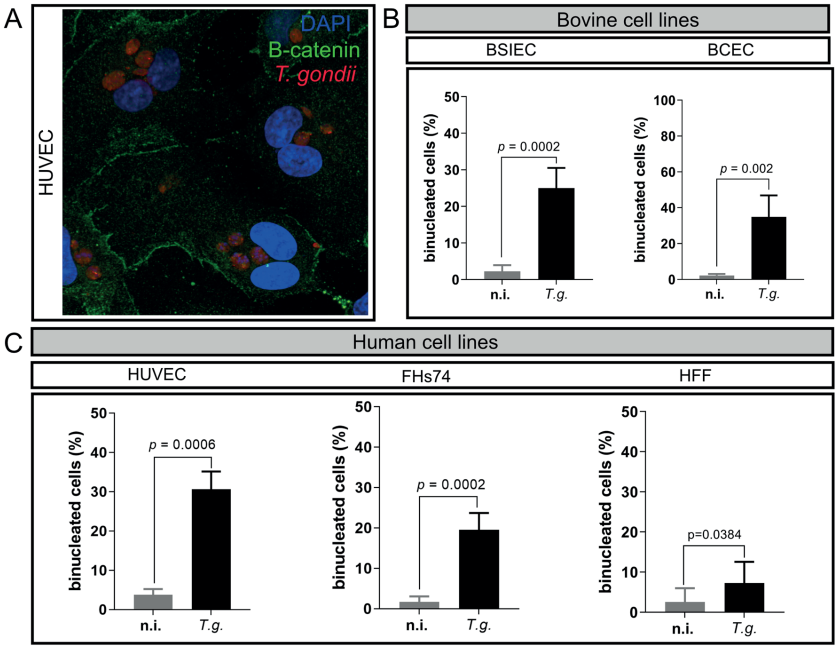


Fig. 4 *Toxoplasma gondii* induces cytokinesis failure in human and bovine host cells. HUVEC, HFF, FHs74, BSIEC and BCEC were infected with *T. gondii* tachyzoites, and fixed 24 h p.i. with PFA and stained against DAPI (nuclear marker, blue), β-catenin (membrane marker, green) and *T. gondii* (red). As in the scheme, binucleated cells were those cells with more than one nucleus per cell (**A**). The total number of binucleated cells was normalized to the total number of cells counted and presented as a percentage in the graphs. The results showed that all tested cell lines increased the percentage of binucleated cells after infection with *T. gondii* (**B**, **C**). Graph bars represent the median ± SD of three biological replicates

cytokinesis inhibition resulting in 18–34% of the host cells with more than two nuclei in all cell types (Fig. 4). Taken together, the current data suggest that *T. gondii*-induced chromosome segregation errors and cytokinesis failure are neither host species- nor host cell type-dependent. However, host cell cycle arrest was only observed in four of the five cell types tested in the current study, suggesting a parasite mechanism that might relate to the cell type.

Conclusions

Toxoplasma gondii is an obligate intracellular parasite that is globally spread and causes severe health problems in humans and animals, such as abortion, severely affecting the progeny welfare [2]. Given that this parasite can infect almost any warm-blooded animal, the mechanism that *T. gondii* uses to develop inside the host cell is of interest to scientists worldwide. In the last year, increasing evidence has shown that *T. gondii* modulates the host cell cycle, but all studies involved only one or two types of cells, using different experimental approaches, MOIs or infection time. Also, some studies worked with immortalized or tumour cells, which are well known to lose the cell cycle checkpoint control that a primary cell has. Therefore, we wanted to determine whether *T. gondii* uses the control of the host cell cycle as a mechanism to ensure its intracellular development, independently of the species or cell type used as a host. In order to study the responses closest to a real infection scenario, we worked only with primary cells at a maximum of four passages after isolation. The results showed that the cell cycle of only one cell type (BCEC) was not influenced after infection. However, it is important to highlight that this cell line was isolated and monitored in our laboratory, with normal growth like the others, and normal intracellular development of *T. gondii* tachyzoites during infection as well. Therefore, the results might be analysed according to specific proteins that the parasite modulates

in order to arrest the host cell cycle but that are not expressed in this cell type. Nevertheless, further experiments are needed to identify why *T. gondii* infection cannot control cell cycle progression in BCEC cells, but this is beyond the scope of the current work.

Regarding the mitosis and binucleated cell percentages, the results showed no differences between human and bovine cell lines. Therefore, we consider that both pathways controlled by *T. gondii* infection probably involved molecules or proteins that are not specific to a cell type or a determinate species. Similarly, it occurred with chromosome segregation errors, which were observed equally in all studied cell lines and species tested in the current study. Mitosis and cytokinesis need the cytoskeleton to function, specifically from the tubulin fibres which form the mitotic spindle or the midbody formation, respectively [22, 23]. Interestingly, *T. gondii* is well known to modulate the host cell tubulin cytoskeleton by relocating it around the parasitophorous vacuole (PV) [5]. After 1 h, infected cells showed a relocation of aster microtubules around the PV, and longer times of infection showed cells with multiple γ-tubulin foci suggesting critical microtubule dynamics in infected cells [5]. Therefore, our results can be explained by the control that *T. gondii* infection does on the host cell cytoskeleton and highlight that the failure cytokinesis process and the problems in the chromosome segregation are mechanisms that *T. gondii* uses in all infections, not depending on the host cell origin or cell type.

Our research indicates that *T. gondii* modulates host cell cycle progression, chromosome segregation and cytokinesis in primary cells independently of the host and cell type. Therefore, we suggest that these mechanisms represent a basal control of the parasite over the host cell. For future studies, it would be interesting to identify whether these *T. gondii*-induced phenotypes are all interconnected or if they represent a specific control that *T. gondii* separately exerts in pathways.

Acknowledgements
We would like to thank all members of Anja Taubert's lab for the helpful discussion of the current work as well as all the technical staff of the Institute of Parasitology. The authors thank A. Wehrend (Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus Liebig University, Giessen, Germany) for the continuous supply of bovine umbilical cords.

Author contributions
Conceptualization: ZDV. Main experimentation: LRB and ZDV. Formal analysis: LRB and ZDV. Statistical analyses: LRB and ZDV. Resources and funding: AT and CH. Data curation: ZDV. Writing—original draft: ZDV. Writing—review and editing: LRB, CH, AT and ZDV.

Funding
Open Access funding enabled and organized by Projekt DEAL. The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

Availability of data and materials
The original contributions presented in the study are included in the article/Supplementary material.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors have no competing interests to declare.

Author details
[1]Institute of Parasitology, Biomedical Research Center Seltersberg, Justus Liebig University of Giessen, Giessen, Germany.

Received: 2 January 2024 Accepted: 11 March 2024
Published online: 05 April 2024

References

1. Nayeri T, Sarvi S, Moosazadeh M, Amouei A, Hosseininejad Z, Daryani A. The global seroprevalence of anti-*Toxoplasma gondii* antibodies in women who had spontaneous abortion: a systematic review and meta-analysis. PLoS Negl Trop Dis. 2020;14:e0008103.
2. Dubey JP. Toxoplasmosis of animals and humans. Boca Raton: CRC Press; 2021.
3. Brunet J, Pfaff AW, Abidi A, Unoki M, Nakamura Y, Guinard M, et al. *Toxoplasma gondii* exploits UHRF1 and induces host cell cycle arrest at G2 to enable its proliferation. Cell Microbiol. 2008;10:908–20.
4. Molestina RE, El-Guendy N, Sinai AP. Infection with *Toxoplasma gondii* results in dysregulation of the host cell cycle. Cell Microbiol. 2008;10:1153–65.
5. Walker ME, Hjort EE, Smith SS, Tripathi A, Hornick JE, Hinchcliffe EH, et al. *Toxoplasma gondii* actively remodels the microtubule network in host cells. Microb Infect Institut Pasteur. 2008;10:1440.
6. Cai Y, Chen H, Mo X, Tang Y, Xu X, Zhang A, et al. *Toxoplasma gondii* inhibits apoptosis via a novel STAT3-miR-17-92-Bim pathway in macrophages. Cell Signal. 2014;26:1204–12.
7. Coppens I. Exploitation of auxotrophies and metabolic defects in *Toxoplasma* as therapeutic approaches. Int J Parasitol. 2014;44:109–20.
8. Saeij JP, Frickel E-M. Exposing *Toxoplasma gondii* hiding inside the vacuole: a role for GBPs, autophagy and host cell death. Curr Opin Microbiol. 2017;40:72–80.
9. Mosca W, Briceño L, Hernández MI. Cell mediated immunity in Chagas' disease: *Trypanosoma cruzi* antigens induce suppression of the in vitro proliferative response of mononuclear cells. Mem Inst Oswaldo Cruz. 1991;86:147–52.
10 Scanlon M, Shaw AP, Zhou CJ, Visvesvara GS, Leitch GJ. Infection by microsporidia disrupts the host cell cycle. J Eukaryot Microbiol. 2000;47:525–31.
11. Kuzmenok OI, Chiang S-C, Lin Y-C, Lee ST. Retardation of cell cycle progression of macrophages from G1 to S phase by ICAM-L from *Leishmania*. Int J Parasitol. 2005;35:1547–55.
12. Costales JA, Daily JP, Burleigh BA. Cytokine-dependent and–independent gene expression changes and cell cycle block revealed in *Trypanosoma cruzi*-infected host cells by comparative mRNA profiling. BMC Genomics. 2009;10:252.
13. von Schubert C, Xue G, Schmuckli-Maurer J, Woods KL, Nigg EA, Dobbelaere DAE. The transforming parasite *Theileria* co-opts host cell mitotic and central spindles to persist in continuously dividing cells. PLoS Biol. 2010;8:e1000499.
14. Velásquez ZD, Conejeros I, Larrazabal C, Kerner K, Hermosilla C, Taubert A. *Toxoplasma gondii*-induced host cellular cell cycle dysregulation is linked to chromosome missegregation and cytokinesis failure in primary endothelial host cells. Sci Rep. 2019;9:12496.

15. Hanson KK, March S, Ng S, Bhatia SN, Mota MM. In vitro alterations do not reflect a requirement for host cell cycle progression during *Plasmodium* liver stage infection. Eukaryot Cell. 2015;14:96–103.
16. Kim MJ, Jung B-K, Cho J, Song H, Pyo K-H, Lee JM, et al. Exosomes secreted by *Toxoplasma gondii*-infected L6 cells: their effects on host cell proliferation and cell cycle changes. Korean J Parasitol. 2016;54:147–54.
17. Franco M, Panas MW, Marino ND, Lee M-CW, Buchholz KR, Kelly FD, et al. A novel secreted protein, MYR1, is central to *Toxoplasma*'s manipulation of host cells. mBio. 2016;7:e02231-15.
18. Velásquez ZD, Rojas-Barón L, Larrazabal C, Saliermo M, Gärtner U, Pervizaj-Oruqaj L, et al. *Neospora caninum* infection triggers S-phase arrest and alters nuclear characteristics in primary bovine endothelial host cells. Front Cell Dev Biol. 2022;10:946335.
19. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9:676–82.
20 Pierre-Louis E, Etheridge MG, de Paula Baptista R, Khan A, Chasen NM, Etheridge RD. Disruption of *Toxoplasma gondii*-induced host cell DNA replication is dependent on contact inhibition and host cell type. mSphere. 2022;7:e0016022.
21. Hwang A, Maity A, McKenna WG, Muschel RJ. Cell cycle-dependent regulation of the cyclin b1 promoter. J Biol Chem. 1995;270:28419–24.
22. Kline-Smith SL, Walczak CE. Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. Mol Cell. 2004;15:317–27.
23. Normand G, King RW. Understanding cytokinesis failure. Adv Exp Med Biol. 2010;676:27–55.

Publisher's Note

2.2. *TOXOPLASMA GONDII* INFECTION-INDUCED HOST CELLULAR DNA DAMAGE IS STRAIN-DEPENDENT AND LEADS TO THE ACTIVATION OF THE ATM-DEPENDENT HOMOLOGOUS RECOMBINATION PATHWAY

Rojas-Barón L., Hermosilla C., Taubert A., Velásquez Z. (2024).

Front. Cell. Infect. Microbiol. 2024 Mar 8;14:1374659. doi: 10.3389/fcimb.2024.1374659. PMID: 38524184; PMCID: PMC10957594.

Own part in the publication:

Project planning:	60%, together with co-authors and supervisors
Experimentation:	80%, mainly independent
Data evaluation:	80%, mainly independent
Manuscript writing:	80%, mainly independent

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 08 March 2024
DOI 10.3389/fcimb.2024.1374659

OPEN ACCESS

EDITED BY
Martin Craig Taylor,
University of London, United Kingdom

REVIEWED BY
Richard McCulloch,
University of Glasgow, United Kingdom
Julia Romano,
Johns Hopkins University, United States

*CORRESPONDENCE
Zahady D. Velásquez
zahady.velasquez@vetmed.uni-giessen.de

RECEIVED 22 January 2024
ACCEPTED 26 February 2024
PUBLISHED 08 March 2024

CITATION
Rojas-Barón L, Hermosilla C, Taubert A and Velásquez ZD (2024) *Toxoplasma gondii* infection-induced host cellular DNA damage is strain-dependent and leads to the activation of the ATM-dependent homologous recombination pathway. *Front. Cell. Infect. Microbiol.* 14:1374659. doi: 10.3389/fcimb.2024.1374659

Toxoplasma gondii infection-induced host cellular DNA damage is strain-dependent and leads to the activation of the ATM-dependent homologous recombination pathway

Lisbeth Rojas-Barón, Carlos Hermosilla, Anja Taubert and Zahady D. Velásquez*

Institute of Parasitology, Biomedical Research Center Seltersberg, Justus Liebig University Giessen, Giessen, Germany

Toxoplasma gondii is a globally occurring apicomplexan parasite that infects humans and animals. Globally, different typical and atypical haplotypes of *T. gondii* induce varying pathologies in hosts. As an obligate intracellular protozoon, *T. gondii* was shown to interfere with host cell cycle progression, leading to mitotic spindle alteration, chromosome segregation errors and cytokinesis failure which all may reflect chromosomal instability. Referring to strain-dependent virulence, we here studied the potential of different *T. gondii* strains (RH, Me49 and NED) to drive DNA damage in primary endothelial host cells. Utilizing microscopic analyses, comet assays and γ-H2AX quantification, we demonstrated a strain-dependent induction of binucleated host cells, DNA damage and DNA double strand breaks, respectively, in *T. gondii*-infected cells with the RH strain driving the most prominent effects. Interestingly, only the NED strain significantly triggered micronuclei formation in *T. gondii*-infected cells. Focusing on the RH strain, we furthermore demonstrated that *T. gondii*-infected primary host cells showed a DNA damage response by activating the ATM-dependent homologous recombination (HR) pathway. In contrast, key molecules of the nonhomologous DNA end joining (NHEJ) pathway were either not affected or downregulated in RH-infected host cells, suggesting that this pathway is not activated by infection. In conclusion, current finding suggests that *T. gondii* infection affects the host cell genome integrity in a strain-dependent manner by causing DNA damage and chromosomal instability.

KEYWORDS

***Toxoplasma gondii*, DNA damage, chromosome instability, micronuclei, double-stranded DNA breaks, DNA repair pathways**

Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate apicomplexan parasite capable of infecting all nucleated cells of all warm-blooded animals (Tenter et al., 2000; Brunet et al., 2008; Dubey, 2009). In humans, this parasite infects up to a third of the global population, but infection in most cases remains asymptomatic resulting in chronicity (Bigna et al., 2020; Fernández-Escobar et al., 2022). However, pregnant and immunocompromised individuals represent risk groups and may develop abortion and severe neurological/ocular disorders, respectively (Jones et al., 2003; Shobab et al., 2013; Robert-Gangneux et al., 2018; Rostami et al., 2020).

As an obligate intracellular parasite, *T. gondii* has developed multiple mechanisms to manipulate a broad range of biological processes in its host cell, such as metabolism, autophagy or apoptosis, to guarantee its intracellular replication and survival (Brunet et al., 2008; Zhuang et al., 2020). Also, it has been described that *T. gondii*-driven cell cycle perturbation thereby mainly documenting an arrest in early cell cycle phases, i. e. before progression to mitosis. Human foreskin fibroblasts infected with *T. gondii* tachyzoites accumulate at the G2/M boundary (Molestina et al., 2008) while human trophoblast cell line and in human dermal fibroblasts (Brunet et al., 2008) or even both (L6 rat myoblast cell line (Kim et al., 2016), were reported to be arrested in the G2-phase. Immortalized human fibroblasts, HFF, were arrested in the S-phase after 6 h p.i. with *T. gondii* tachyzoites (Pierre-Louis et al., 2022). The G2 phase arrest in human dermal fibroblasts or a human trophoblast cell line was linked to the downregulation of cyclin B1 (Brunet et al., 2008). Interestingly, cyclin B1 gene promotor silencing was shown to be mediated by *T. gondii*-secreted ROP16 targeting host cellular transcription factor UHRF1 and triggering host cell cycle arrest by inducing a series of epigenetic reactions (deacetylation, methylation of histone H3 around the cyclin B1 promotor) (Sabou et al., 2019). Cell cycle arrest during S phase at G2/M boundary in *T. gondii*-infected human foreskin fibroblasts was accompanied by a delayed or lacking increase of cyclin A and cyclin B1 in combination with an early elevation of cyclin E1 when compared to EGF stimulation, thereby indicating a missing exit from S phase and failure to progress toward mitosis in *T. gondii* infection (Molestina et al., 2008). This kind of cell cycle perturbation depended on parasite viability and proliferation since replication-defective tachyzoites hardly influenced the host cell cycle (Molestina et al., 2008). In the murine RAW264.7 cell line, *T. gondii* infections caused an enhanced proportion of polyploid cells (8n), most probably reflecting DNA replication without subsequent cytokinesis (Franco et al., 2016). Nonetheless *T. gondii* not only modulates the host cell cycle but also host cell chromosome segregation and cytokinesis in infected primary endothelial cells (Velásquez et al., 2019). In general, chromosome segregation and cytokinesis impairment are reported to be related to chromosomal instability (Holland and Cleveland, 2009), a phenomenon which is linked to a variety of chromosomal aberrations, such as loss, gain or disarrangement of chromosomes (Bakhoum and Cantley, 2018). Likewise, chromosomal instability is known to induce DNA damage, resulting in DNA strand breaks and cellular stress during DNA replication (Wilhelm et al., 2020). However, to date, little is known on *T. gondii*-driven DNA damage in host cells and even less data is available on mechanisms, which are activated by host cells in response. Recent data showed that *T. gondii* RH strain-infected tumor cells experienced parasite-driven DNA double-strand breaks and reacted by activating the homologous recombination repair pathway (Zhuang et al., 2020). Given that DNA damage of host cells may be involved in *T. gondii* pathogenicity, strain-dependent reactions seem of high interest, but related data are currently missing. It is well-known that *T. gondii* strains show significant genetic, phenotypic and pathogenic diversity. Nowadays, three main lineages (types I-III) are described according to their virulence and mortality for laboratory mice (Fernández-Escobar et al., 2021). *T. gondii* strains circulating in Europe mainly belong to type II strains and, to a less extent, to type III strain, both in humans and animals (Khan et al., 2007; Lorenzi et al., 2016; Fernández-Escobar et al., 2022). The *T. gondii* RH strain, widely used in *Toxoplasma*-related research, belongs to type I strains, which are classified as highly virulent leading to widespread parasite dissemination and lethal infection in mice (100% cumulative mortality) (Sanchez and Besteiro, n.d.). To address strain-dependent host cell DNA damage and chromosomal instability induction, we here tested representatives of haplotype I-III by using RH (type I), Me49 (type II) and NED (type III) strains.

To avoid artifacts eventually driven by the immortalization status of (tumor) cells, we here studied reactions of primary (endothelial) host cells. Overall, strain-related data partially mirrored strain pathogenicity. Thus, RH strain infections most potently induced the formation of both, binucleated host cells and host cellular DNA damage foci whilst the Me49 strain failed to do so. Interestingly, NED infections also moderately triggered binucleated cells, DNA damage foci and micronuclei formation. Focusing on DNA damage-related responses in RH strain infections, especially the ATM-related branch of the homologous recombination (HR) pathway was activated in primary host cells whilst molecules of the non-homologous end joining (NHEJ) pathway were either not affected or even found downregulated.

Materials and methods

Primary bovine umbilical vein endothelial cell isolation and culture

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated from umbilical veins obtained from calves born by *sectio caesarea* at the Justus Liebig University Giessen, Germany. Umbilical cords were maintained at 4°C in 0.9% HBSS-HEPES buffer (pH 7.4; Gibco, Grand Island, NY, USA) supplemented with 1% penicillin (500 U/mL; Sigma St. Louis, MO, USA) and streptomycin (500 µg/mL; Sigma) for a maximum of 12 h before use. Isolation of endothelial cells was performed by using 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in Pucks solution (Gibco) and infused into the lumen of ligated umbilical veins for 20 min at 37°C in 5% CO_2 atmosphere.

After gently massaging umbilical veins, the cell suspension was collected in medium and supplemented with 1 mL fetal calf serum (FCS; Gibco) to inactivate collagenase. After two washes (350 *xg*, 12 min, RT), cells were resuspended in complete endothelial cell growth medium (ECGM, PromoCell, supplemented with 10% FCS), plated in 25 cm^2 tissue plastic culture flasks (Greiner) and incubated at 37°C and 5% CO_2 atmosphere. BUVEC were cultured in modified ECGM medium [ECGM, diluted at 30% in M199 medium, supplemented with 5% FCS (Greiner) and 1% penicillin and streptomycin] with medium changes every 2-3 days. All biological isolates were used for *in vitro* experiments at a maximum of 4 passages. Experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], Institutional Ethics Commission of Justus Liebig University Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU.

Parasite maintenance

Tachyzoites of *Toxoplasma gondii* (RH, Me49, NED strains corresponding to haplotypes I, II, III, respectively), were maintained by serial passages in HFF cells (human foreskin fibroblast), using DMEM (1X) + GlutaMAX medium (Gibco 61965-026) supplemented with 10% FCS, 1% penicillin (500 U/mL; Sigma St. Louis, MO; USA) and streptomycin (500 µg/mL; Sigma). Tachyzoite stages were obtained by scraping the monolayer and filtering through a syringe with a 25G needle to release tachyzoites from their parasitophorous vacuoles in the host cells. The parasite suspension was centrifuged at 400 *xg* for 15 seconds to remove cell debris and the supernatant containing tachyzoites was collected. A second centrifugation step was performed to sediment the parasites at 400 *xg* for 10 min. Tachyzoites were counted in a Neubauer chamber, suspended in a modified ECGM medium, and used for BUVEC infections and subsequent experiments.

Comet assays

For cellular DNA damage assessment, three BUVEC isolates were infected with *T. gondii* RH, NED or Me49 tachyzoites using an MOI of 1:2. At 24 h p. i., non-infected and infected cells were gently removed from plates by scraping and centrifuged at 700 *xg* for 2 min. The cell pellet was washed with 1X ice-cold PBS buffer (magnesium and calcium-free). Cells were resuspended at $1x10^5$ cells/mL in ice-cold PBS and analyzed via comet assays according to the manufacturer´s instructions (Abcam, ab238544). In brief, cells were first encapsulated in a low melting agarose suspension at a 1/10 ratio (v/v) and transferred onto the top of an agarose-based layer previously prepared in special glass comet slides, thereby maintaining the cell suspension at 37°C to avoid gelation. After gelation, cell samples were incubated in 1X lysis buffer [NaCl, EDTA solution, 10X lysis solution (provided by manufacturer), DMSO] for 1 h at 4°C to remove cell membranes, cytoplasm and nucleoplasm, and to solubilise nuclear packaging proteins. Thereafter, samples were treated with a pre-chilled alkaline solution (300 mM NaOH, 1 mM EDTA) for 30 min at 4°C, allowing DNA loops to be unwound. Samples were then subjected to electrophoresis at 12 V (1 volt/cm according to the chamber used), 240 mA for 30 min at 4°C under alkaline conditions, washed three times with pre-chilled destilled water for 2 min and once with cold 70% ethanol for 5 min. In a final step, DNA was visualized by 1X Vista Green DNA staining (15 min at RT), an intercalating DNA dye provided by the manufacturer. Based on their differential migratory behavior, intact DNA (= "comet head") can be distinguished from DNA with single-stranded and double-stranded DNA breaks, resulting in "comet tail" structures. Comets (head + tails) were analyzed by the OpenComet Software allowing for automated analysis of comet assay images. The DNA damage was quantified by measuring the displacement between intact nuclear DNA (comet head) and the resulting tail, resulting from single- and double-strand DNA breaks. Hence, the tail length (µm) was graphed for this assay.

Immunofluorescence assays

Three BUVEC isolates were seeded in 12-well plates with 12 mm coverslips previously precoated with fibronectin (1:400, Sigma: F1141). At subconfluency, BUVEC were infected with *T. gondii* RH, Me49 or NED tachyzoites at an MOI of 1:2. At 24 h p. i., samples were fixed in 4% paraformaldehyde (PFA, 15 min, RT) and washed three times in 1X PBS buffer. Samples were then blocked/permeabilized (1X PBS buffer, 3% BSA, 0.3% Triton X-100, all from Carl Roth) for 1 h at RT and incubated in primary antibody solutions (Table 1) at 4°C in a humidified chamber, overnight. Thereafter, samples were washed three times with 1X PBS and incubated in secondary antibody solutions (Table 1) for 30 min at RT and darkness. Cell nuclei were stained with 4´, 6-diamidin-2-phenylindole (DAPI) present in mounting medium solution (Fluoromount G-DAPI, Thermo Fischer Scientific, Cat. N° 495952) and analyzed with a ReScan Confocal microscope instrumentation (RCM 1.1 Visible, Confocal.nl) combined with a Nikon Ti-2 Eclipse microscope.

Protein extraction from *T. gondii*-infected host cells

Six primary BUVEC isolates were infected with *T. gondii* RH tachyzoites (MOI 1:2). At 24 h p. i., cells were washed with 1X PBS buffer, detached from the plate using trypsin/EDTA solution [0.25% (w/v) trypsin; 0.53 mM EDTA, 37°C, 5 min] and pelleted at 400 *xg* for 5 min. The cell pellet was washed with 1X PBS buffer and resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF; all Roth) supplemented with a protease inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate tyrosine phosphatase inhibitor (Abcam ab120386) and 1 mM phenylmethylsulphonyl

TABLE 1 Primary and secondary antibodies used for immunofluorescencs assays.

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies				
T. gondii	ThermoFisher	PA1-7256	Goat	1:100
H2AvD (pS137)	Cell Signalling	80312	Mouse	1:300
H2AvD (pS137)	Rockland	600-401-914S	Rabbit	1:300
	Secondary antibodies			
Antigen/Conjugate	Company	Cat. number	Host/target	Dilution
Alexa Fluor 488	ThermoFisher	A11055	goat	1:500
Alexa Fluor 594	ThermoFisher	A-11058	goat	1:500
Alexa Fluor 594	ThermoFisher	R37117	rabbit	1:500
Alexa Fluor 647	ThermoFisher	A-21244	rabbit	1:500
Alexa Fluor 647	ThermoFisher	A-21235	mouse	1:500

fluoride, a serine protease inhibitor (Abcam ab141032). Protein extracts were sonicated for five cycles of 20 s sonication and 20 s resting and then centrifuged (10,000 *x g*, 10 min, 4°C) to sediment intact cells, membranes, and nuclei. The supernatants were analyzed for protein content via BCA Protein Assay (Pierce BCA Protein Assay Kit, Thermo Scientific, cat. number 23225) following the manufacturer's instructions. Protein concentration was quantified in a Varioskan plate reader by measuring the absorbance at 562 nm.

SDS-PAGE and immunoblotting

Protein extracts were diluted in loading buffer with 6 M urea (10% SDS, 12.5% 2-mercaptoethanol, 25% glycerol, 150 mM Tris-HCl pH 6.8) and boiled at 95°C for 5 min. Samples (40 µg protein/slot) were loaded on 6% polyacrylamide gels and subjected to SDS-PAGE electrophoresis (200 V; approx. 50 min, BioRad). Proteins were transferred to PVDF membranes (Millipore) at 300 mA for 2 h in a wet-tank transfer system (BioRad) and then blocked for 1 h at RT [3% BSA in TBS buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.6)]. Afterwards, membrane-bound proteins were reacted with primary antibodies (diluted in blocking solution: TBS buffer, 0.1% Tween-20, 3% BSA, 4°C, overnight) directed against key proteins of the homologous recombination repair pathway (ATM, BRCA1, p-BRCA1, BRCA2, Rad54, Mre11, p-Mre11, p95NBS1), non-homologous end joining (DNA-PKcs, p-DNA-PKcs, Ku70, Artemis, DNA ligase IV) and vinculin (used as a loading control for the normalization of the samples) (Table 2). Thereafter, the membranes were washed three times with TBS-Tween (0.1%) and incubated in secondary antibody solutions (Table 2) for 30 min at RT. After three washings in TBS-Tween (0.1%), protein detection was performed using an enhanced chemiluminescence detection system (ECL Prime, Amersham). Images were taken using the Science Imaging Instrument (INTAS) applying the INTAS ChemoStar Imager software. A protein ladder was used to estimate protein masses (HiMark Pre-stained Protein Standard #LC5699, Thermo Fisher Scientific). Protein band intensities were analyzed by the Fiji Gel Analyzer plugin.

Quantification of binucleated and γH2AX-positive cells

All quantifications were performed in three biological BUVEC replicates infected with tachyzoites of *T. gondii* RH, Me49 or NED strains. Samples were collected at 24 h p. i. and stained for DNA (nuclear staining, DAPI, blue, Invitrogen), DNA damage foci (γH2AX, green, Table 1) and tachyzoites (anti-*Toxoplasma* antibodies, red, Table 1). For immunofluorescence assays, please refer to the section above. In all experimental settings, five random images were acquired at 60X with an epifluorescence microscope (Nikon Ti-2 Eclipse) and a total of 1800 cells were analyzed per condition. For binucleated cell estimation, the total number of binucleated cells was normalized by the total number of cells in the same field of view. The DNA damage quantification was performed by the detection of γH2AX protein inside of the nuclear area. The histone variant H2AX is phosphorylated in Ser 139 when cells display DNA double strand breaks induction forming DNA damage foci inside the nucleus that can vary in number from one to hundred. Therefore, we consider a positive cell to be one that shows at least one focus of DNA damage positive for γH2AX. First, we proceeded with the segmentation of the DAPI channel in each image and the respective ROIs were merged with the green channel (γH2AX staining) to allocate the nuclear region in the image with the DNA damage foci staining. Each γH2AX-positive cell was counted as one independently on the number of DNA damage foci per nucleus. All γH2AX-positive cells were counted and normalized to the total number of cells in the same field of view. Micronuclei were defined as roundish DAPI-positive structures localized in juxtaposition but separate from the nuclear area. The total number of micronuclei was counted and normalized to the total number of cells in the same field of view.

TABLE 2 Primary and secondary antibodies used for Western Blotting.

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies				
ATM (D2E2)	Cell Signalling	2873	Rabbit	1:1000
BRCA1	Cell Signalling	14823	Rabbit	1:1000
p-BRCA1	Cell Signalling	9009	Rabbit	1:1000
BRCA2	Cell Signalling	10741	Rabbit	1:1000
Rad54	Cell Signalling	15016	Rabbit	1:1000
Mre1l	Cell Signalling	4887	Rabbit	1:1000
p-Mre1l	Cell Signalling	4859	Rabbit	1:1000
P95/NBS1	Cell Signalling	14956	Rabbit	1:1000
DNA-PKcs	Cell Signalling	38168	Rabbit	1:1000
Ku70	Cell Signalling	4588	Rabbit	1:1000
Artemis (D708V)	Cell Signalling	13381	Rabbit	1:1000
DNA ligase IV	Cell Signalling	14649	Rabbit	1:1000
Vinculin	Santa Cruz	sc-73614	Mouse	1:500
	Secondary antibodies			
Antigen/Conjugate	Company	Cat. number	Host/target	Dilution
Goat anti-mouse IgG Peroxidase conjugated	Pierce	31430	Goat/mouse	1:40,000
Goat anti-rabbit IgG Peroxidase conjugated	Pierce	31460	Goat/rabbit	1:40,000

Image acquisition and reconstruction

Fluorescence images were acquired with a ReScan Confocal microscope instrumentation (RCM 1.1 Visible, Confocal.nl) equipped with a fixed 50 µm pinhole size and combined with a Nikon Ti-2 Eclipse microscope equipped with a motorized Z-stage (DI1500, Nikon). The RCM unit was connected to a Toptica CLE laser with the following excitations: 405/488/561/640 nm. Images were taken via an sCMOS camera (PCO edge) using a CFI Plan Apochromat X60 lambda-immersion oil objective (NA 1.4/0.13; Nikon). The instrument was operated by the NIS-Elements software (version 5.11). To calculate the total number of cells and the number of binucleated cells present within one cell layer, all images were first segmented using the Otsu thresholding algorithm. Identical brightness and contrast conditions were applied for each data set within one experiment. The total number of cells was obtained using the Fiji plugin "Analyze particles" applying a size of 10 µm.

Statistical analysis

The data were expressed as mean ± SD from independent experiments. For cell number quantification experiments, one-way analysis of variance (non-parametric ANOVA) with Kruskal-Wallis´s post-test was performed using GraphPad Prism 9.3.1 software applying a significance level of 5%. For WB-based analyses, unpaired two-tailed *t*-tests were performed comparing controls vs infected cells at a 95% confidence interval. All graphs and statistical analyses were performed using GraphPad Prism 9 software.

Results

T. gondii-driven host cell DNA damage and binucleated cell formation is haplotype-dependent

T. gondii RH infections have been reported to induce the host cell cycle arrest and to cause alterations in chromosome segregation and cytokinesis in host cells (Velásquez et al., 2019). These aberrant processes include chromosomal instability, resulting from chromosome loss, gain or inadequate DNA rearrangements (Bakhoum and Cantley, 2018). Chromosomal instability can trigger DNA damage by causing DNA strand breaks or by inducing cell stress during DNA replication (Wilhelm et al., 2020). Given that *T. gondii* indeed affects chromosomal segregation (Velásquez et al., 2019), we aimed here to analyze whether *T. gondii* infections induce host cellular DNA damage. In a first experimental approach, we analyzed the DNA strand integrity by using conventional comet assays. This method is

well-accepted for general DNA damage detection in eukaryotic cells but cannot distinguish between single and double-strand breaks (Ostling and Johanson, 1984). Here, cells with intact DNA only show a comet head and - due to altered migratory behavior in the electric field - damaged DNA strands form the comet tail (Figure 1A). Current data revealed that *T. gondii* RH, NED and Me49 infections of BUVEC in principle all resulted in a significantly increased proportion of cells experiencing DNA damage in comparison to non-infected controls (infected cells vs. controls for RH, NED and Me49: $p \leq 0.0001$; $p \leq 0.0001$; $p \leq 0.0001$, respectively, Figure 1B). However, by far, the strongest reactions were induced by the RH strain (Figure 1B).

Given that cellular damage responses induced by DNA single- or double-strand breaks involve different signaling pathways and protein factors (Ma and Dai, 2018) and considering that comet assays cannot distinguish between single- and double-strand breaks, we here applied a second experimental approach by assessing γ-H2AX signals in *T. gondii*-infected cells. In higher eukaryotic cells, DNA double-strand breaks trigger the phosphorylation of the histone H2A variant H2AX at serine 139 to generate γ-H2AX (Kinner et al., 2008). Nuclear γ-H2AX signals are therefore used as a marker for double-strand break events being represented as DNA damage foci. In the current study, BUVEC were infected with RH, Me49 or NED strains of *T. gondii*, stained for DNA by DAPI to identify the nuclear area and co-labeled for γ-H2AX at 24 h p. i. (Figure 1C). Overall, the proportion of cells experiencing DNA damage foci (= γ-H2AX-positive BUVEC) increased with parasite infections, a finding that revealed *T. gondii* haplotype-dependent. Thus, a significant parasite-driven induction of DNA double-strand breaks was detected in both RH- (15.1% positive cells, $p < 0.0001$) and NED- (10.6% positive cells, $p = 0.0001$) infected cells. In contrast, only 2.4% of Me49-infected cells showed DNA damage foci thereby failing to differ significantly from non-infected controls (0.5%, Figure 1D). Given that the - by far - strongest effects were again found in RH strain-infected cells, this finding may mirror strain pathogenicity (Figure 1D). Altogether, these findings suggest that infections with the *T. gondii* strains NED and RH both induced double strand DNA breaks while Me49 infections do not.

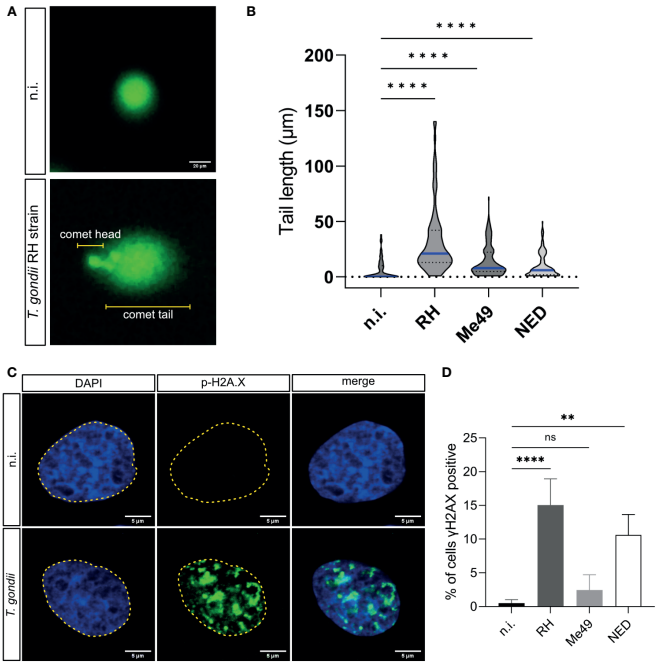


FIGURE 1
Induction of DNA damage by *Toxoplasma gondii* RH, Me49 and NED infections in primary bovine endothelial cells. BUVEC monolayers ($n = 3$) were infected with RH, Me49 or NED tachyzoites (MOI 1:2; 24 h p.i.) to analyze infection-driven DNA damage using comet assays. **(A)** Exemplary illustration of comet assays on *T. gondii* RH-infected and uninfected cells. The round shape (comet head) identifies cells lacking DNA strand breaks while the comet tail reflects DNA with single or double DNA strand breaks. **(B)** The comet tail length was measured and plotted to compare *T. gondii* RH, Me49 and NED-infected cells with non-infected controls. The data showed that all *T. gondii* strains increased the number of host cells experiencing single- or double-stranded DNA breaks. **(C, D)** Detection of double-strand break-based DNA damage foci by γH2AX immunostaining. The data revealed a strain-dependent induction of DNA damage foci. The scale bar represents 20 or 5 µm. ns: not significant, **p < 0.01, ****p < 0.0001.

Chromosomal instability is structural or numerical due to chromosome segregation impairment, chromosome condensation defects or abnormal cytokinesis (Siri et al., 2021). Given that we demonstrated that *T. gondii*-induced DNA damage is haplotype-dependent, we also assessed the influence of these haplotypes on cytokinesis failure on the level of infection-driven binucleated host cell formation. In agreement to DNA damage foci-related data, only infections with the RH (38.1%, $p < 0.0001$) and NED (10.5%, $p = 0.002$) strains led to a significant increase in the formation of binucleated cells and, again, RH-infected cells showed the overall strongest effects (Figure 2A). Accordingly, when estimating the percentages of binucleated infected cells showing DNA damage foci (γ-H2AX-positive cells), a significantly increased proportion of 6.8% ($p < 0.0001$) and 2.3% ($p = 0.029$) was found in RH- and NED-strain-infected BUVEC, respectively, whilst neither Me49-infected cells nor non-infected control binucleated cells showed a significant induction of DNA double-strand breaks (Figure 2B). Hence, these findings suggest that DNA damage may indeed be associated with the binucleated phenotype induced by *T. gondii*.

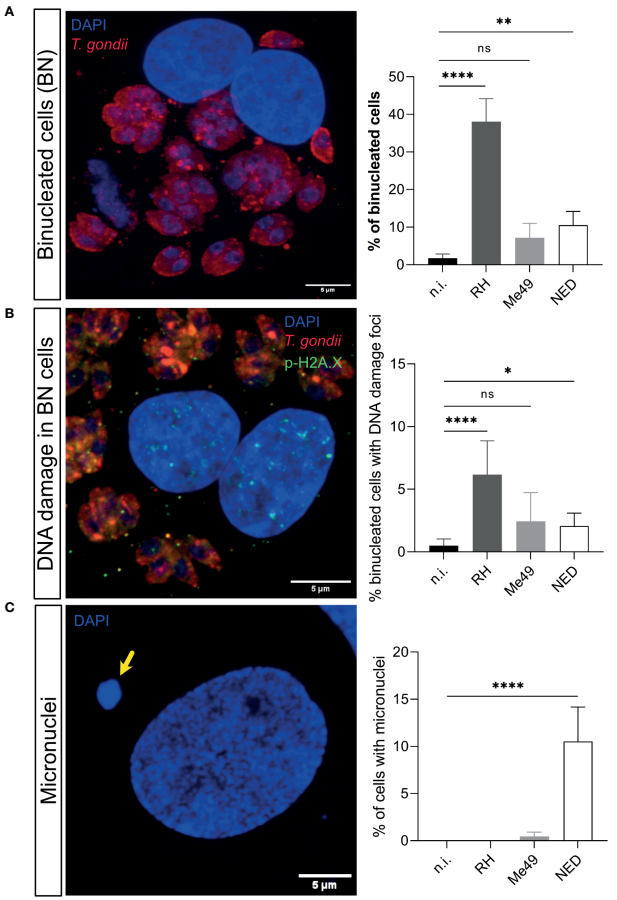


FIGURE 2
Effects of *Toxoplasma gondii* RH, Me49 and NED infections on BUVEC in binucleated host cell and micronuclei formation. BUVEC isolates (n =3) were infected with RH, Me49 or NED tachyzoites at an MOI 1:2 during 24 h **(A)** The presence of binucleated cells was estimated in *T. gondii*-infected cells. Data showed that RH and NED strains induced host binucleated phenotypes at different quantitative levels whilst this phenomenon was not observed in Me49-infected cells or controls. **(B)** The percentage of DNA damage foci-positive binucleated cells was analyzed showing that only RH and NED infections induced both findings at a time. **(C)** Detection of micronuclei formation indicated that exclusively NED-infected host cells revealed a significant increase. The scale bar represents 5 µm. ns: not significant, *p ≤ 0.05, **p < 0.01, ****p < 0.0001.

Besides causing cytokinesis impairment, chromosome condensation and segregation failures may also result in a lack or displacement of chromosomes or in the formation of chromosome bridges and micronuclei (Fenech et al., 2011; Ye et al., 2019; Siri et al., 2021). Micronuclei are typically represented by DAPI-positive globular structures which are localized close but separate from the cell nucleus. Here, we estimated the presence of micronuclei in RH-, Me49- and NED-infected BUVEC. As an interesting finding, a significantly enhanced proportion of host cells with micronuclei was exclusively found in NED-infected cells (10.5%, $p < 0.0001$) (Figure 2C).

T. gondii-infected host cells activate the homologous recombination pathway (HR) to repair parasite-driven DNA damage

Cells under genotoxic stress show a DNA damage response (DDR) and therefore activate different intracellular repair pathways depending on the type of DNA damage. Hence, DNA strand breaks either activate the homologous recombination (HR) or the non-homologous end joining (NHEJ) pathway. Interestingly, the HR pathway works cell cycle-dependent and primarily operates during the S- and G2-phase, whilst NHEJ mediated repair mechanisms are primarily activated after detection of DNA damage during the G1-phase. Nevertheless, this pathway may be activated throughout the cell cycle (Li and Heyer, 2008; Watanabe and Lieber, 2022) (Figure 3). Given that the current findings proved most prominent effects on DNA double-strand breaks in the case of the *T. gondii* RH strain, we here focused on RH strain infections and studied the expression of several key molecules of both, HR and NHEJ pathways by Western blotting (Figure 4). On a quantitative level we demonstrated that most molecules of the NHEJ pathway were not affected by infection (DNA ligase IV, artemis) or even down-regulated (Ku70, DNA-PKcs), indicating that *T. gondii* RH infections do not activate the NHEJ pathway (Figure 4). In contrast, several key molecules of the HR repair pathway were significantly induced in *T. gondii* RH-infected host cells (Figure 5). As schematically depicted in Figure 3, the HR repair pathway splits into two branches (ATM- and ATR-related pathways) depending on type of damaged DNA strand. Thus, the ATM-related pathway is mainly induced by DNA double-strand breaks, whilst the ATR-related pathway is activated by single strain breaks or by stalled replication forks. To distinguish the induction of ATM- or ATR-mediated signaling, we here studied the expression of several characteristic molecules of both pathways. Overall, *T. gondii* RH

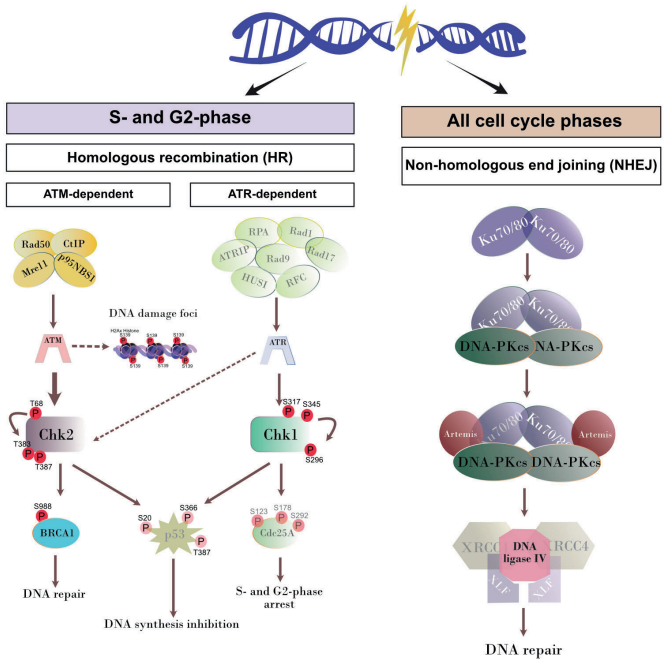


FIGURE 3
Schematic illustration of DNA repair pathways. The proteins of the pathways that were not analyzed in the current study are shown attenuated.

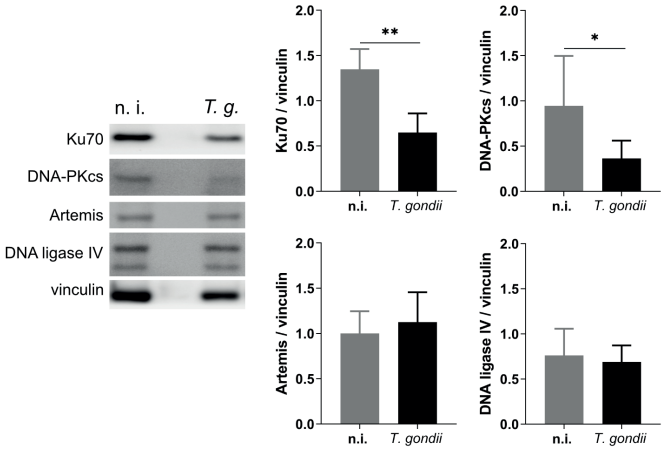


FIGURE 4
Effects of *T. gondii* RH infections on the NHEJ repair pathway. BUVEC isolates (n = 6) were infected with *T. gondii* RH strain (MOI 1:2). At 24 h p. i., samples were analyzed by Western Blotting for the expression of key proteins involved in the NHEJ pathway. The results showed that *T. gondii* RH infection does not activate the NHEJ pathway but downregulates the expression of Ku70 and DNA-PKcs. Bars represent the mean ± SD of six biological replicates. *p ≤ 0.05, **p < 0.01.

infections exclusively up-regulated molecules of the ATM-related pathways (ATM, Mre11, Rad50, and pBRCA) (Figure 5) whilst proteins involved in the ATR-related pathways either did not show any changes in expression (Figure 5) or – in case of BRCA2 – were even downregulated. Altogether, these findings confirm that *T. gondii* RH-infected host cells suffer from infection-driven DNA double-strand breaks and elicit a DNA damage response by activating the S-/G2-phase-related ATM-dependent branch of the HR pathway.

Discussion

Genome integrity is an essential feature of cellular homeostasis and is a prerequisite for preserving the genetic information delivered to the offspring. Consequently, genome instability has a direct impact on cell, tissue or animal survival. Eukaryotic cells maintain genome integrity by activating different DNA damage response (DDR) pathways upon endogenous and environmental genotoxic stress promoting the repair of different types of DNA lesions. Moreover, activation of DDR controls cell cycle progression, thereby delivering time for cells to repair the damaged DNA (Campos and Clemente-Blanco, 2020; Petsalaki and Zachos, 2020). Previous results on primary host cells have shown that *T. gondii* infection affects the chromosomal stability of the host cells by inducing binucleated cell formation and chromosome segregation alterations (Velásquez et al., 2019). Interestingly, all of these features are hallmarks of chromosome instability (Geigl et al., 2008) and it appears likely that these events may be linked to the pathogenicity of *T. gondii* infections. On a global level, different *T. gondii* haplotypes with varying virulence occur. Whilst mainly the classical haplotypes I-III with an estimated moderate to low virulence circulate in Europe, atypical and mixed haplotypes eventually bearing high pathogenicity are spread in South America (Galal et al., 2022). To address a potential influence of different haplotypes on binucleated host cells, micronuclei formation and DNA-damage related cellular events, we here analyzed infection-driven effects of three different *T. gondii* strains (RH, Me49, NED strains corresponding to haplotypes I, II, III, respectively) on primary host endothelial cells.

In all experiments we worked with different donors of primary endothelial host cells to avoid cell cycle-related artifacts that may be present in permanent cell lines or in cells of tumor origin. Considering the three *T. gondii* strains we showed in a first experimental series that the RH strain - which is reported as the most pathogenic one among these three strains - most prominently induced the formation of binucleated cells thereby confirming recent RH-related findings and indicating cell cycle impairment (Velásquez et al., 2019). Interestingly, infections-driven binclucleated cell formation proved strain-dependent, since the NED strain triggered this event on a minor level whilst ME49 infections had no significant effect on binucleated phenotypes. Given that binucleated cell formation may be linked to chromosome instability and DNA damage, we then assessed a general overview on the presence of DNA damage in infected host cells via comet assays. Here, we confirmed strain-dependent effects and showed that the strongest DNA damage was again driven by the *T. gondii* RH infections. The NED strain induced low levels of DNA damage whilst the Me49 strain failed to do so. Given that comet assays cannot distinguish between single- and double-strand breaks, we additionally analyzed

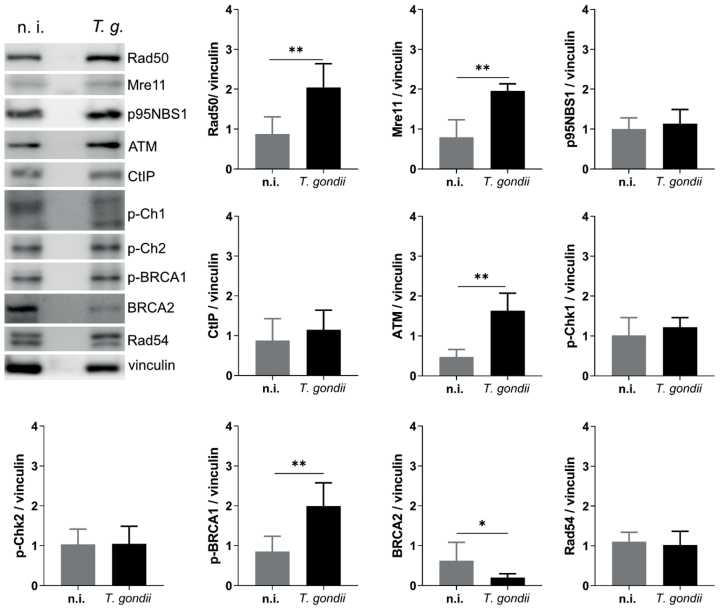


FIGURE 5
T. gondii infection activates the ATM-mediated HR repair pathway. BUVEC monolayers (n =6) were infected with *T. gondii* RH tachyzoites at an MOI 1:2. At 24 h p. i., protein extracts were analyzed via Western Blotting to analyze the expression of key proteins involved in the HR repair pathway. Results show an up-regulation of several proteins involved in the ATM-dependent HR pathway. Bars represent the mean ± SD of six biological replicates. *p ≤ 0.05, **p < 0.01.

the presence of γ-H2AX-based DNA damage foci, which are indicative for DNA double-strand breaks. In agreement to the data mentioned above and referring to RH-, Me49- and NED-infected cell layers, strongest effects related to DNA damage foci were again attributed to the RH strain. The findings on the RH strain are in line to recent data of Zhuang et al (Zhuang et al., 2020), who reported on the induction of double-strand breaks in *T. gondii* RH-infected HeLa, HEK293T and Vero cells. Another hallmark of chromosome instability is the formation of so-called micronuclei. In contrast to our expectation, we here exclusively detected an enhanced proportion of host cells experiencing micronuclei formation in the case of NED strain infections.

Whenever a cell experiences genotoxic events and DNA damage, it activates a DNA damage response (DDR) via different repair pathways to preserve the correct genetic information to be passed to daughter cells. The repair pathway to be induced depends on type and extent of DNA insult, therefore single- and double-strand break repair pathways are both part of the DDR of a cell (Figure 3). Single-strand repair pathways include both excision repair modes (nucleotide excision repair and base excision repair) and mismatch repair. DNA double-strand insults may either induce the NHEJ or the homologous recombination (HR) repair pathways, the latter of which is linked to S- and G2 phases of cell cycle. Given that the current data confirmed DNA double-strand breaks in *T. gondii*-infected cells and revealed strongest effects in RH infections, we here studied the DNA damage response on the level of the HR and NHEJ pathways exclusively in RH-infected BUVEC. Overall, we found a *T. gondii*-induced activation of the HR pathway which indirectly supports former findings on a *T. gondii*-driven cell cycle arrest between S- and G2 phase in different types of host cells (Molestina et al., 2003; Brunet et al., 2008; Kim et al., 2016) and especially in BUVEC (Velásquez et al., 2019). In the case of the NHEJ pathway, the upstream molecules Ku70 and DNA-PKcs were found down-regulated in infected host cells indicating that this pathway may actively be blocked by parasite infection. Interestingly, Ku70 was reported to be involved in viral DNA recognition in human cells promoting type I and type III interferon/proinflammatory cytokine production (Sui et al., 2021), a reaction that is assumed to adverse affect *T. gondii* development. Moreover, Ku70 was shown to interact with Bax, thereby inhibiting Bax-mediated apoptosis (Cohen et al., 2004). Considering these aspects, infection-driven Ku70 downregulation might represent an DNA damage-independent event in *T. gondii*-infected host cells. Besides Ku70, the DNA-PKcs holoenzyme was also found downregulated in *T. gondii* RH infected cells. This protein forms a complex with Ku70/80 in response to DNA damage (Fell and Schild-Poulter,

2015). Moreover, it was shown that cells with low DNA-PKcs levels undergo accelerated cellular senescence (Liu et al., 2019). To our knowledge, there is currently no evidence that *T. gondii* modulates host cell senescence. The HR repair pathway includes different branches. In line with Zhuang et al. (2020) we here confirmed for a primary host cell type that ATM-mediated repair signaling is induced in *T. gondii* RH-infected BUVEC whilst the ATR-dependent pathway was not affected by infection.

Undoubtedly, the molecular mechanism of DNA damage induction driven by *T. gondii* seems of high interest, especially since it proved haplotype-dependent. However, the identification of parasite molecules triggering DNA double-strand breaks and their eventual molecular interactions with host cells are beyond the scope of the current study. Several *T. gondii*-secreted molecules, such as the ROP18 kinase, which was reported to interact with DNA repair-related host proteins (Cheng et al., 2012), may be good candidates in this respect and should be investigated in this respect in future.

Overall, the current findings underline the effects of *T. gondii* infections on host cell cycle and DNA integrity and demonstrate haplotype-dependent effects that may be linked to strain pathogenicity. Furthermore, current data confirm DNA damage responses of primary host cells on the level of ATM-dependent HR repair pathways.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], Institutional Ethics Commission of Justus Liebig University Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LR-B: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. CH: Funding acquisition, Writing – review & editing. AT: Funding acquisition, Resources, Supervision, Writing – review & editing. ZV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank Axel Wehrend and all staff members from the Clinic for Obstetrics, Gynaecology and Andrology of Large and Small Animals at the Justus Liebig University, Giessen, Germany, for the valuable collaboration in the supply of bovine umbilical cords, as well all the technical staff and colleagues of the Institute of Parasitology involved in the isolation of bovine primary cells used in this study. Thanks to Gereon Schares (Friedrich Loeffler Institute, FLI, Germany) for providing the *T. gondii* Me49 and NED tachyzoites.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Bakhoum, S. F., and Cantley, L. C. (2018). The multifaceted role of chromosomal instability in cancer and its microenvironment. *Cell* 174, 1347–1360. doi: 10.1016/j.cell.2018.08.027

Bigna, J. J., Tochie, J. N., Tounouga, D. N., Bekolo, A. O., Ymele, N. S., Youda, E. L., et al. (2020). Global, regional, and country seroprevalence of Toxoplasma gondii in pregnant women: a systematic review, modelling and meta-analysis. *Sci. Rep.* 10, 12102. doi: 10.1038/s41598-020-69078-9

Brunet, J., Pfaff, A. W., Abidi, A., Unoki, M., Nakamura, Y., Guinard, M., et al. (2008). Toxoplasma gondii exploits UHRF1 and induces host cell cycle arrest at G2 to enable its proliferation. *Cell Microbiol.* 10, 908–920. doi: 10.1111/j.1462-5822.2007.01093.x

Campos, A., and Clemente-Blanco, A. (2020). Cell cycle and DNA repair regulation in the damage response: protein phosphatases take over the reins. *Int. J. Mol. Sci.* 21, E446. doi: 10.3390/ijms21020446

Cheng, L., Chen, Y., Chen, L., Shen, Y., Shen, J., An, R., et al. (2012). Interactions between the ROP18 kinase and host cell proteins that aid in the parasitism of Toxoplasma gondii. *Acta Trop.* 122, 255–260. doi: 10.1016/j.actatropica.2012.02.001

Cohen, H. Y., Lavu, S., Bitterman, K. J., Hekking, B., Imahiyerobo, T. A., Miller, C., et al. (2004). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol. Cell* 13, 627–638. doi: 10.1016/S1097-2765(04)00094-2

Dubey, J. P. (2009). History of the discovery of the life cycle of Toxoplasma gondii. *Int. J. Parasitol.* 39, 877–882. doi: 10.1016/j.ijpara.2009.01.005

Fell, V. L., and Schild-Poulter, C. (2015). The Ku heterodimer: Function in DNA repair and beyond. *Mutat. Research/Reviews Mutat. Res.* 763, 15–29. doi: 10.1016/j.mrrev.2014.06.002

Fenech, M., Kirsch-Volders, M., Natarajan, A. T., Surralles, J., Crott, J. W., Parry, J., et al. (2011). Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* 26, 125–132. doi: 10.1093/mutage/geq052

Fernández-Escobar, M., Calero-Bernal, R., Regidor-Cerrillo, J., Vallejo, R., Benavides, J., Collantes-Fernández, E., et al. (2021). *In vivo* and in *vitro* models show unexpected degrees of virulence among Toxoplasma gondii type II and III isolates from sheep. *Vet. Res.* 52, 82. doi: 10.1186/s13567-021-00953-7

Fernández-Escobar, M., Schares, G., Maksimov, P., Joeres, M., Ortega-Mora, L. M., and Calero-Bernal, R. (2022). Toxoplasma gondii genotyping: A closer look into europe. *Front. Cell. Infection Microbiol.* 12. doi: 10.3389/fcimb.2022.842595

Franco, M., Panas, M. W., Marino, N. D., Lee, M.-C. W., Buchholz, K. R., Kelly, F. D., et al. (2016). A novel secreted protein, MYR1, is central to Toxoplasma's manipulation of host cells. *mBio* 7, e02231–e02215. doi: 10.1128/mBio.02231-15

Galal, L., Ariey, F., Gouilh, M. A., Dardé, M.-L., Hamidović, A., Letourneur, F., et al. (2022). A unique Toxoplasma gondii haplotype accompanied the global expansion of cats. *Nat. Commun.* 13, 5778. doi: 10.1038/s41467-022-33556-7

Geigl, J. B., Obenauf, A. C., Schwarzbraun, T., and Speicher, M. R. (2008). Defining 'chromosomal instability.'. *Trends Genet.* 24, 64–69. doi: 10.1016/j.tig.2007.11.006

Holland, A. J., and Cleveland, D. W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat. Rev. Mol. Cell Biol.* 10, 478–487. doi: 10.1038/nrm2718

Jones, J., Lopez, A., and Wilson, M. (2003). Congenital toxoplasmosis. *Am. Fam Physician*. 67, 2131–2138.

Khan, A., Fux, B., Su, C., Dubey, J. P., Darde, M. L., Ajioka, J. W., et al. (2007). Recent transcontinental sweep of Toxoplasma gondii driven by a single monomorphic chromosome. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14872–14877. doi: 10.1073/pnas.0702356104

Kim, M. J., Jung, B.-K., Cho, J., Song, H., Pyo, K.-H., Lee, J. M., et al. (2016). Exosomes secreted by Toxoplasma gondii-infected L6 cells: their effects on host cell proliferation and cell cycle changes. *Korean J. Parasitol.* 54, 147–154. doi: 10.3347/kjp.2016.54.2.147

Kinner, A., Wu, W., Staudt, C., and Iliakis, G. (2008). γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res.* 36, 5678–5694. doi: 10.1093/nar/gkn550

Li, X., and Heyer, W.-D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res.* 18, 99–113. doi: 10.1038/cr.2008.1

Liu, Y., Efimova, E. V., Ramamurthy, A., and Kron, S. J. (2019). Repair-independent functions of DNA-PKcs protect irradiated cells from mitotic slippage and accelerated senescence. *J. Cell Sci.* 132, jcs229385. doi: 10.1242/jcs.229385

Lorenzi, H., Khan, A., Behnke, M. S., Namasivayam, S., Swapna, L. S., Hadjithomas, M., et al. (2016). Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic Toxoplasma gondii genomes. *Nat. Commun.* 7, 10147. doi: 10.1038/ncomms10147

Ma, A., and Dai, X. (2018). The relationship between DNA single-stranded damage response and double-stranded damage response. *Cell Cycle* 17, 73–79. doi: 10.1080/15384101.2017.1403681

Molestina, R. E., El-Guendy, N., and Sinai, A. P. (2008). Infection with Toxoplasma gondii results in dysregulation of the host cell cycle. *Cell. Microbiol.* 10, 1153–1165. doi: 10.1111/j.1462-5822.2008.01117.x

Molestina, R. E., Payne, T. M., Coppens, I., and Sinai, A. P. (2003). Activation of NF-κB by Toxoplasma gondii correlates with increased expression of antiapoptotic genes and localization of phosphorylated IκB to the parasitophorous vacuole membrane. *J. Cell Sci.* 116, 4359–4371. doi: 10.1242/jcs.00683

Ostling, O., and Johanson, K. J. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* 123, 291–298. doi: 10.1016/0006-291X(84)90411-X

Petsalaki, E., and Zachos, G. (2020). DNA damage response proteins regulating mitotic cell division: double agents preserving genome stability. *FEBS J.* 287, 1700–1721. doi: 10.1111/febs.15240

Pierre-Louis, E., Etheridge, M. G., de Paula Baptista, R., Khan, A., Chasen, N. M., and Etheridge, R. D. (2022). Disruption of toxoplasma gondii-induced host cell DNA replication is dependent on contact inhibition and host cell type. *mSphere* 7 (3), e0016022. doi: 10.1128/msphere.00160-22

Robert-Gangneux, F., Meroni, V., Dupont, D., Botterel, F., Garcia, J. M. A., Brenier-Pinchart, M.-P., et al. (2018). Toxoplasmosis in transplant recipients, Europe 2010–2014. *Emerg. Infect. Dis.* 24, 1497–1504. doi: 10.3201/eid2408.180045

Rostami, A., Riahi, S. M., Gamble, H. R., Fakhri, Y., Nourollahpour Shiadeh, M., Danesh, M., et al. (2020). Global prevalence of latent toxoplasmosis in pregnant women: a systematic review and meta-analysis. *Clin. Microbiol. Infect.* 26, 673–683. doi: 10.1016/j.cmi.2020.01.008

Sabou, M., Doderer-Lang, C., Leyer, C., Konjic, A., Kubina, S., Lennon, S., et al. (2019). Toxoplasma gondii ROP16 kinase silences the cyclin B1 gene promoter by hijacking host cell UHRF1-dependent epigenetic pathways. *Cell. Mol. Life Sci.* 77, 2741–2756. doi: 10.1007/s00018-019-03267-2

Shobab, L., Pleyer, U., Johnsen, J., Metzner, S., James, E. R., Torun, N., et al. (2013). Toxoplasma serotype is associated with development of ocular toxoplasmosis. *J. Infect. Dis.* 208, 1520–1528. doi: 10.1093/infdis/jit313

Siri, S. O., Martino, J., and Gottifredi, V. (2021). Structural chromosome instability: types, origins, consequences, and therapeutic opportunities. *Cancers (Basel)* 13, 3056. doi: 10.3390/cancers13123056

Sui, H., Hao, M., Chang, W., and Imamichi, T. (2021). The role of ku70 as a cytosolic DNA sensor in innate immunity and beyond. *Front. Cell. Infection Microbiol.* 11. doi: 10.3389/fcimb.2021.761983

Tenter, A. M., Heckeroth, A. R., and Weiss, L. M. (2000). Toxoplasma gondii: from animals to humans. *Int. J. Parasitol.* 30, 1217–1258. doi: 10.1016/S0020-7519(00)00124-7

Velásquez, Z. D., Conejeros, I., Larrazabal, C., Kerner, K., Hermosilla, C., and Taubert, A. (2019). Toxoplasma gondii-induced host cellular cell cycle dysregulation is linked to chromosome missegregation and cytokinesis failure in primary endothelial host cells. *Sci. Rep.* 9, 12496. doi: 10.1038/s41598-019-48961-0

Watanabe, G., and Lieber, M. R. (2022). Dynamics of the artemis and DNA-PKcs complex in the repair of double-strand breaks. *J. Mol. Biol.* 434, 167858. doi: 10.1016/j.jmb.2022.167858

Wilhelm, T., Said, M., and Naim, V. (2020). DNA replication stress and chromosomal instability: dangerous liaisons. *Genes (Basel)* 11, 642. doi: 10.3390/genes11060642

Ye, C. J., Sharpe, Z., Alemara, S., Mackenzie, S., Liu, G., Abdallah, B., et al. (2019). Micronuclei and genome chaos: changing the system inheritance. *Genes (Basel)* 10, 366. doi: 10.3390/genes10050366

Zhuang, H., Yao, C., Zhao, X., Chen, X., Yang, Y., Huang, S., et al. (2020). DNA double-strand breaks in the Toxoplasma gondii-infected cells by the action of reactive oxygen species. *Parasit Vectors* 13, 490. doi: 10.1186/s13071-020-04324-7

2.3. *TOXOPLASMA GONDII* ME49 AND NED STRAINS ARREST HOST CELL CYCLE PROGRESSION AND ALTER CHROMOSOME SEGREGATION IN A STRAIN-INDEPENDENT MANNER

Rojas-Barón L., Hermosilla C., Taubert A., Velásquez Z. (2024).

Front. Microbiol., 21 February 2024. Sec. Infectious Agents and Disease. Volume 15 – 2024 │https://doi.org/10.3389/fmicb.2024.1336267

Own part in the publication:

Project planning:	50%, together with co-authors and supervisors
Experimentation:	80%, mainly independent
Data evaluation:	80%, mainly independent
Manuscript writing:	80%, mainly independent

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 21 February 2024
DOI 10.3389/fmicb.2024.1336267

OPEN ACCESS

EDITED BY
Saurabh Mishra,
Cornell University, United States

REVIEWED BY
Hasan Tarık Atmaca,
Balıkesir University, Türkiye
Dong-Hui Zhou,
Fujian Agriculture and Forestry University, China

*CORRESPONDENCE
Lisbeth Rojas-Barón
✉ lisbeth.cecilia.rojas.baron@vetmed.uni-giessen.de

RECEIVED 10 November 2023
ACCEPTED 02 February 2024
PUBLISHED 21 February 2024

CITATION
Rojas-Barón L, Hermosilla C, Taubert A and Velásquez ZD (2024) *Toxoplasma gondii* Me49 and NED strains arrest host cell cycle progression and alter chromosome segregation in a strain-independent manner. *Front. Microbiol.* 15:1336267. doi: 10.3389/fmicb.2024.1336267

Toxoplasma gondii Me49 and NED strains arrest host cell cycle progression and alter chromosome segregation in a strain-independent manner

Lisbeth Rojas-Barón*, Carlos Hermosilla, Anja Taubert and Zahady D. Velásquez

Institute of Parasitology, Biomedical Research Center Seltersberg, Justus Liebig University Giessen, Giessen, Germany

Toxoplasma gondii is an obligate intracellular parasite that modulates a broad range of host cell functions to guarantee its intracellular development and replication. *T. gondii* includes three classical clonal lineages exhibiting different degrees of virulence. Regarding the genetic diversity of *T. gondii* circulating in Europe, type II strains and, to a lesser extent, type III strains are the dominant populations, both in humans and animals. Infections with the type I strain led to widespread parasite dissemination and death in mice, while type III is considered avirulent. Previously, we demonstrated that primary endothelial cells infected with the *T. gondii* RH strain (haplotype I) were arrested in the G2/M-phase transition, triggering cytokinesis failure and chromosome missegregation. Since *T. gondii* haplotypes differ in their virulence, we here studied whether *T. gondii*-driven host cell cycle perturbation is strain-dependent. Primary endothelial cells were infected with *T. gondii* Me49 (type II strain) or NED (type III strain), and their growth kinetics were compared up to cell lysis (6–30 h p. i.). In this study, only slight differences in the onset of full proliferation were observed, and developmental data in principle matched those of the RH strain. FACS-based DNA quantification to estimate cell proportions experiencing different cell cycle phases (G0/1-, S-, and G2/M-phase) revealed that Me49 and NED strains both arrested the host cell cycle in the S-phase. Cyclins A2 and B1 as key molecules of S- and M-phase were not changed by Me49 infection, while NED infection induced cyclin B1 upregulation. To analyze parasite-driven alterations during mitosis, we demonstrated that both Me49 and NED infections led to impaired host cellular chromosome segregation and irregular centriole overduplication. Moreover, in line with the RH strain, both strains boosted the proportion of binucleated cells within infected endothelial cell layers, thereby indicating enhanced cytokinesis failure. Taken together, we demonstrate that all parasite-driven host cell cycle arrest, chromosome missegregation, and binucleated phenotypes are *T. gondii*-specific but strain independent.

KEYWORDS

***Toxoplasma gondii*, haplotypes, Me49 strain, NED strain, cell cycle arrest, cell cycle dysregulation**

1 Introduction

Toxoplasma gondii is a major zoonotic obligate intracellular apicomplexan parasite and the etiological agent of toxoplasmosis, which may cause harmful effects mainly in pregnant and immunocompromised hosts. *T. gondii* modulates a broad range of host cell functions to guarantee its intracellular development and replication (Velásquez et al., 2019; Fernández-Escobar et al., 2022). As a polyxenous and cosmopolitan zoonotic parasite, *T. gondii* can infect all warm-blooded animals as intermediate hosts (humans, domestic and wild mammals, and birds) and domestic and wild felines as definitive hosts (Gubbels et al., 2008; White and Suvorova, 2018). Hence, its life cycle is a complex transmission process, and detailed molecular knowledge is of importance not only for public health but also for the livestock industry and wildlife management programs (Calero-Bernal et al., 2022).

Worldwide studies have shown that *T. gondii* possesses significant genetic and phenotypic diversity; at present, three main lineages (types I–III) are described, which vary in virulence and mortality for laboratory mice (Fernández-Escobar et al., 2021). Concerning the genetic diversity of *T. gondii* strains circulating in Europe, type II strains and, to a lesser extent, type III strains are the dominant populations, both in humans and animals (Khan et al., 2007; Lorenzi et al., 2016; Fernández-Escobar et al., 2022). The *T. gondii* type I strain is classified as highly virulent, leading to widespread parasite dissemination and lethal infection in mice (100% cumulative mortality). In contrast, mouse mortality and tachyzoite dissemination induced by type II or III strains are considerably lower (30%), with type III strains generally being considered avirulent for mice (Sibley and Boothroyd, 1992; Su et al., 2002; Dardé et al., 2014; Calero-Bernal et al., 2022). The main representatives for each lineage are *T. gondii* RH and GT1 for haplotype I, PRU and Me49 for haplotype II, and CEP and NED for haplotype III (Wu et al., 2022). However, the most studied strain in *in vitro* research is the *T. gondii* RH strain, while the NED strain is commonly used in murine *in vivo* models (Croken et al., 2014; Wang and Sibley, 2020).

It is well known that apicomplexan coccidian parasites extensively modulate their host cells to guarantee successful intracellular development and proliferation. As such, these parasites were reported to affect numerous host cellular processes, such as apoptosis, autophagy, cytoskeleton, metabolism, immune reactions, and cell cycle (Alberts et al., 2007; Dardé et al., 2014; Velásquez et al., 2019). Referring to the latter, the *T. gondii* RH strain arrested the host cell cycle in S-phase or at G2/M-phase transition, which was accompanied by a binucleated host cell phenotype and cytokinesis failure (Molestina et al., 2008; Velásquez et al., 2019). Moreover, RH strain-infected primary endothelial host cells showed supernumerary centrosomes in mitotic spindles, a displacement of single chromosomes from the equatorial plane, and dramatic chromosome missegregation errors (Velásquez et al., 2019). In mammals, the cell cycle is tightly regulated by several cell cycle-dependent cyclins and cyclin-dependent protein kinases (CDKs) that control cell cycle progression from G0/1 to M-phase (Alberts et al., 2007). During cell cycle, the formation of centrioles, the mitotic spindle, and the arrangement of chromosomes are also highly controlled to guarantee the correct genetic information to be inherited by daughter cells (Miettinen et al., 2019). Even tiny errors in these cellular processes might result in genome/chromosome instability or even cell death (Alberts et al., 2007). Former data on *T. gondii* RH infections stated cell type-dependent variations since human foreskin fibroblasts (HFFs) were arrested at the G2-to-M-boundary, while human trophoblast cells, human dermal fibroblasts, and L6 rat myoblasts showed stasis in G2-phase (Brunet et al., 2008; Molestina et al., 2008; Kim et al., 2016). In these studies G2-phase arrest was linked to cyclin B1 downregulation, while other G2/M-phase checkpoint-related molecules, such as p53, p21, and CDK1, were not changed in expression (Brunet et al., 2008; Velásquez et al., 2019). We recently demonstrated that *T. gondii* RH strain-infected primary endothelial cells experienced aberrant mitosis with supernumerary centrosome (and centriole) formation, resulting in impaired cytokinesis (Velásquez et al., 2019). Given that these data referred to haplotype I-driven host cell modulation, we aimed to determine whether cell cycle-related *T. gondii*-driven effects were haplotype/strain-dependent. For direct comparison and to avoid cell type-driven effects, *in vitro* infections with *T. gondii* haplotypes II and III (i.e., Me49 and NED strain, respectively) were performed in the same bovine primary endothelial host cell (BUVEC) as previously used for RH strain.

2 Materials and methods

2.1 Primary bovine umbilical vein endothelial cell isolation and culture

Primary bovine umbilical vein endothelial cells BUVEC were isolated from umbilical veins obtained from calves born by *section caesarea* at the Justus Liebig University in Giessen, Germany. Umbilical cords were maintained at 4°C in sterile 0.9% HBSS-HEPES buffer (pH 7.4; Gibco, Grand Island, NY, United States) supplemented with 1% penicillin (500 U/mL; Sigma, St. Louis, MO, United States) and streptomycin (500 µg/mL; Sigma) for a maximum of 12 h before use. Isolation of endothelial cells was performed by using 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in Pucks solution (Gibco) and infused into the lumen of ligated umbilical veins for 20 min at 37°C in a 5% CO_2 atmosphere. After gently massaging umbilical veins, the cell suspension was collected in medium and supplemented with 1 mL of fetal calf serum (FCS; Gibco) to inactivate collagenase. After two washes (350 x *g*, 12 min, RT), cells were resuspended in complete endothelial cell growth medium (ECGM, PromoCell, supplemented with 10% FCS), plated in 25 cm^2 tissue plastic culture flasks (Greiner), and incubated at 37°C and 5% CO_2 atmosphere. BUVEC monolayers were cultured in modified ECGM medium [ECGM, diluted at 30% in M199 medium, supplemented with 5% FCS (Greiner) and 1% penicillin and streptomycin] with medium changes every 2–3 days. All biological isolates were used for *in vitro* experiments at a maximum of four passages, as previously described in Velásquez et al. (2019). Experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], the Institutional Ethics Commission of Justus Liebig University of Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU.

2.2 Parasite maintenance

Tachyzoites of *T. gondii* Me49 and NED strains were maintained by serial passages in MARC-145 (African green monkey kidney epithelial cells) using DMEM medium (D6429, Sigma) supplemented with 5% FCS, 1% penicillin (500 U/mL; Sigma St. Louis, MO, United States), and streptomycin (500 µg/mL; Sigma). The number of passages for MARC-145 cells and *T. gondii* tachyzoites was controlled to compare our previous results on RH strains with those described here (Velásquez et al., 2019). *T. gondii* tachyzoites were obtained by monolayer scraping and centrifugation (400 x *g*, 1 min) to remove cell debris. A second centrifugation step was performed to sediment the parasites at 800 x *g* for 12 min. Tachyzoites were counted in a Neubauer chamber, suspended in a modified ECGM medium, and used for BUVEC infections.

2.3 Kinetics of *Toxoplasma gondii* infections

Confluent BUVEC layers ($n = 3$) were infected with *T. gondii* Me49 or NED tachyzoites (MOI 1:2) and incubated at 37°C in a 5% CO_2 atmosphere. By counting the number of tachyzoites per parasitophorous vacuole (PV) every 6 h up to 30 h p. i., parasite infection kinetics in the same primary endothelial cell isolates were analyzed to estimate Me49- or NED-specific division cycles. Therefore, cells were fixed at each time point with 4% paraformaldehyde for 15 min at room temperature (RT) and then washed three times with 1X PBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, and 12 mM total phosphate in the form of hydrogen phosphate and dihydrogen phosphate). All fixed cells were stored at 4°C until further use. To assess parasite development in endothelial host cells, cell nuclei, and tachyzoites were labeled with 4′,6′-diamidin-2-phenyllindol (DAPI) and a specific *T. gondii* antibody, respectively (Table 1), allowing us to count the total number of tachyzoites/PV formed during infection.

2.4 Immunofluorescence assays

Three BUVEC isolates were seeded in 12-well plates with coverslips precoated with fibronectin (1:400, Sigma-Aldrich, F1141-2MG) and infected either with *T. gondii* Me49 or NED tachyzoites at sub-confluency (MOI 1:2). At 24 h p. i., all samples were fixed in 4% paraformaldehyde (15 min, RT) and washed three times in sterile PBS. The samples were incubated in a blocking/permeabilization solution (PBS with 3% BSA and 0.3% Triton X-100) for 1 h at RT. Thereafter, they were incubated in primary antibody solutions (Table 1) at 4°C in a humidified chamber overnight. The samples were then washed three times with 1X PBS and incubated in secondary antibody solutions (Table 1) for 30 min at RT and darkness. Host cell nuclei were labeled with DAPI present in the mounting medium solution (Fluoromount G-DAPI, Thermo Fisher, cat. Number 495952). The samples were analyzed with ReScan Confocal instrumentation (RCM 1.1 Visible, Confocal.nl) combined with a Nikon Eclipse Ti2-A inverted microscope.

2.5 Protein extraction

Six BUVEC isolates were infected with either *T. gondii* Me49 or NED tachyzoites (MOI 1:2). At 24 h p. i., cells were washed with 1X PBS buffer, detached from the plate using trypsin/EDTA solution [0.25% (w/v) Trypsin; 0.53 mM EDTA, 37°C, 5 min], and pelleted (400 *x g*, 5 min). The cell pellet was washed with 1X PBS buffer and resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF; all Roth) supplemented with a protease inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate tyrosine phosphatase inhibitor (Abcam, ab120386), and 1 mM phenylmethylsulphonyl fluoride, a serine protease inhibitor (Abcam, ab141032). Protein extracts were sonicated for five cycles of 20 s sonication and 20 s resting and then centrifuged (10,000 x *g*, 10 min, 4°C) to sediment intact cells, membranes, and nuclei. The supernatants were analyzed for protein content via BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, cat. Number 23225) following the

TABLE 1 Primary and secondary antibodies used in the current study.

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies				
T. gondii	Thermo Fisher	PA1-7256	Goat	1:100
γ-Tubulin	Abcam	Ab1795030	Rabbit	1:100
Vinculin	Santa Cruz	sc-73614	Mouse	1:1000
Cyclin A2	Abcam	Ab38	Mouse	1:1000
Cyclin B1	Abcam	Ab32053	Rabbit	1:3000

Antigen/Conjugate	Company	Cat. number	Host/target	Dilution
Secondary antibodies				
Alexa Fluor 594	Thermo Fisher	A-21468	Goat	1:500
Alexa Fluor 647	Thermo Fisher	A-21244	Rabbit	1:500
Goat anti-mouse IgG Peroxidase conjugated	Pierce	31430	Goat/mouse	1:40,000
Goat anti-rabbit IgG Peroxidase conjugated	Pierce	31460	Goat/rabbit	1:40,000

manufacturer's instructions. Sample analysis was performed on a Varioskan plate reader, measuring the absorbance at 562 nm.

2.6 SDS-PAGE and immunoblotting

Protein extracts were diluted in loading buffer with 6 M urea (10% SDS, 12.5% 2-mercaptoethanol, 25% glycerol, 150 mM Tris–HCl pH 6.8) and boiled at 95°C for 5 min. Samples (40 µg protein/slot) were loaded on 12% polyacrylamide gels and subjected to SDS-PAGE electrophoresis (100 V; approx. 1.5 h; Bio-Rad). Proteins were transferred to PVDF membranes (Millipore) (300 mA, 2 h) in a wet-tank transfer system and then blocked for 1 h at RT [3% BSA in TBS buffer (50 mM Tris–HCl, 150 mM NaCl; pH 7.6)]. Afterwards, proteins on membranes were incubated in primary antibodies (4°C, overnight) directed against cyclin A2, cyclin B1, and vinculin (Table 1) and diluted in blocking solution (TBS buffer, 0.1% Tween-20, 3% BSA). Vinculin detection was used as a loading control for sample normalization. After primary antibody probing, membranes were washed three times with TBS-Tween (0.1%) and incubated in secondary antibody solutions (Table 1) for 30 min at RT. After three washings (TBS-Tween 0.1%), protein detection was performed using a chemiluminescence detection system (ECL Prime, Amersham). Images were taken using the INTAS Science Imaging Instrument and the INTAS ChemoStar Imager software. A protein ladder was used to estimate protein sizes (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific). Protein band intensities were analyzed using the Fiji Gel Analyzer plugin (Schindelin et al., 2012).

2.7 Flow cytometry-based analysis of cell cycle phases

For flow cytometry (FACS)-based analysis of the cellular DNA content, non-infected and infected host cell layers ($n = 6$) were washed with 1X PBS buffer and detached from the plate by trypsin/EDTA (0.25%) treatments at 37°C for 5 min. Then, cells were pelleted at 400 x *g* for 5 min. The cell pellet was resuspended, washed in 1X PBS buffer, and centrifuged at 400 x *g* for 5 min. Thereafter, cells were fixed with ice-cold absolute ethanol for 3 min at −20°C and pelleted again (400 x *g*, 5 min). Samples were stained with the commercial FxCycle PI/RNAse kit following the manufacturer's instructions (Thermo Fisher, F10797). The samples were analyzed by an Accuri C6 Plus Flow Cytometer analyzer (Becton-Dickinson, Heidelberg, Germany) applying 535/5 nm excitation and emission collected in a 617/20 band-pass. The cells were gated according to their size and granularity. Data analysis was performed via FlowJo LLC software (Ashland, OR).

2.8 Image acquisition and reconstruction

Fluorescence images were acquired with a ReScan Confocal instrumentation (RCM 1.1 Visible, Confocal, The Netherlands) equipped with a fixed 50 µm pinhole and combined with a Nikon Eclipse Ti2-A inverted microscope with a motorized Z-stage (DI1500, Nikon). The RCM unit was connected to a Toptica CLE laser with the following excitation modes: 405/488/561/640 nm. Images were taken via an sCMOS camera (PCO edge) using a CFI Plan Apochromat X60 lambda-immersion oil objective (NA 1.4/0.13; Nikon). The instrument was operated by the NIS-Elements software (version 5.11). Images were acquired via a z-stack optical series with a step size of 0.1 microns to cover all structures of interest. To estimate both the total number of cells and the number of binucleated cells present in one cell layer, all images were first segmented using the Otsu thresholding algorithm. Identical brightness and contrast conditions were applied for each data set within one experiment. The total number of cells was obtained using the Fiji plugin "Analyzes particles" with a size of 10 µm (Schindelin et al., 2012).

2.8.1 Mitosis quantification

The percentage of cells that underwent mitosis was calculated by normalizing it with the total number of cells in each field of view. Mitosis was evaluated from the prophase until the telophase. Cytokinesis was evaluated in terms of the percentage of binucleated cells. The prophase was defined as the chromosome condensation in the nuclear region using DAPI staining of the chromosomes. Metaphase cells were those with all chromosomes in the equatorial line, and anaphase cells were when chromosomes left the central plane to migrate into each mitosis pole. Cells in anaphase with a DAPI-positive signal in between were identified as chromosome bridges. Finally, cells in telophase were counted when chromosomes had completely migrated to each mitosis pole.

2.9 Statistical analysis

The data were expressed as the mean ± SD of independent experiments. For cell number- and FACS-based experiments, one-way analysis of variance (non-parametric ANOVA) with Kruskal-Wallis post-test was performed using GraphPad Prism 9.3.1 software, applying a significance level of 5%. For immunoblot-based analyses, unpaired two-tailed *t*-tests were performed comparing controls vs. infected cells, with a 95% confidence interval. All graphs and statistical analyses were performed using GraphPad Prism 9 software.

3 Results

3.1 *Toxoplasma gondii* Me49 and NED intracellular development show comparable kinetics in primary endothelial cells

The current aim was to evaluate if variable *T. gondii* strains may differentially affect the host cell cycle during tachyzoite intracellular development. Given that diverse *T. gondii* haplotypes bear different virulence in the murine system, which may be linked to varying speed in development and cell lysis, we first analyzed the developmental characteristics of Me49 and NED strains (haplotypes II and III, respectively) in primary BUVEC layers. The rationale to choose a primary bovine endothelial cell type as host cells was: (i) to avoid immortalization-driven effects on cell cycle regulation as commonly reported for permanent tumor-based cell lines, (ii) to be as close as possible to the *in vivo* scenario, and (iii) to perform current studies in exactly the same cell type as reported before for the *T. gondii* RH strain [haplotype I, (Velásquez et al., 2019)] thereby allowing for direct data

10.3389/fmicb.2024.1336267

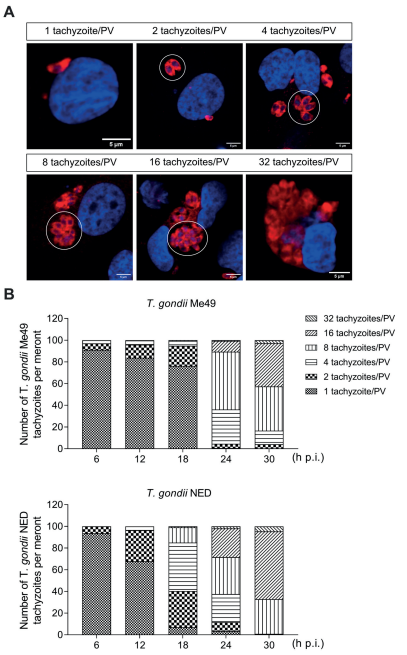


FIGURE 1
Toxoplasma gondii Me49 and NED tachyzoite replication kinetics in primary bovine umbilical vein endothelial cells (BUVEC). BUVEC isolates ($n = 3$) were simultaneously infected at subconfluence with either Me49 or NED tachyzoites (MOI 1:2) and monitored for parasite proliferation by analyzing the numbers of tachyzoites/parasitophorous vacuole (PV, white circle) from 6 to 30 h p. i. **(A)** Representative illustration of different stages of proliferation. Host cell nuclei were stained by DAPI (blue) and tachyzoites by *T. gondii*-specific antibodies (red). **(B)** Estimation of the proliferation status of Me49 and NED strains at 6, 12, 18, 24 and 32 h p. i. The scale bar represents 5 µm.

comparison. Therefore, BUVEC and MARC-145 passages, as well as *T. gondii* tachyzoites, were carefully controlled.

To study the kinetics of parasite development, identical BUVEC isolates were simultaneously infected with tachyzoites of *T. gondii* Me49 and NED strains, and the total intracellular development of each strain was thoroughly assessed by counting the number of tachyzoites per PV every 6 h from 6 to 30 h p. i. (Figure 1). Given that the host cells used in the current study were of primary origin and therefore not immortalized, three biological isolates at a maximum of four passages were used to avoid potential age-dependent changes in cell division times and further to ensure preservation of the endothelial phenotype. All host cells were seeded and infected at the same time, using the same batch of tachyzoites from each strain. A rosette was defined as when the PV contained 32 tachyzoites. All samples were analyzed before 32 h p. i. to avoid cell lysis-driven artifacts.

When analyzing in total 895 and 770 single infected host cells for NED and Me49 strains, respectively, at 24 h p. i., the overall infection rates differed moderately but not substantially (Me49: 81.9 ± 3.1%; NED: 70.1 ± 7.7%). The onset of tachyzoite division revealed equal in both strains at 6 h p. i. 2.5% of infected BUVEC showed two tachyzoites per PV for both strains (Figures 1A,B). However, at 12 and 18 h p. i., the NED strain proceeded slightly faster in development than the ME49 strain, since a higher proportion of PV contained four and eight tachyzoites (ME49: 1.3 and 5.7%, respectively; NED: 2 and 16.9%, respectively) (Figures 1A,B). Nevertheless, towards 30 h p. i., the NED strain caught up in development, thus resulting in a comparable proportion of PV with 32 tachyzoites (ME49: 1.4%; NED: 1%) (Figures 1A,B). Of note, BUVEC lysis started at comparable time points at 33 h p. i., thereby denying any relevant differences in the *in vitro* virulence of these two *T. gondii* strains. Based on these overall findings, it appeared eligible to perform cell cycle-related experiments on both strains at the same time points after parasite infection.

3.2 *Toxoplasma gondii* Me49 and NED tachyzoite infections both induce binucleated host cells and affect centriole formation

It was previously described that *T. gondii* RH strain infection in BUVEC layers resulted in an enhanced proportion of bi/multi-nucleated host cells (thereby indicating cytokinesis failure) and an alteration of mitosis progression by inducing supernumerary centrosome formation and chromosome segregation errors (Molestina et al., 2008; Velásquez et al., 2019). In this study, we intended to study whether these findings also applied to other *T. gondii* haplotypes (i.e., Me49 and NED). Therefore, the number of bi/multi-nucleated host endothelial cells (i.e., with ≥ 2 nuclei per cell) was counted and normalized against the total number of cells present in the field of view. As depicted in Figure 2A, both Me49 and NED infections of BUVEC induced a significantly enhanced proportion of bi/multi-nucleated host cells (Me49: 12.7%; NED: 8.1%), while only 0.7% of non-infected cells revealed the binucleated phenotype. The mitotic rate was also analyzed by estimating the total number of cells in mitosis vs. the total number of cells in the field of view. The results showed that only the NED strain reduced statistically significant mitosis percentages (Figure 2B). To verify whether mitotic failures may be linked to inadequate mitotic spindle and centrosome formation, we additionally stained chromosomes with DAPI (blue) and centrosomes with γ-tubulin (green) (Figure 2C) and analyzed the different phases of mitosis in *T. gondii* Me49- and NED-infected BUVEC. The γ-tubulin staining was used as a centrosome marker to visualize the mitosis spindle localization. As expected, most of the mitotic cells displayed a normal mitosis progression with only two centrosome poles. However, approximately 10% of infected mitotic cells showed an altered chromosome arrangement (Figure 2C, prophase and metaphase). A low number of host cells displayed chromosome bridges between the two poles of the mitotic spindle (Figure 2C, asterisks, and Supplementary Video S2). Interestingly, the centrosomes seem to be composed of more than one spot (Figure 2C, and Supplementary Video S1). To corroborate this observation, we drew a line over the centrosomes in mitotic cells, and we plotted the information in a histogram. The results showed that in most of the cases, the histogram exhibited two brightness dots, suggesting a possible centriole duplication at each centrosome (Figure 2C, histogram plots, and Supplementary Video S1).

10.3389/fmicb.2024.1336267

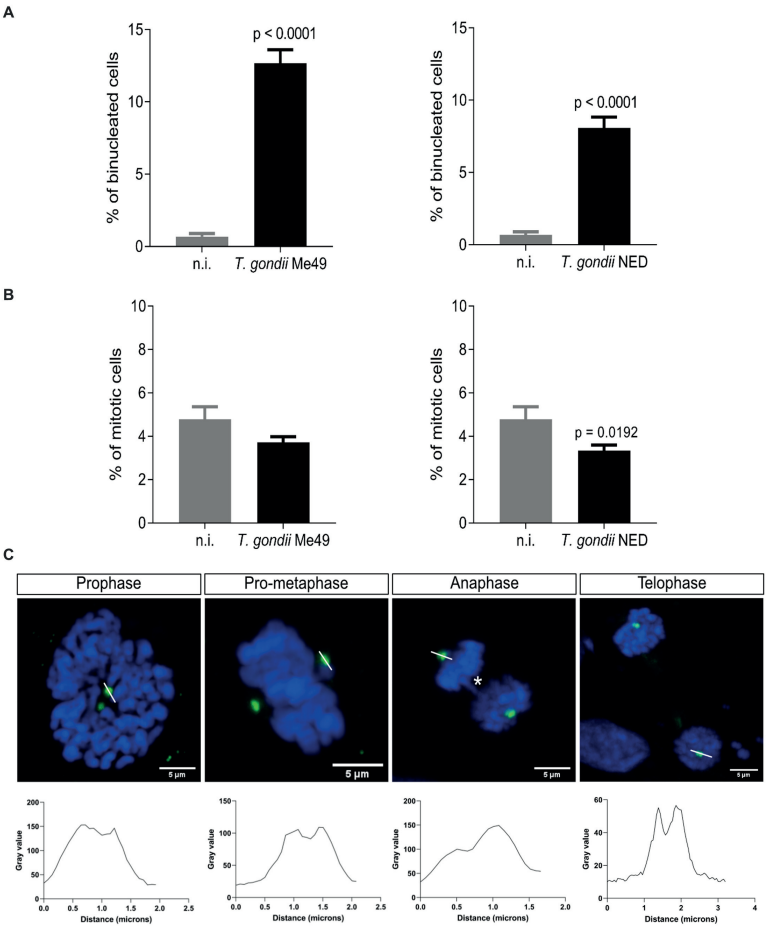


FIGURE 2
Toxoplasma gondii Me49 and NED infections induce a binucleated phenotype and centriole overduplication in infected host cells. Subconfluent BUVEC isolates ($n = 3$) were infected with either Me49 or NED tachyzoites at an MOI 1:2. After 24 h p. i., samples were stained for DNA/chromosomes (DAPI, blue) and centrioles (γ-tubulin, green). **(A)** Percentages of host cells with a binucleated phenotype. **(B)** Mitotic rate (cells undergoing mitosis/total number of cells) of *T. gondii*-infected BUVEC and control cells. **(C)** Exemplary illustration of the main aberrant mitotic structures observed for both Me49 and NED strain infections. Additionally, the intensity of centriole-related signals was assessed and plotted as a graph showing intensity value vs. distance (white line). Yellow arrows: centriole overduplication; asterisks: chromosome bridge. The scale bar represents 5 µm.

3.3 *Toxoplasma gondii* ME49 and NED infections both promote host cell cycle arrest in S-phase

To analyze whether *T. gondii* Me49 or NED strains cause dysregulation of the host cell cycle progression in BUVEC, a FACS-based analysis was performed to estimate total DNA content. The cell population was gated according to their size and granularity, and PI-based DNA staining was used to define distinct categories of cell cycle phases (G0/1, S, and G2/M) according to the DNA amount per cell (Figure 3A). In this classical method, the first DNA peak is assigned to G0/1-phase, the second peak represents cells in G2/M-phase, and the cell population between both peaks corresponds to cells in the S-phase (Figure 3A). Here, *T. gondii* Me49- and NED-infected cells both showed a significant decrease of cells in G0/1-phase when compared to non-infected cells. Simultaneously, the proportion of *T. gondii*-infected cells in the S-phase increased, thereby suggesting a parasite-driven host cell arrest in the S-phase (Figure 3B).

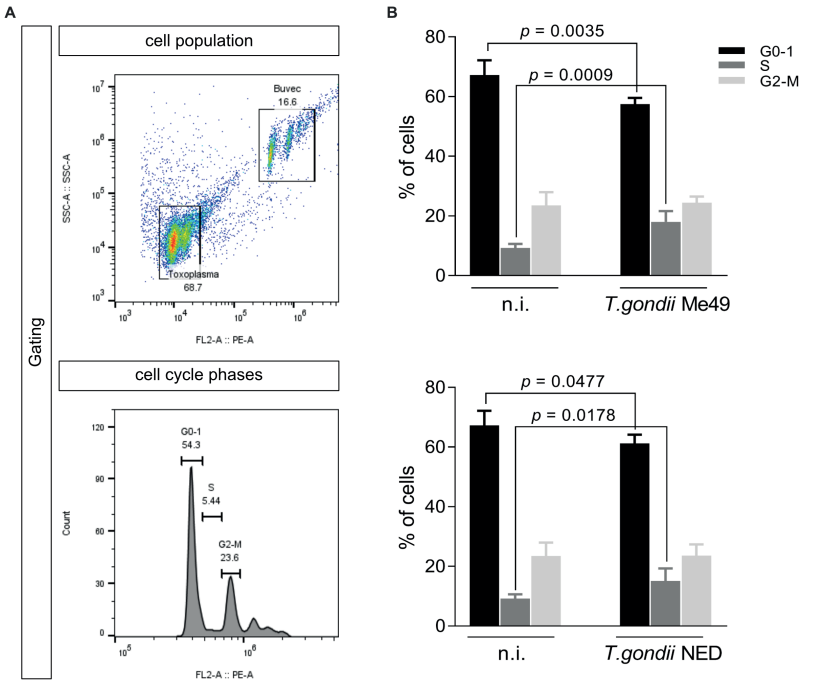


FIGURE 3
Toxoplasma gondii Me49 and NED infections arrest the host cell cycle in S-phase. Subconfluent BUVEC isolates ($n = 6$) were infected with either Me49 or NED tachyzoites at an MOI 1:2 and evaluated for DNA content at 24 h p. i. **(A)** Exemplary flowchart of FACS analysis showing the total number of host cells in G0-1 (one genomic DNA copy), G2- (two genomic copies), and S-phase (DNA synthesis, the cell population in between both phases). The number of cells positive for DNA staining (PI) was graphed as a histogram to obtain the total number of cells in each peak. **(B)** Percentages of Me49- and NED-infected BUVEC and control cells in each cell cycle phase were estimated via FACS-based DNA quantification. Bars represent the median ± SD.

Since cellular DNA content-based analyses do not allow for discrimination between all single phases (such as G0/G1 or G2/M) and based on current data suggesting that *T. gondii*-infected host cells accumulate in the S-phase, we additionally analyzed the expression of cyclins A2 and B1 (Figure 4), which signify key regulatory proteins of S-phase control and of M-phase enter and progression, respectively. Western blotting analyses of six ME49- and NED-infected BUVEC isolates showed that only the NED strain induces cyclin B1 overexpression (Figure 4B). Cyclin A2 expression was not affected by any of the strains (Figures 4A,B).

4 Discussion

In a recent study, we demonstrated that *T. gondii* tachyzoites of haplotype I (RH strain) induced host cell cycle arrest, chromosome missegregation, multipolar spindle, and cytokinesis failure concomitant with an increased percentage of binucleated primary host endothelial cells *in vitro* (Velásquez et al., 2019). To assess eventual strain-driven effects, we compare our previous published data with infections using two other *T. gondii* haplotypes, II and III (Me49 and NED, respectively). We evaluated the host cell cycle control, progression, and mitosis after 24 h h p. i. with *T. gondii* tachyzoites Me49 and NED. To be able to compare our results with previously published data, the same host cell type was used and parasite strain passages were tightly controlled. Furthermore, we followed the same experimental approach used in Velásquez et al. (2019).

To our best knowledge, available data on *T. gondii*-driven host cell cycle modulation currently refer to haplotype I tachyzoites (RH strain) and indicate that this clonal lineage might control host cell cycle progression to ease its intracellular asexual development (Brunet et al., 2008; Molestina et al., 2008; Velásquez et al., 2019; Wong et al., 2020; Pierre-Louis et al., 2022). Since several typical and atypical clonal lineages of *T. gondii* occur worldwide and show variable pathogenicity (Dardé et al., 2014; Miller et al., 2023), we aimed to compare recent RH data (haplotype I) (Velásquez et al., 2019) with haplotypes II and III (Me49 and NED, respectively) by infecting the same host cell type. In our hands, the replication times of the ME49 and NED strains in BUVEC proved comparable to those of the RH strain, allowing us to use this primary cell culture system for comparative approaches. Our

10.3389/fmicb.2024.1336267

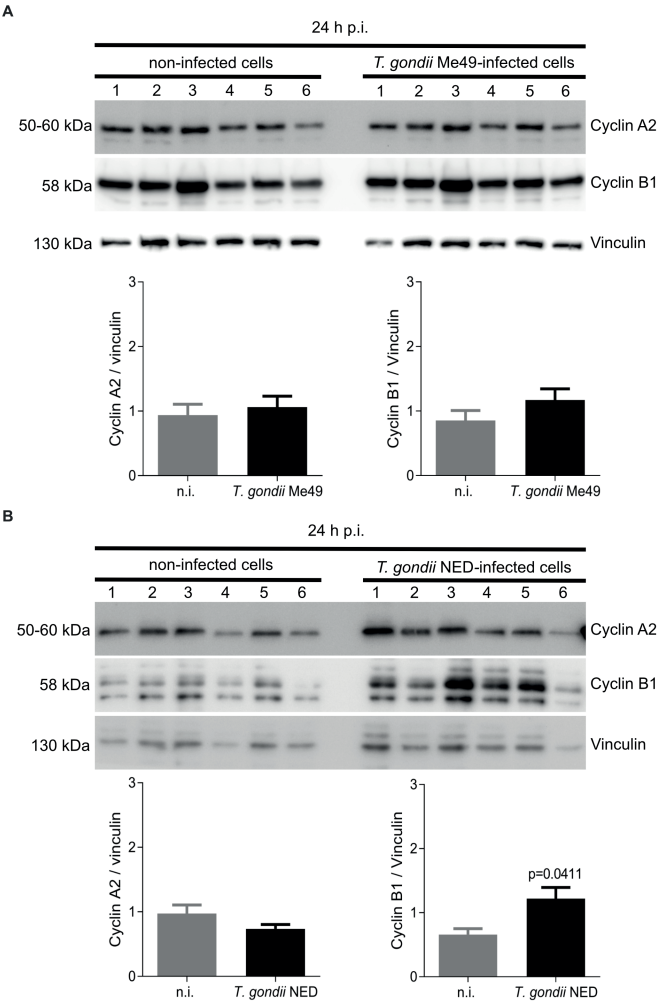


FIGURE 4
Cyclin A2 and cyclin B1 expression in *Toxoplasma gondii* Me49 and NED strain-infected BUVEC. Confluent BUVEC isolates ($n = 6$) were infected with either Me49 **(A)** or NED **(B)** tachyzoites. At 24 h p. i., protein extracts were analyzed by Western blotting to estimate cyclin A2 (indicative for S-phase) and cyclin B1 (mitosis marker) expression. Densities of protein signals were quantified and graphed as a relative ratio to vinculin expression (loading control). Bars represent the median ± SD of six biological replicates.

results showed that the NED strain reduced the mitosis rate and led to an overexpression of cycin B1, in contrast to the Me49 strain. This suggests that the NED strain modulates the host cell cycle throughout the mitosis checkpoint, while ME49 and RH appear to modulate whitin S-phase progression itself. Since neither *T. gondii* RH nor ME49 controls the mitosis checkpoint cyclin, even though both arrest the host cell cycle, *T. gondii* tachyzoite has evolved into several clonal lineages, with the most prevalent ones being types I, II, and III. While all lineages have the potential to infect both humans and animals, types I and II are more prevalent in immunocompromised or pregnant humans than in animals. Interestingly, type III primarily infects animals as opposed to people (Howe and Sibley, 1995). In our findings, the NED strain was the one to have distinct behavior about the cell cycle and mitosis control. Therefore, this slight modification may suggest that the NED

strain evolved into a better-intermediated host adaptation, modifying the type of the host cell cycle and mitosis control. However, further experiments need to be done in order to answer this hypothesis.

Referring to parasite-mediated modulation of cell cycle progression, the current data showed that both NED and ME49 strains induced host cell cycle arrest in S-phase, but they differed in the control, thereby denying any haplotype-dependent reactions. Interestingly, S-phase arrest has been studied by other groups worldwide, suggesting that arrested cells were not able to incorporate new DNA molecules (Pierre-Louis et al., 2022). Given that *T. gondii* arrests the host cell cycle equally in primary, immortalized, and tumor cells, we could suggest that it is a parasite strategy that has been maintained throughout haplotype evolution (Brunet et al., 2008; Molestina et al., 2008; Kim et al., 2016; Velásquez et al., 2019; Pierre-Louis et al., 2022). The mitosis progression was shown to be modulated differently in NED- or ME49-infected cells compared to previous data published on the RH strain since infected cells displayed multipolar spindle formation (Velásquez et al., 2019). In this study, we showed that neither ME49 nor NED strains induce multipolar spindles. However, the centrosome poles seem to have more than one spot of γ-tubulin, suggesting possible centriole duplication. This phenotype was also observed in the RH strain infection, but it always came with a multipolar phenotype. Altogether, this suggests that *T. gondii* modulation of the centriole number can be independent of the strain type, but the multipolar spindle formation may only be induced by the RH strain.

During the process of chromosome segregation and mitotic spindle formation, several specific proteins are needed, such as histones and cohesins, to deliver structural support for chromosomes and to bind sister chromatids to ensure an equal distribution between daughter cells during cell division (Kline-Smith and Walczak, 2004; Mariño-Ramírez et al., 2005; Makrantoni and Marston, 2018). The loss or gain of chromosomes drives chromosomal errors and triggers intracellular pathways that arrest the cell cycle to either repair the damage or eliminate potential aneuploid cells by apoptotic death (Alberts et al., 2007; Levine and Holland, 2018). The same applies to irregular chromosome bridges, which were found here to be induced by *T. gondii* Me49 and NED strains and that have been correlated with DNA damage (Ganem and Pellman, 2012). As described by Pampalona et al. (2016), chromosome bridges are characteristic of tumor cells and are mainly observed in mid-late anaphase, eventually persisting throughout mitosis, but are atypical for the early G1 phase. Since the endothelial host cells used in the current study are of primary and non-tumoral origin and, consequently, should follow physiological cell cycle control, chromosome bridge-related findings should be associated with *T. gondii* infection. However, considering that chromosome bridging is better monitored by live-cell imaging, future experimentation will focus on this method to reliably correlate these findings with *T. gondii*-mediated effects.

In line with recent data on the *T. gondii* RH strain (Velásquez et al., 2019), we demonstrated that ME49 and NED infections of BUVEC also induced an increased percentage of binucleated cells, which directly correlates with enhanced cytokinesis failure. Cytokinesis represents the last pivotal step of cell division. Hence, cytokinesis includes cytoplasmic division finally giving rise to two daughter cells, even when some exceptions have been described in the early embryonic stages of the fruit fly model *Drosophila* (Schejter and Wieschaus, 1993; Kiseleva et al., 2001). In mammals, megakaryocytes (blood platelets), hepatocytes, and heart muscle cells perform nuclear division without cytokinesis, leading to a high proportion of multi-nucleated cells (Nagata et al., 1997; Ahuja et al., 2007; Alberts et al., 2007; Margall-Ducos et al., 2007; Lordier et al., 2008). However, the endothelium of vessels *in vivo* physiologically does not include binucleated phenotypes, therefore evidencing that our findings are directly correlated with *T. gondii* infections. Given that—with the current study—this phenotype is now described for haplotypes I–III *in vitro*, binucleated phenotypes and cytokinesis failure may be considered a specific hallmark of *T. gondii* tachyzoite replication. Whether this phenomenon is indeed not linked to strain virulence seems likely but should be tested *in vivo* for further clarification.

Taking all the data together, we can suggest that host cell cycle modulation, chromosome segregation, and cytokinesis failure are intrinsic mechanisms of *T. gondii* tachyzoite infection and are independent of the parasite haplotype or virulence.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], Institutional Ethics Commission of Justus Liebig University of Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LR-B: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. CH: Funding acquisition, Resources, Writing – review & editing. AT: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. ZV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors would like to thank all members of AT lab for helpful discussions on the current study, as well as all the

technical staff of the Institute of Parasitology. The authors thank A. Wehrend (Clinic for Obstetrics, Gynecology, and Andrology of Large and Small Animals, Justus Liebig University, Giessen, Germany) for the continuous supply of bovine umbilical cords and Dr. Gereon Schares (Friedrich Loeffler Institute, FLI, Germany) for providing the *T. gondii* Me49 and NED tachyzoites.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1336267/full#supplementary-material

References

Ahuja, P., Sdek, P., and MacLellan, W. R. (2007). Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol. Rev.* 87, 521–544. doi: 10.1152/physrev.00032.2006

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2007). "The cell cycle" in *Molecular biology of the cell 5th edition, Garland Science,* (New York: W.W. Norton & Company).

Brunet, J., Pfaff, A. W., Abidi, A., Unoki, M., Nakamura, Y., Guinard, M., et al. (2008). *Toxoplasma gondii* exploits UHRF1 and induces host cell cycle arrest at G2 to enable its proliferation. *Cell. Microbiol.* 10, 908–920. doi: 10.1111/j.1462-5822.2007.01093.x

Calero-Bernal, R., Fernández-Escobar, M., Katzer, F., Su, C., and Ortega-Mora, L. M. (2022). Unifying virulence evaluation in *Toxoplasma gondii*: a timely task. *Front. Cell. Infect. Microbiol.* 12:868727. doi: 10.3389/fcimb.2022.868727

Croken, M. M., Ma, Y., Markillie, L. M., Taylor, R. C., Orr, G., Weiss, L. M., et al. (2014). Distinct strains of *Toxoplasma gondii* feature divergent transcriptomes regardless of developmental stage. *PLoS One* 9:e111297. doi: 10.1371/journal.pone.0111297

Dardé, M.-L., Ajzenberg, D., and Su, C. (2014). "Chapter 3 - molecular epidemiology and population structure of *Toxoplasma gondii*" in *Toxoplasma gondii*. eds. L. M. Weiss and K. Kim. *2nd* ed (Boston: Academic Press), 61–97.

Fernández-Escobar, M., Calero-Bernal, R., Regidor-Cerrillo, J., Vallejo, R., Benavides, J., Collantes-Fernández, E., et al. (2021). In vivo and in vitro models show unexpected degrees of virulence among *Toxoplasma gondii* type II and III isolates from sheep. *Vet. Res.* 52:82. doi: 10.1186/s13567-021-00953-7

Fernández-Escobar, M., Schares, G., Maksimov, P., Joeres, M., Ortega-Mora, L. M., and Calero-Bernal, R. (2022). *Toxoplasma gondii* genotyping: a closer look into Europe. *Front. Cell. Infect. Microbiol.* 12:842595. doi: 10.3389/fcimb.2022.842595

Ganem, N. J., and Pellman, D. (2012). Linking abnormal mitosis to the acquisition of DNA damage. *J. Cell Biol.* 199, 871–881. doi: 10.1083/jcb.201210040

Gubbels, M.-J., White, M., and Szatanek, T. (2008). The cell cycle and *Toxoplasma gondii* cell division: tightly knit or loosely stitched? *Int. J. Parasitol.* 38, 1343–1358. doi: 10.1016/j.ijpara.2008.06.004

Howe, D. K., and Sibley, L. D. (1995). *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172, 1561–1566. doi: 10.1093/infdis/172.6.1561

Khan, A., Fux, B., Su, C., Dubey, J. P., Darde, M. L., Ajioka, J. W., et al. (2007). Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. *Proc. Natl. Acad. Sci. USA* 104, 14872–14877. doi: 10.1073/pnas.0702356104

Kim, M. J., Jung, B.-K., Cho, J., Song, H., Pyo, K.-H., Lee, J. M., et al. (2016). Exosomes secreted by *Toxoplasma gondii*-infected L6 cells: their effects on host cell proliferation and cell cycle changes. *Korean J. Parasitol.* 54, 147–154. doi: 10.3347/kjp.2016.54.2.147

Kiseleva, E., Rutherford, S., Cotter, L. M., Allen, T. D., and Goldberg, M. W. (2001). Steps of nuclear pore complex disassembly and reassembly during mitosis in early Drosophila embryos. *J. Cell Sci.* 114, 3607–3618. doi: 10.1242/jcs.114.20.3607

Kline-Smith, S. L., and Walczak, C. E. (2004). Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. *Mol. Cell* 15, 317–327. doi: 10.1016/j.molcel.2004.07.012

Levine, M. S., and Holland, A. J. (2018). The impact of mitotic errors on cell proliferation and tumorigenesis. *Genes Dev.* 32, 620–638. doi: 10.1101/gad.314351.118

Lordier, L., Jalil, A., Aurade, F., Larbret, F., Larghero, J., Debili, N., et al. (2008). Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and rho/rock signaling. *Blood* 112, 3164–3174. doi: 10.1182/blood-2008-03-144956

Lorenzi, H., Khan, A., Behnke, M. S., Namasivayam, S., Swapna, L. S., Hadjithomas, M., et al. (2016). Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. *Nat. Commun.* 7:10147. doi: 10.1038/ncomms10147

Makrantoni, V., and Marston, A. L. (2018). Cohesin and chromosome segregation. *Curr. Biol.* 28, R688–R693. doi: 10.1016/j.cub.2018.05.019

Margall-Ducos, G., Celton-Morizur, S., Couton, D., Brégerie, O., and Desdouets, C. (2007). Liver tetraploidization is controlled by a new process of incomplete cytokinesis. *J. Cell Sci.* 120, 3633–3639. doi: 10.1242/jcs.016907

Mariño-Ramírez, L., Kann, M. G., Shoemaker, B. A., and Landsman, D. (2005). Histone structure and nucleosome stability. *Expert Rev. Proteomics* 2, 719–729. doi: 10.1586/14789450.2.5.719

Miettinen, T. P., Kang, J. H., Yang, L. F., and Manalis, S. R. (2019). Mammalian cell growth dynamics in mitosis. *eLife* 8:e44700. doi: 10.7554/eLife.44700

Miller, M. A., Newberry, C. A., Sinnott, D. M., Batac, F. I., Greenwald, K., Reed, A., et al. (2023). Newly detected, virulent *Toxoplasma gondii* COUG strain causing fatal steatitis and toxoplasmosis in southern sea otters (*Enhydra lutris nereis*). *Front. Mar. Sci.* 10:1116899. doi: 10.3389/fmars.2023.1116899

Molestina, R. E., El-Guendy, N., and Sinai, A. P. (2008). Infection with *Toxoplasma gondii* results in dysregulation of the host cell cycle. *Cell. Microbiol.* 10, 1153–1165. doi: 10.1111/j.1462-5822.2008.01117.x

Nagata, Y., Muro, Y., and Todokoro, K. (1997). Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *J. Cell Biol.* 139, 449–457. doi: 10.1083/jcb.139.2.449

Pampalona, J., Roscioli, E., Silkworth, W. T., Bowden, B., Genescà, A., Tusell, L., et al. (2016). Chromosome bridges maintain kinetochore-microtubule attachment throughout mitosis and rarely break during anaphase. *PLoS One* 11:e0147420. doi: 10.1371/journal.pone.0147420

Pierre-Louis, E., Etheridge, M. G., de Paula Baptista, R., Khan, A., Chasen, N. M., and Etheridge, R. D. (2022). Disruption of *Toxoplasma gondii*-induced host cell DNA replication is dependent on contact inhibition and host cell type. *mSphere* 7:e0016022. doi: 10.1128/msphere.00160-22

Schejter, E. D., and Wieschaus, E. (1993). Functional elements of the cytoskeleton in the early Drosophila embryo. *Annu. Rev. Cell Biol.* 9, 67–99. doi: 10.1146/annurev.cb.09.110193.000435

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Sibley, L. D., and Boothroyd, J. C. (1992). Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* 359, 82–85. doi: 10.1038/359082a0

Su, C., Howe, D. K., Dubey, J. P., Ajioka, J. W., and Sibley, L. D. (2002). Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. *Proc. Natl. Acad. Sci.* 99, 10753–10758. doi: 10.1073/pnas.172117099

Velásquez, Z. D., Conejeros, I., Larrazabal, C., Kerner, K., Hermosilla, C., and Taubert, A. (2019). *Toxoplasma gondii*-induced host cellular cell cycle dysregulation is linked to chromosome missegregation and cytokinesis failure in primary endothelial host cells. *Sci. Rep.* 9:12496. doi: 10.1038/s41598-019-48961-0

Wang, Q., and Sibley, L. D. (2020). Assays for monitoring *Toxoplasma gondii* infectivity in the laboratory mouse. In: *Toxoplasma gondii. Methods in Molecular Biology*. Ed. C. Tonkin. New York, NY: Humana. vol 2071. doi: 10.1007/978-1-4939-9857-9_5

White, M. W., and Suvorova, E. S. (2018). Apicomplexa cell cycles: something old, borrowed, lost, and new. *Trends Parasitol.* 34, 759–771. doi: 10.1016/j.pt.2018.07.006

Wong, Z. S., Borrelli, S. L. S., Coyne, C. C., and Boyle, J. P. (2020). Cell type- and species-specific host responses to *Toxoplasma gondii* and its near relatives. *Int. J. Parasitol.* 50, 423–431. doi: 10.1016/j.ijpara.2020.05.001

Wu, M., Liu, S., Chen, Y., Liu, D., An, R., Cai, H., et al. (2022). Live-attenuated ME49Δcdpk3 strain of *Toxoplasma gondii* protects against acute and chronic toxoplasmosis. *NPJ Vaccines* 7, 98–10. doi: 10.1038/s41541-022-00518-5

2.4. *NEOSPORA CANINUM* INFECTION TRIGGERS S-PHASE ARREST AND ALTERS NUCLEAR CHARACTERISTICS IN PRIMARY BOVINE ENDOTHELIAL CELLS

Velásquez Z., **Rojas-Barón L.**, Larrazabal C., Salierno M., Gärtner U., Pervizaj-Oruqaj L., Herold S., Hermosilla C., Taubert A. (2022).

Front Cell Dev Biol. 10:1025995. doi: 10.3389/fcell.2022.1025995. PMID: 36111335; PMCID: PMC9469085.

Own part in the publication:

Project planning:	50%, together with co-authors and supervisors
Experimentation:	50%, together with co-authors
Data evaluation:	50%, together with co-authors
Manuscript writing:	50%, together with co-authors

frontiers | Frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 05 August 2022
doi: 10.3389/fcell.2022.946335

Neospora caninum Infection Triggers S-phase Arrest and Alters Nuclear Characteristics in Primary Bovine Endothelial Host Cells

Zahady D. Velásquez[1], Lisbeth Rojas-Barón[1], Camilo Larrazabal[1], Marcelo Salierno[2], Ulrich Gärtner[3], Learta Pervizaj-Oruqaj[4,5,6], Susanne Herold[4,5,6], Carlos Hermosilla[1] and Anja Taubert[1]*

[1]Institute of Parasitology, Biomedical Research Center Seltersberg, Justus Liebig University Giessen, Giessen, Germany, [2]Centre for Developmental Neurobiology, MRC Centre for Neurodevelopmental Disorders, King's College London, London, United Kingdom, [3]Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Giessen, Germany, [4]Department of Medicine V Internal Medicine Infectious Diseases and Infection Control Universities of Giessen and Marburg Lung Center (UGMLC) Member of the German Center for Lung Research (DZL) Justus-Liebig University Giessen, Giessen, Germany, [5]Institute for Lung Health (ILH), Giessen, Germany, [6]Excellence Cluster Cardipulmonary Institute (CPI), Giessen, Germany

OPEN ACCESS

Edited by:
Elena Goncharova, University of California, Davis, United States

Reviewed by:
Soumen Choudhury, U.P. Pandit Deen Dayal Upadhyaya Veterinary University, India
Faheem Ahmed Khan, Huazhong University of Science and Technology, China

***Correspondence:**
Zahady D. Velásquez
zahady.velasquez@vetmed.uni-giessen.de

Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

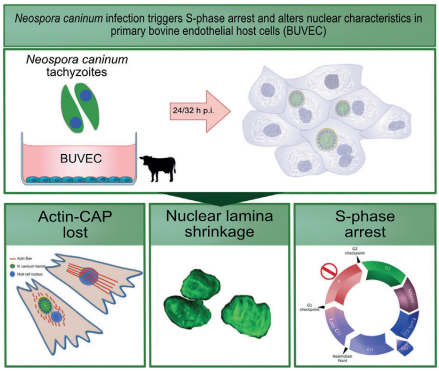
Received: *17 May 2022*
Accepted: *23 June 2022*
Published: *05 August 2022*

Citation:
Velásquez ZD, Rojas-Barón L, Larrazabal C, Salierno M, Gärtner U, Pervizaj-Oruqaj L, Herold S, Hermosilla C and Taubert A (2022) Neospora caninum Infection Triggers S-phase Arrest and Alters Nuclear Characteristics in Primary Bovine Endothelial Host Cells. Front. Cell Dev. Biol. 10:946335. doi: 10.3389/fcell.2022.946335

Neospora caninum represents a major cause of abortive disease in bovines and small ruminants worldwide. As a typical obligate intracellular apicomplexan parasite, *N. caninum* needs to modulate its host cell for successful replication. In the current study, we focused on parasite-driven interference with host cell cycle progression. By performing DNA content-based cell cycle phase analyses in *N. caninum*-infected primary bovine umbilical vein endothelial cells (BUVEC), a parasite-driven S-phase arrest was detected at both 24 and 32 h p. i., being paralleled by fewer host cells experiencing the G0/G1 cell cycle phase. When analyzing S-subphases, proliferation cell nuclear antigen (per PCNA)-based experiments showed a reduced population of BUVEC in the late S-phase. Analyses on key molecules of cell cycle regulation documented a significant alteration of cyclin A2 and cyclin B1 abundance in *N. caninum*-infected host endothelial cells, thereby confirming irregularities in the S-phase and S-to-G2/M-phase transition. In line with cell cycle alterations, general nuclear parameters revealed smaller nuclear sizes and morphological abnormalities of BUVEC nuclei within the *N. caninum*-infected host cell layer. The latter observations were also confirmed by transmission electron microscopy (TEM) and by analyses of lamin B1 as a marker of nuclear lamina, which illustrated an inhomogeneous nuclear lamin B1 distribution, nuclear foldings, and invaginations, thereby reflecting nuclear misshaping. Interestingly, the latter finding applied to both non-infected and infected host cells within parasitized BUVEC layer. Additionally, actin detection indicated alterations in the perinuclear actin cap formation since typical nucleo-transversal filaments were consistently lacking in *N. caninum*-infected BUVEC, as also documented by significantly decreased actin-related intensities in the perinuclear region. These data indicate that *N. caninum* indeed alters host cell cycle progression and severely affects the host cell nuclear phenotype in primary bovine endothelial host cells. In summary, these findings add novel data on the complex *N. caninum*-specific modulation of host cell and nucleus, thereby demonstrating clear differences in cell

cycle progression modulation driven by other closely related apicomplexans like *Toxoplasma gondii* and *Besnotia besnoiti*.

Keywords: *Neospora caninum*, apicomplexan parasites, cell cycle arrest, nuclear lamina, actin-cap



Graphical Abstract |

INTRODUCTION

Neospora caninum is an obligate intracellular protozoan parasite belonging to the phylum Alveolata (subphylum Apicomplexa) and causing neosporosis in a wide range of warm-blooded mammals, including domestic and wild animals (Dubey et al., 2007). Neosporosis is a worldwide emerging parasitosis and is usually related to reproductive disorders (i. e., infertility, abortion, and neonatal mortality), particularly in cattle, small ruminants, and dogs (Dubey et al., 2007). Consistently, neosporosis represents a major cause of bovine abortion, resulting in high losses of over a billion dollars in the cattle industry worldwide (Dubey et al., 2007; Goodswen et al., 2013). The mode of transmission in cattle is mainly vertical by fast replicating *N. caninum* tachyzoites, causing a high prevalence in affected cattle herds (Dubey et al., 1992; Barr et al., 1993; Barr et al., 1994). *N. caninum* tachyzoites share fast proliferative properties with other parasite genera of the Sarcocystidae family with veterinary and human medicine relevance, such as *Toxoplasma*, *Besnoitia,* and *Sarcocystis*. However, unlike polyxenous *Toxoplasma gondii*, *N. caninum* is vastly host species-specific and its development is restricted to distinct host cell types *in vivo*, such as endothelial cells, neurons, epithelial cells, and fibroblasts (Hemphill et al., 1996; Lei et al., 2005). In general, apicomplexan parasites significantly modulate their host cells to sustain intracellular development and proliferation. Consequently, these parasites were described to alter numerous host cell functional categories, such as apoptosis (Goebel et al., 2001; Molestina et al., 2003; Lang et al., 2009), cytoskeleton (Dobrowolski and Sibley, 1996; Hermosilla et al., 2008), host cell membrane composition (Graewe et al., 2011), carbohydrate or cholesterol metabolism (Coppens et al., 2000; Nishikawa et al., 2005), innate immune reactions (Hermosilla et al., 2006; Taubert et al., 2006) and cell cycle progression (Brunet et al., 2008; Molestina et al., 2008; Velásquez et al., 2019; Velásquez et al., 2020a; Velásquez et al., 2020b). Likewise, it was demonstrated that *N. caninum* infections effectively alter host cellular immune responses by inducing distinct chemokine and adhesion molecule transcription in bovine endothelial cells (Taubert et al., 2006) or by triggering a pro-inflammatory gene transcription profile in bovine macrophages and trophoblasts (Jiménez-Pelayo et al., 2019; García-Sánchez et al., 2020). Additionally, global proteomic and transcriptomic analyses revealed a multitude of functional categories to be modulated by *N. caninum* in bovine trophoblasts, such as protein synthesis/turnover, metabolism, mitochondrial function, stress response, and host cell cycle (Horcajo et al., 2017; Regidor-Cerrillo et al., 2020). However, no data are currently available on either *Neospora hughesi* nor *N. caninum*-triggered alteration of this cellular function even though species-specific reactions are meanwhile well documented. Likewise, we recently reported that *T. gondii, B. besnoiti,* and *E. bovis* differentially affect cell cycle progression in the same host cell type [i. e. primary bovine umbilical vein endothelial cells (BUVEC)]. Thus, *T. gondii*-infected BUVEC showed G2/M-phase arrest, chromosome miss-segregation, and cytokinesis failure (Velásquez et al., 2019). In contrast, *E. bovis* and *B. besnoiti* infected BUVEC did not show chromosome segregation or cytokinesis impairment but failed to progress from G1-phase, suggesting a parasite-driven host cell cycle stasis in G1-phase or at G1-to-S-phase transition, possibly being related to specific

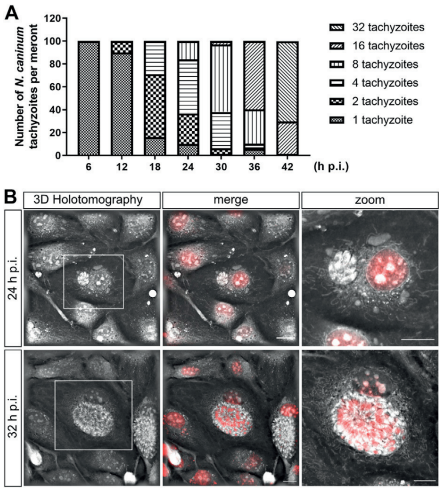


FIGURE 1 | *N. caninum* tachyzoite intracellular development in primary bovine umbilical vein endothelial cells (BUVEC). Sub-confluent BUVEC were infected with *N. caninum* tachyzoites at an MOI 3:1 and analyzed from 6 to 42 h p. i. **(A)** Fixed cells were stained against *N. caninum* tachyzoite, and the tachyzoite numbers/parasitophorous vacuole (PV) were counted every 6 h during 42 h of infection. Mature tachyzoites were observed between 36 and 42 h p. i. The graph shows the distribution of the number of tachyzoites per meront at each time point. In BUVEC, the first duplication of *N. caninum* tachyzoites was observed only after 6 h p. i. Cells were lysed after 42 h p. i, thereby releasing newly formed tachyzoites into the medium. **(B)** Live cell 3D-holotomography images of *N. caninum* tachyzoite-infected BUVEC at 24 and 32 h p. i. nuclei of both, i. e., BUVEC and parasites, were stained with DRAQ5 (vital DNA staining, red). Bars: 5 µm.

requirements for their intracellular development (Velásquez et al., 2020a; Velásquez et al., 2020b).

Given that respective data are completely lacking on *N. caninum*-infected host cells, we here demonstrated the influence of *N. caninum* infection on host cell cycle progression and used the same primary host cell type (BUVEC) as before for *T. gondi*-, *E. bovis*- and *B. besnoiti*-related studies to generate comparative data. To the best of our knowledge, we here showed for the first time that *N. caninum* infections induce an S-phase arrest in host cell cycle progression, controlling not only the DNA amount but also some key proteins which are involved in the regulation of various cell cycle phases. As an interesting finding, we here documented that nuclei of *N. caninum*-infected BUVEC were reduced in size and showed altered morphologies over time. Moreover, the structure of their nuclear lamina changed, probably as a consequence of lamin B1 defects or due to actin-cytoskeleton disruptions (actin cap) occurring around the parasitophorous vacuole (PV) containing *N. caninum* tachyzoites.

RESULTS

N. caninum Tachyzoite Development in Primary Bovine Umbilical Vein Endothelial Cells

Given that the developmental kinetics of apicomplexan parasites depend not only on the species but also on the host cell type, we here thoroughly monitored *N. caninum* tachyzoite formation over time in BUVEC. As expected, proliferation of *N. caninum* tachyzoites took slightly longer than that of *T. gondii* or *B. besnoiti* tachyzoites in the same host endothelial cell type (Velásquez et al., 2019; Velásquez et al., 2020a). To strictly avoid variations due to the seeding process, BUVEC isolate or tachyzoite batch, we here seeded identical BUVEC isolates at the same time point and used tachyzoites from the same isolation for host cell infections. By sticking to this procedure, we estimated tachyzoite numbers per meront throughout *in vitro* infection (i. e., at 6, 12, 18, 24, 30, 36, and 42 h p. i.). Analyses were restricted to 32 h p. i., since thereafter cell lysis was common. In each sample, a total of 100 host cells were analyzed. At the stage of 32 tachyzoites/PV, the meront was considered mature (**Figure 1B**). Overall, by applying an MOI of 1:1, we achieved a mean infection rate of 46.2 ± 3.4% in BUVEC. Developmental monitoring revealed that the first tachyzoite duplication took place between 6 and 12 h p. i., whilst the second and third steps of replication occurred between 12 and 18 h p. i. (**Figure 1A**). At 24 h p. i., most tachyzoites (90 ± 4.7%) had divided at least once. Consequently, PVs showed 2 (26.7 ± 1.4%), 4 (47.3 ± 1%), or 8 (16 ± 0.7%) tachyzoites (**Figure 1A**). At 30 h p. i., PVs mainly contained 8 (59 ± 0.3%) tachyzoites and, to less degree, 2 (1.33 ± 0.19%), 4 (31.2 ± 1.2%) or 16 (3 ± 0.3%) tachyzoites. At 36 and 42 h p. i., most PVs carried 16 (35.2 ± 0.4%) or 32 tachyzoites, respectively (**Figure 1A**). In order to monitor intracellular development of *N. caninum* under live cell conditions, we additionally performed live cell 3D-holotomographic microscopy covering up to 42 h of infection. Here, parasite development and nucleus formation were documented via vital DNA staining with the cell-permeable fluorescent DNA probe DRAQ5 (**Figure 1B**). Based on the formation of parasite rosettes over time and a concurrent lack of host cell lysis, we chose the time points of 24 and 32 h p. i. for further analyses on the impact of *N. caninum* replication on host cell cycle progression.

N. caninum Infection Arrests the Host Cell Cycle in the S-Phase

In a first experimental approach, cell cycle progression in *N. caninum*-infected BUVEC was evaluated at 24 and 32 h p. i. by flow cytometry-based analysis of the cellular DNA content, allowing for the discrimination of the main three periods of the cell cycle: G0/G1-, S-, and G2/M-phase. This is a well-established method which proved suitable for other apicomplexan parasite-infected BUVEC (Velásquez et al., 2019; Velásquez et al., 2020a). An exemplary illustration for *N. caninum*-infected BUVEC gating is shown in **Supplementary**

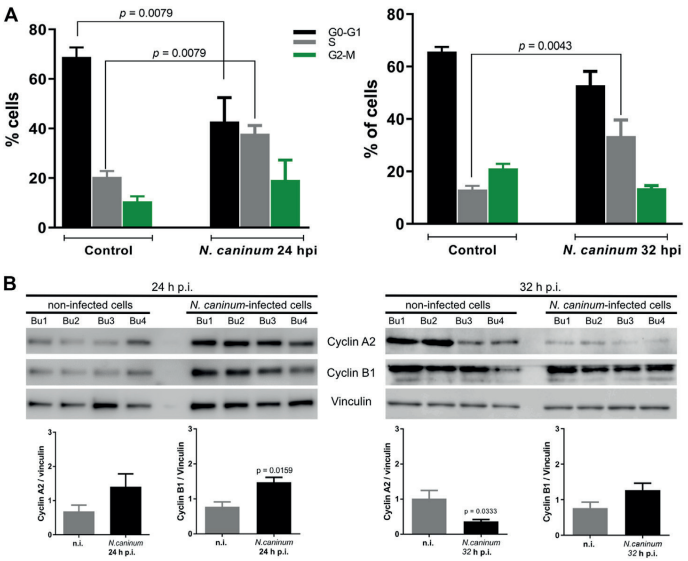


FIGURE 2 | Effects of *N. caninum* infection on the host cellular cell cycle and on cyclin A2 and B1 abundance. Sub-confluent BUVEC ($n = 6$) were infected with tachyzoites of *N. caninum* at an MOI of 3:1 and examined for DNA contents at 24 and 32 h p. i. by FACS-based analyses. **(A)** Cell population was first gated in order to eliminate debris from the analysis. Furthermore, the DNA channel vs. the population histogram was used to obtain the total number of cells in each peak. Data were plotted as a percentage of total cells vs. DNA amounts found in each G0/G1-, S- and G2/M-phase, respectively. **(B)** Analysis of cell cycle-related molecule expression in *N. caninum*-infected BUVEC. Four biological replicates of BUVEC were infected with *N. caninum* tachyzoites and analyzed by Western blotting for the abundance of the cell cycle-related molecules cyclin A2 (S-, G2-phase transition), and cyclin B1 (M-phase), at 24 and 32 h p. i. The density of protein signals was quantified and graphed as a relative ratio to vinculin (loading control). Bars represent the median ±SD of four biological replicates.

Figure S1). The data revealed a significantly increased proportion of infected host cells experiencing S-phase at both 24 and 32 h p. i. (infected vs. control cells: 24 h p. i.: $p = 0.0079$; 32 h p. i.: $p = 0.0043$) being accompanied by a simultaneous decrease of *N. caninum*-infected BUVEC in G0/G1-phase (24 h p. i.: $p = 0.0079$; 32 h p. i.: $p = 0.049$) when compared to non-infected control cells, thereby overall indicating a parasite-driven arrest of host cells in S-phase during full parasite proliferation (**Figure 2A**).

In a second experimental series, we measured the cellular abundance of selected key molecules of the cell cycle by analyzing four different *N. caninum*-infected and control BUVEC isolates via Western blotting to discriminate cell cycle phases in more detail (**Figure 2B**). Therefore, cyclin B1 and cyclin A2 abundance were monitored at both 24 and 32 h p. i. In line with current DNA content-based data, the abundance of cyclin A2, which is typically increased throughout S-phase peaking in G2-phase, was reduced in the M-phase at 32 h p. i., but was not altered at 24 h p. i. ($p = 0.1229$). Furthermore, cyclin B1, which starts to be upregulated in S-phase and peaks at the G2/M border, was found significantly enhanced in *N. caninum*-infected BUVEC at 24 h p. i. ($p = 0.0159$) but showed no significant changes in its abundance at 32 h p. i. when compared to control cells ($p = 0.200$). These cyclin-related data support results in **Figure 2A** about *N. caninum*-infected host cells failing to enter into the G2/M phase and arresting cells in the S-phase of the cell cycle.

N. caninum Infection Alters S-Subphase Distribution in Host Endothelial Cells

Cell cycle progression is tightly controlled by different checkpoints regulating the entry into the subsequent phase or the exit from the current individual cell cycle phase. This regulation drives cells to either proceed to the next phase or to return to the last step. Irregularities occurring within each cell cycle phase may also block cell cycle progression. As shown above, *N. caninum*-infected BUVECs were arrested in S-phase, which may also result from irregularities during S-phase itself. Therefore, we monitored different subphases of S-phase by detecting PCNA protein, which is involved in DNA replication/repair and additionally has cell cycle-dependent properties. Of note, the characteristic nuclear PCNA distribution pattern is generally accepted as indicative of different S-subphases and therefore allows discrimination of early, mid, and late S-phase (Schönenberger et al., 2015). To analyze the nuclear PCNA pattern in *N. caninum*-infected BUVEC and controls, host endothelial cells were submitted to simultaneous PCNA immunodetection and nuclear staining (by DAPI)

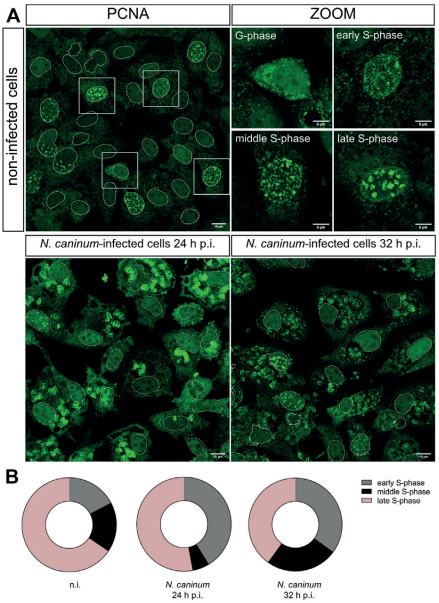


FIGURE 3 | Effects of *N. caninum* infection on S-subphases. **(A)** *N. caninum*-infected BUVEC and non-infected cells were fixed at 24 ad 32 h p. i and stained for DAPI and proliferation cell nuclear antigen (PCNA; green) and analyzed *via* confocal microscopy. The nuclear PCNA localization and pattern allow one to discriminate the S-phase steps (early, middle, and late). The host cell nuclei were automatically segmented on the DAPI channel, the nuclear region was selected, and the coordinates were overlayed into the PCNA channel to identify the nuclear zone (white circles around the nuclei). **(B)** Quantification of nuclear PCNA-related pattern in non-infected cells in comparison with *N. caninum*-infected cells at 24 and 32 h p. i. The graph represents the percentages of cells in each S-phase step, early (gray), middle (black), and late (pink). The scale bar represents 5 μm.

(**Figure 3A**). In non-infected BUVEC monolayers, most cells were in late S-phase (65.5 ± 9.1%), whilst fewer cells experienced early (17.2 ± 3.3%) or mid S-phase (17.2 ± 4.3%) (**Figures 3A,B**). However, considerable differences in S-subphases were detected in *N. caninum*-infected host cell layers. Thus, at 24 h p. i., a shift in S-subphases was apparent, thereby leading to an almost complete lack of host cells in mid S-phase (5.88 ± 2.4%) and a reduced proportion of BUVEC in late S-phase (52.94 ± 10.36%) but to an increase of host cells experiencing early S-phase (41.2 ± 8.7%) (**Figure 3B**). At 32 h p. i., infected host cells still showed a reduced proportion of host cells in the late S phase (40 ± 6.1%) when compared to control cells, whilst more host cells had again proceeded into the mid S-phase (25 ± 3.8%) (**Figure 3B**). Overall, it seemed that parasite-infected cells experienced difficulties in their progress into the late S-phase, which may then lead to an impairment in the transfer into the G2/M phase.

N. caninum Infection Affects the Nuclear Structure and Perinuclear Actin Structures in Host Cell Layers

When analyzing PCNA distribution within the nuclei of fixed *N. caninum*-infected BUVEC, we noticed irregular nuclear morphologies in both *N. caninum*-infected cells and non-infected cells within the same infected cell monolayer. Overall, the nuclear shape and volume play a central role in cellular and developmental processes and are maintained by mechanical forces mediated via cytoskeletal elements from inside and outside the cell. Typically, there is a correlation between cell shape and nuclear size, which is preserved during the entire cell life, and it is also inherited by daughter cells (Khatau et al., 2009). To verify the above mentioned morphological observations and to exclude fixation-based artefacts, we illustrated the nuclear morphology in both living and PFA-fixed cells via DNA staining with Hoechst 33342 and DAPI, respectively (**Figure 4A**). As expected, the nuclei of fixed cells from non-infected cell layers mainly showed a regular, oval shape with smooth cell borders, and a few cells displayed slight nuclear depressions (**Figure 4A**), the latter of which was practically absent in living (non-fixed), non-infected BUVEC monolayers (**Figure 4A**). In contrast, in PFA-fixed *N. caninum*-infected host cell layers, an increased proportion of cells showed nuclear abnormalities. These host cells no longer presented smooth borders and regular shapes of the nuclei but instead displayed irregular nuclear morphologies with inconsistencies, corners, strictures, and dentings (**Figure 4A**, white arrows), some of them unveiling nuclear fragmentation (**Figures 4A,B**, white arrow). In line, similar effects were observed in living cells, where nuclei showed invaginations and condensed lines within the nuclear area (**Figures 4A–C**, white arrow). Interestingly, we additionally observed several nuclei presenting a half-moon shape, which was not found in fixed cells (**Figures 4A–D**, white arrows). To confirm these morphological alterations, the nuclear structures were additionally illustrated by TEM analysis. In line with the above observations, *N. caninum*-infected host cells showed irregular, deformed nuclei at 24 and 32 h p. i. with invaginations and stretches of nuclear membrane disintegration (**Figure 4B**–space in between yellow arrows). Interestingly, we also observed a *N. caninum* tachyzoite being located within a nuclear invagination but seemingly lacking a PV (**Figure 4B**–asterisk 32 h p. i.).

To assess some general nuclear parameters and to confirm infection-driven nuclear changes on a quantitative level, we further measured the area, circularity, and nuclear axis ratio in both non-infected and infected cells. These analyses showed that the nuclear area was 11.7% smaller in *N. caninum*-infected host cells at 32 h p. i. when compared to control cells (**Figure 5A**), whilst the nuclear circularity was not affected by infection (**Figure 5B**). Furthermore, the axes ratio was revealed to be 4.9% larger for *N. caninum*-infected cells when compared to non-infected cells (**Figure 5C**).

To study nuclear elements in more detail, we additionally analyzed the staining pattern of lamin B1 as a marker of the nuclear lamina. In general, the nuclear shape is maintained by

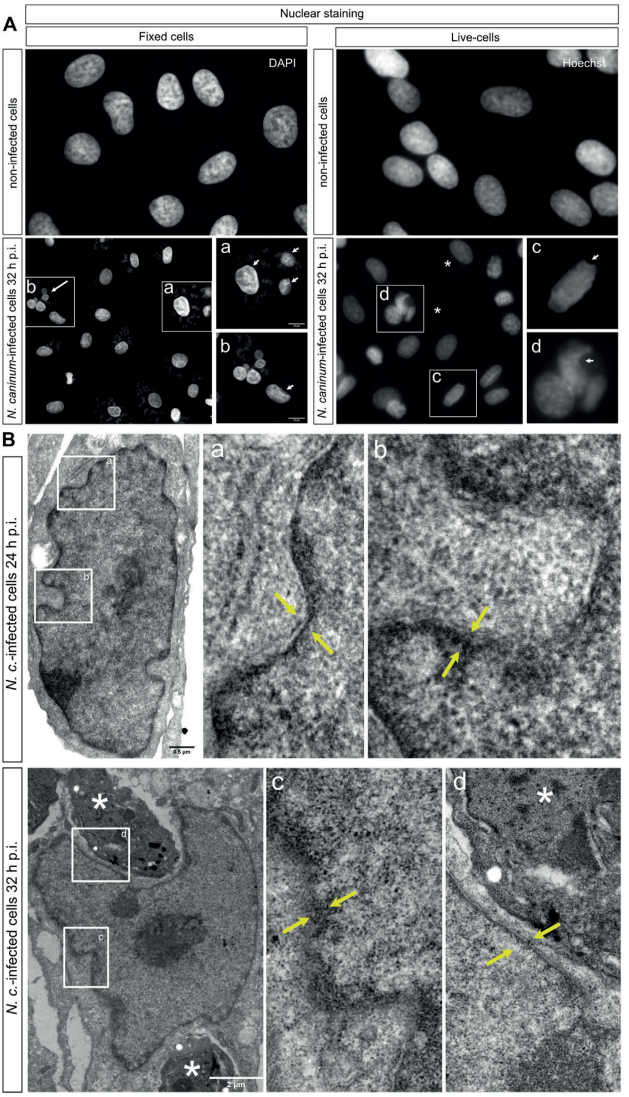


FIGURE 4 | Morphology of cell nuclei in *N. caninum*-infected BUVEC layers. **(A)** Three BUVEC biological replicates were infected with *N. caninum* tachyzoites, and the host cell nuclear shape was compared between PFA-fixed and living cells after 32 h p. i. After fixation, cells were permeabilized and stained with a DAPI probe to allow host cell nucleus recognition as well as to detect the nucleus of the parasite (little gray dots surrounding the host cell nucleus). Live-cell imaging was developed with Hoechst nuclear staining, which allows a good resolution of the host cell nuclei but deficient parasitic nuclei, for that, the localization of the parasite is shown with an asterisk. **(B)** TEM analysis of *N. caninum*-infected host cells shows a nuclear compartment with depression and altered by tachyzoite localization (asterisk). The inspection of the nuclear membrane displayed zones without the detection of double membranes (space between yellow arrows). **(a, b)** Inset of the image into the left corner in order to show in more detail the nuclear membrane structure. The scale bar represents 5 µm.

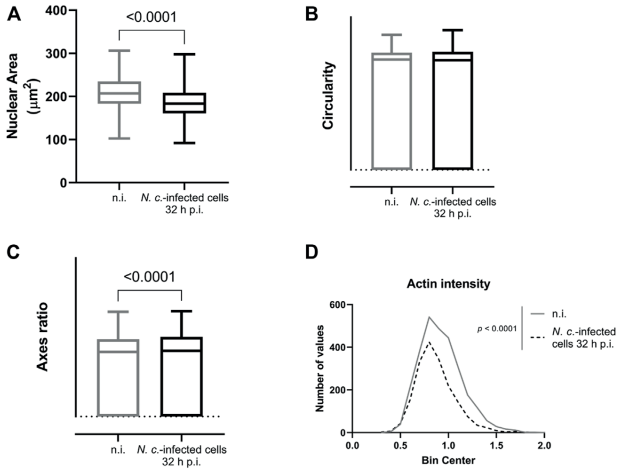


FIGURE 5 | Effects of *N. caninum* infection on classical nuclear parameters. Non-infected and *N. caninum*-infected host endothelial cells (BUVEC) were stained with DAPI, and random pictures were taken at 20X for measuring the host cell nuclear area **(A)**, circularity **(B)**, nuclear axis ratio **(C)**, and the actin intensity **(D)**. Bars represent the median ±SD of three biological replicates.

several factors, which include both nuclear and cytoplasmic molecules. The nuclear envelope is formed by two concentric membranes being equipped with nuclear pore complexes and supported by an underlying lamina composed of a meshwork of nuclear intermediate filaments formed by A- and B-type lamins (Hetzer, 2010). Lamin B1 forms part of the nuclear lamina but also interacts directly with chromatin (Brunet et al., 2019). To analyze whether nuclear alterations were based on an irregular nuclear lamina formation, we analyzed the distribution pattern of lamin B1 in non-infected and *N. caninum*-infected (32 h p. i.) cell monolayers. As expected and in line with the above mentioned observations on DAPI-stained nuclei, non-infected cells showed a homogenous distribution of lamin B1 over the total nuclear area and only a very few cells eventually displayed tiny membrane foldings (**Figure 6A**). In contrast, a much higher proportion of host cells revealed altered nuclear lamin B1-related distribution patterns within *N. caninum*-infected BUVEC layers, which were characterized by inhomogeneous staining showing several nuclear foldings and invaginations, thereby reflecting abnormal nuclear shapes and irregular silhouettes (**Figure 6A**–white arrow in *N. caninum*-infected host cells **Supplementary Video S1**). In some nuclei, bubble-like protrusions (**Figure 6A** -white head arrow) were detected and further confirmed by 3D reconstructions (**Supplementary Video S1**). When assessing these nuclear abnormalities on a quantitative level, a significantly increased proportion of both *N. caninum*-infected (18.8 ± 6.9%) and non-infected (81.2 ± 6.9%) cells within an infected cell layer (infection rate: 25.96 ± 14.73%) showed inadequate lamin B1 distribution patterns and misshaped nuclei (**Figure 6B**) when compared to cells from non-infected cell layers (9.3 ± 2.8%) (infected/non-infected cells from infected layer vs. non-infected cell layer: both $p = 0.0078$).

The shape of the total cell but also of its nucleus completely depends on the presence of distinct filamentous proteins, such as actin and intermediate filaments, as well as microtubules. Of special interest for nuclear shaping and function is the so-called perinuclear actin cap, which is composed of thick, parallel, and highly contractile actomyosin filament bundles with their ends being anchored in focal adhesions and the central part spanning over and being physically attached to the nucleus and nuclear lamina by the linker of nucleoskeleton and cytoskeleton (LINC) complex (Khatau et al., 2009; Kim et al., 2013; Maninova et al., 2017). To detect cytoskeletal actin filaments in non-infected and *N. caninum*-infected (32 h p. i.) host cells, we here used phalloidin staining (**Figure 7A**). When considering the total actin-based cytoskeletal network of cells, seemingly similar phenotypes were observed in both conditions (**Figure 7A**). However, the area in juxtaposition to nuclei appeared considerably affected in *N. caninum*-infected cells. Thus, actin filaments typically transversing the nuclei and being anchored to the nuclear periphery–as commonly observed in non-infected cells (**Figure 7A**–indicated by an arrow, **Supplementary Video S2**) - were consistently absent from the nuclei of *N. caninum*-infected cells (**Figure 7A**–indicated by asterisk). Consequently, the remnants of the respective filaments seemed fragmented or eventually without anchorage. Interestingly, actin quantification by FACS showed decreased abundance in *N. caninum*-infected cells ($p = 0.0240$), thereby mirroring the observation that perinuclear actin intensities seemed reduced in *N. caninum*-infected cells when compared to control cells.

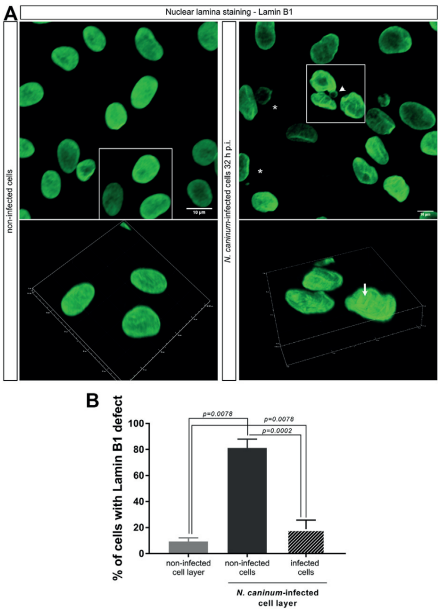


FIGURE 6 | Nuclear lamin B1 detection in *N. caninum*-infected BUVEC layers. **(A)** Nuclear lamina analysis by the staining of lamin B1 (green). Fixed cells were stained against lamin B1 for the nuclear lamina and DAPI for the nuclear area recognition (not shown in the image). A white arrowhead in the 3D volume projection image: circular structures protruding from the nucleus; asterisks: parasite localization; white arrow: lamin B1 folding. **(B)** Quantification of the percentage of host cells with distorted lamin B1 localization in the total *N. caninum*-infected BUVEC monolayer (dark and light gray). The scale bar represents 10 µm. Bars represent the median ±SD of three biological replicates.

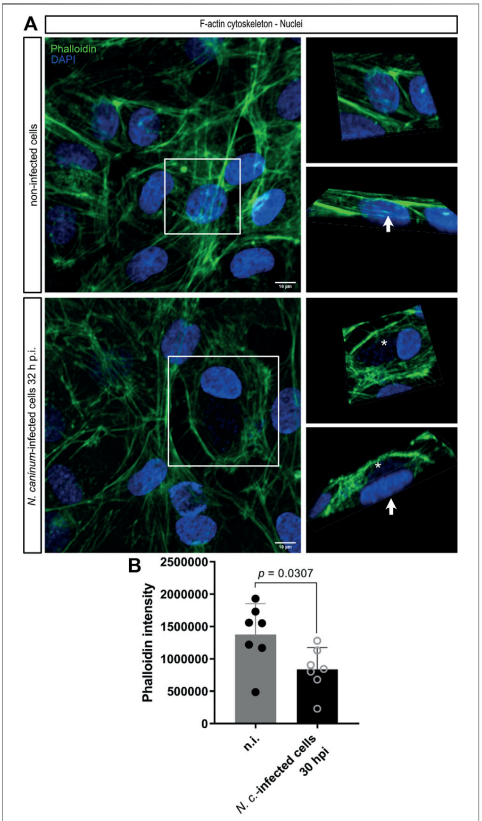


FIGURE 7 | F-actin-based cytoskeletal nuclear structures in *N. caninum*-infected BUVECs. **(A)** Three biological BUVEC replicates were infected with *N. caninum* tachyzoites and, after 32 h p. i., submitted to F-actin (green) and DAPI (blue) staining. Pictures of the whole cell were taken in order to visualize the actin network in both cell conditions. In the right panel, the 3D volume image reconstruction of only one host cell is shown to display in detail the actin filament localization inside the cell. The white arrows show the nuclear region covered by the actin cytoskeleton, and the asterisks point out the parasite localization. **(B)** FACS-based quantification of the F- actin in both non-infected and *N. caninum*-infected host cells. Scale bar represents 10 µm. Bars represent the median ±SD of six biological replicates.

In contrast to lamin B1-related data, the actin cap-based changes were not exclusively found in infected cells, thereby questioning this finding as an exclusive mechanistic basis of nuclear malshaping. However, these overall observations suggest that *N. caninum* intracellular development might interfere with the host cellular actin cytoskeleton in the perinuclear region and most likely lead to nuclear membrane destabilization, thereby contributing to abnormal nuclear shaping.

Nuclear lamin B1-related data showed that non-infected host cells within infected monolayers revealed a higher percentage with abnormal lamin B1 distribution in comparison to *N. caninum*-infected ones. To test for *N. caninum*-driven paracrine effects on nuclear lamin B1 in non-infected cells, we tested whether supernatants from *N. caninum*-infected BUVEC monolayers would lead to nuclear lamin B1 abnormalities in non-infected BUVEC layers. Therefore, non-infected BUVEC were supplemented with infection-conditioned medium (i. e. filtered supernatants of *N. caninum* tachyzoite-infected BUVEC after 32 h p. i.) or with supernatants from non-infected BUVEC (controls). The results demonstrated that supernatants from *N. caninum*-infected BUVEC indeed induced S-phase arrest (p = 0.0256) with a concomitant G0-G1 phase reduction (**Figure 8A**) in non-infected BUVEC. However, cyclin B1 abundance was not significantly influenced by differential supernatant treatments (**Figure 8B**). Moreover, phalloidin-based actin quantification showed no effect of differential supernatant treatments on

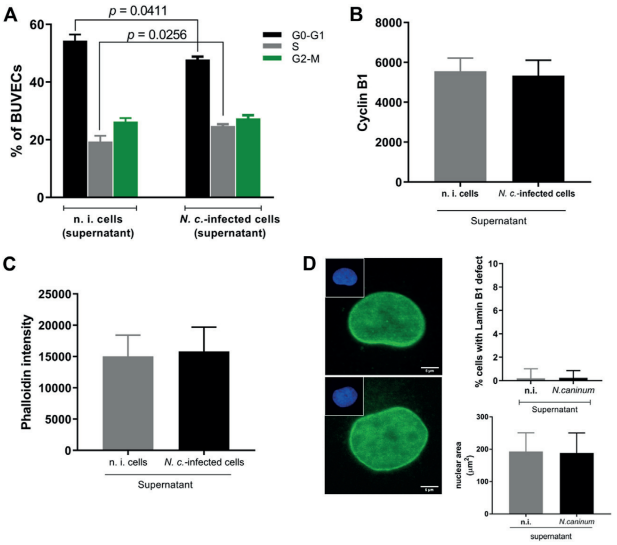


FIGURE 8 | Analysis of *N. caninum* infection-derived supernatant paracrine effects on host cell cycle progression and the nuclear shape of non-infected host cells. Six biological BUVEC ($n = 6$) replicates were infected with *N. caninum* tachyzoites and, after 32 h p. i., the medium (i. e., supernatant) was isolated and filtered to erase any contamination with tachyzoites/cell debris. The non-infected and *N. caninum*-infected host cell supernatant was immediately transferred to non-infected monolayers and incubated for 32 h in order to analyze paracrine effects on host cell cycle progression and nuclear shape. **(A)** BUVEC populations were first gated, and the histograms for propidium iodide (PI) were conducted in order to differentiate the mean peaks of the histograms. Data were plotted as the percentage of total cells vs. DNA amounts found in each G0/G1-, S- and G2/M-phase. **(B, C)** Analyses of cyclin B1 and F-actin by FACS-based quantification. **(D)** Lamin B1 detection by immunofluorescence (green) and the nuclear area by DAPI (left inset) staining. The nuclear size was quantified using the DAPI channel and represented in the graph. Bars represent the median ±SD of six biological replicates. The scale bar represents 5 µm.

actin-mediated nuclear shapes. Thus, neither actin-derived effects on nuclear shapes nor on nuclear sizes were observed in supernatant-treated cell monolayers (**Figures 8C,D**).

DISCUSSION

The cell cycle is a well-conserved and tightly regulated process in all eukaryotic cells and is key for cell survival/division. Up to date, studies on apicomplexa-driven cell cycle modulation have unveiled changes in abundance of cyclins critically involved in host cell division, such as G2-specific cyclin B1 or cyclins A/B in combination with cyclin E in case of *T. gondii* (Molestina et al., 2008; Velásquez et al., 2019), cyclin E1 in case of *B. besnoiti*- and *E. bovis*-infected host cells thereby indicating a cell cycle arrest at G1/S transition phase (Velásquez et al., 2020a; Velásquez et al., 2020b). Moreover, apicomplexa-modulated cell cycle progress seems to be parasite species- and cell type-dependent involving up- or downregulation of specific cyclins while performing this influence (Molestina et al., 2008; Taubert et al., 2010; Velásquez et al., 2019; Velásquez et al., 2020a; Velásquez et al., 2020b). Thus, *T. gondii* tachyzoites blocked host cell cycle progression in G2/M-phase, thereby interfering with chromosome segregation and mitotic spindle formation and inducing cytokinesis failure in infected endothelial cells (Velásquez et al., 2019). In contrast, infections of the same host cell type with *B. besnoiti* tachyzoites and *E. bovis* sporozoites led to host cell arrest in G0/G1-phase but altered the abundance of cell cycle-related key molecules in a different manner (Velásquez et al., 2020a; Velásquez et al., 2020b). As such, exclusive *E. bovis* infection triggered premature senescence in BUVEC, in addition to cyclin E1 up-regulation during macromeront formation (Velásquez et al., 2020b). Conversely, and despite the fact that *N. caninum* tachyzoites replicate in bovine endothelial cells of blood and lymph vessels *in vivo*, no data were available on *N. caninum*-mediated cell cycle modulation in host endothelial cells. Therefore, in the current study, we used BUVEC as the host cell system for the following reasons: *1*) to be as close as possible to the *in vivo* situation in bovines, and *2*) to avoid cell type-driven effects and thereby simplify reliable comparisons on parasite species levels when referring to other studies above mentioned. Here, we showed that *N. caninum* tachyzoites indeed interfered with host cell cycle progression by inducing S-phase arrest, nuclear lamin B1 maldistribution, peri-nuclear actin losses, and host cellular

nuclear deformations. More importantly, these findings clearly differed from those alterations driven by different stages of the closely related parasites *T. gondii, B. besnoiti,* and *E. bovis*, indicating that apicomplexa-driven cell cycle modulation is not only a species- and cell type- but also a stage-dependent process.

By the combination of WB- and FACS-based analyses, we here demonstrated that *N. caninum*-infected host endothelial cells were arrested at the DNA synthesis stage when harboring immature meronts with no more than 8 tachyzoites onwards. One plausible explanation for cell cycle arrest in S-phase is that during this phase not only protein-, amino sugar- and pentose phosphate-synthesis are up-regulated but also metabolic pathways, such as glycolysis/gluconeogenesis and nucleotide sugar metabolism, all of them fundamental for both, host cell maintenance and intracellular parasite proliferation (Regidor-Cerrillo et al., 2020; Velásquez et al., 2021; Vélez et al., 2021). Given that tachyzoite production is an energy- and building block-demanding process, the transient or continuous deprivation of essential molecules could indeed induce an energy-lacking status in *N. caninum*-infected host cells, finally leading to a block in DNA synthesis and/or mitotic process. Likewise, recent data showed that *N. caninum* tachyzoites recruit host cell structures to their PV and salvage lipids from organelles (Nolan et al., 2015). Interestingly, treatments of non-infected endothelial cells with infection-conditioned medium also resulted in S-phase stasis, suggesting that either the parasites or *N. caninum*-infected endothelial host cells release soluble factors which act on bystander cells. Likewise, induction of S-phase (based on BrdU incorporation) by infection-conditioned medium was also described for *T. gondii*-driven effects in non-infected human foreskin fibroblasts (HFF) (Lavine and Arrizabalaga, 2009). Interestingly, besides inducing S-phase stasis in host cells, *T. gondii* more readily infected fibroblasts in S-phase and conditioned medium increased the efficiency of invasion in HFF, which led to the assumption that cells in S-phase bear a selective advantage for *T. gondii* (Lavine and Arrizabalaga, 2009).

Important molecular mechanisms involved in adequate cell cycle progression include the accuracy of DNA synthesis and several checkpoint-based controls. The later exist for G1-phase (G1-checkpoint), during the whole S-phase (S-phase checkpoint) and after S-phase (G2/M checkpoint) thereby covering a temporal and spatial program (Hartwell and Weinert, 1989; Kastan and Bartek, 2004). The current data suggests that *N. caninum* infection could lead to S-phase stasis of host cells by affecting the synthesis of proteins linked to distinct cell cycle checkpoints. It is well accepted that cyclin A2 is a key regulator of both mitotic entry and DNA replication, following a coordinated pattern of expression and subcellular localization (Pagano et al., 1992; Petersen et al., 1999; Fung et al., 2007). As such, the complex of cyclin A/cdk2 drives eukaryotic cells in G2-phase to continue into the mitosis phase. Early cyclin A/cdk2 downregulation correlates with S-phase arrest, not allowing cells to enter into the G2/mitosis phase (Furuno et al., 1999). Interestingly, our data showed that *N. caninum*-infected endothelial host cells were stuck in S-phase with a concomitant down-regulation of cyclin A2, suggesting that *N. caninum*-infected host cells were most probably not able to pass the cell cycle checkpoints after S-phase.

Unexpectedly, we observed that *N. caninum* drastically affected the nuclei of infected host cells, i. e., their sizes, the actin cap and the nuclear lamina. Of note, these nuclear features were not exclusively observed in *N. caninum*-infected host cells but also in non-infected bystander cells. The nuclear lamina is formed by lamins A and B, with lamin B being essentially involved in cell viability, DNA replication, DNA repair, RNA polymerase transcription, and chromatin remodeling as epigenetic control. Thus, the specification of distinct lamins and their localization affect the DNA elongation chain, being therefore considered fundamental for DNA synthesis during cell replication (Moir et al., 2000; Spann et al., 2002; Shimi et al., 2008; Shumaker et al., 2008). The current data on lamin B1 and nuclear shape revealed that *N. caninum*-infected host cells showed nuclei that were diminished in size and presented altered lamin B1 distribution, a phenomenon that may also be linked to their inability to overpass S-phase since nuclear lamina defects may hamper accurate DNA repair or DNA replication. As a consequence, critical DNA-related damage checkpoints failed and cells were not able to progress into the mitotic process. Small nuclei originating from lamin-depleted nuclear extracts showed an inhibition of nuclear transport and thereby resulted in cell cycle arrest prior to DNA synthesis (Newport et al., 1990; Meier et al., 1991). Besides lamin B1-related nuclear alterations, we additionally observed actin-related abnormalities in *N. caninum*-infected BUVEC. Thus, the current data indicated that the juxta- and transnuclear actin cytoskeleton was not properly formed and that especially the perinuclear actin cap was lacking in infected cells. Importantly, perinuclear actin cap integrity depends on proper F-actin assembly, which is critically involved in adequate nuclear shaping (Khatau et al., 2009). Given that no F-actin-mediated nuclear shape alterations were detected in non-infected host cells exposed to infection-conditioned medium. Moreover, we suggest that the reduction of host cell nucleus sizes as well as the cell cycle arrest in S-phase could be a consequence of DNA damage caused by fast intracellular meront growth and resulting in isometric shrinkage of host cell nuclei.

Taken together, the current and recent data underline species and even stage-specific effects on host cell cycle progression that our group has published. This suggests that further investigations into parasite species of the family Sarcocystidae are needed to elucidate precise molecular mechanisms, signaling pathways, and cyclins involved in individual modulation of cell cycle progression. In summary, we here demonstrate to our best knowledge for the first time that *N. caninum* tachyzoite infection affects host endothelial cell cycle progression in a species-specific manner by alterations in cyclin A2/B1 abundance and actin-dependent nuclear lamina deformations, resulting in irregularities in S-phase and S-to-G2/M-phase transition.

MATERIALS AND METHODS

Primary Bovine Umbilical Vein Endothelial Cell Isolation and Maintenance

BUVEC were isolated from umbilical veins obtained from calves born by *sectio caesarea* at Justus Liebig University Giessen, Giessen, Germany. Therefore, umbilical cords were kept at 4°C

in 0.9% HBSS-HEPES buffer (pH 7.4; Gibco, Grand Island, NY, United States) supplemented with 1% penicillin (500 U/mL; Sigma, St. Louis, MO, United States) and streptomycin (500 µg/ml; Sigma) for a maximum of 16 h before use. For endothelial cell isolation, 0.025% collagenase type II (Worthington Biochemical Corporation) in Pucks solution (Gibco) was infused into the lumen of ligated umbilical veins and incubated for 20 min at 37°C in a 5% CO_2 atmosphere. After gently massaging the umbilical veins, the cell suspension was collected and supplemented with 1 ml of fetal calf serum (FCS, Gibco) to inactivate collagenase. After two washes (350 x *g*, 12 min, 20°C), cells were suspended in complete endothelial cell growth medium (ECGM, PromoCell, supplemented with 10% FCS), plated in 25 cm^2 tissue culture flasks (Greiner) and kept at 37°C in a 5% CO_2 atmosphere. BUVEC were cultured in modified ECGM medium [EGCM, diluted at 30% in M199 medium (Greiner), supplemented with 5% FCS (Greiner), 1% penicillin and streptomycin (both Greiner)] with medium changes every 2–3 days. BUVEC cell layers were used for infection after 3 passages *in vitro*. All BUVEC isolations were conducted by the Institutional Ethics Commission of Justus Liebig University of Giessen (Germany) and by the current European Animal Welfare Legislation: ART13TFEU.

Parasite Maintenance

N. caninum (strain Nc1) tachyzoites were maintained by serial passages either in BUVEC or African green monkey kidney epithelial cells (MARC-145) according to Taubert et al. (2006). Cell supernatants containing egressed parasites were collected and centrifuged once at 200 x *g* for 2 min to eliminate cellular debris. The supernatant was transferred to a new tube and centrifuged again at 800 x *g* for 6 min. The pellet was resuspended in modified ECGM medium and tachyzoites were then counted in a Neubauer hemocyte chamber.

Depending on the experiment, BUVEC (4-6 biological replicates) were either seeded in µ-dishes of 35 mm diameter (IBIDI, Martinsried, Germany) for live cell 3D-holotomographic microscopy (3D Explorer, Nanolive), in 25 cm^2 culture flasks (Greiner) for FACS-based assays, or in 12-well plates (Greiner) containing fibronectin-coated (2.5 µg/ml, Thermo Fisher) glass coverslips (Nunc) for immunofluorescence assays. In all cases, plates were incubated at 37°C with a 5% CO_2 atmosphere until confluency. Then, host endothelial cells were infected with 2.4 × 10^6 (µ-dishes of 35 mm diameter), 2.4 ×10^5 (12-well plates) or 8.4 × 10^6 (25 cm^2 culture flasks) fresh tachyzoites. Cell culture medium was changed 1 day after infection and thereafter every second day. Infection rates were determined at 1 day post infection (p. i.) microscopically.

Monitoring of Intracellular *N. caninum* Tachyzoite Development in Bovine Umbilical Vein Endothelial Cells

Three BUVEC isolates were simultaneously seeded into 12-well plates containing 15 mm diameter fibronectin-coated (2.5 µg/ml, ThermoFisher) coverslips (Nunc). Three technical replicates were generated for each time point. All wells were infected at the same time. Samples were collected at different time points after infection (6, 12, 18, 24, 30, 36, and 42 h p. i.) and fixed in 4% paraformaldehyde (15 min, Roth). Tachyzoite development was analyzed by immunofluorescence using 4′,6-diamidin-2-phenylindol (DAPI) staining as a nuclear marker and an in-house canine hyperimmune serum to detect *N. caninum* stages. Randomly, the number of tachyzoites/PV were counted in ≥100 host cells per time point, BUVEC isolate and replicate.

Live-Cell 3D-Holotomographic Microscopy Analysis

Refractive index (RI)-based 3D-holotomographic images were obtained by a live cell 3D Cell Explorer-Fluo microscope (Nanolive) equipped with a ×60 magnification (λ = 520 nm, sample exposure 0.2 mW/mm2) and a depth of field of 30 µm. Cells were seeded on µ-dishes (35 mm diameter, IBIDI, Martinsried, Germany) and infected with *N. caninum* tachyzoites (MOI: 1:2). Non-infected and *N. caninum*-infected BUVEC were transferred to a top-stage incubator (IBIDI, Martinsried, Germany) to control temperature, humidity, and CO_2 levels during microscopy. Images were captured and analyzed using STEVE® software (Nanolive) to obtain a RI-based z-stack. For nuclei detection, samples were stained with DRAQ5 Fluorescent Probe® (5 µM, Thermo Fischer). Morphological alterations of host cell nuclei was evaluated by live cell imaging in non-infected and *N. caninum*-infected BUVEC stained with Hoechst 33,342 (0.2 µg/ml, ThermoFisher).

Protein Extraction

Proteins were extracted from infected and non-infected BUVEC by cell sonication (20 s, 5 times) in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF, all Roth) supplemented with a protease inhibitor cocktail (1:200, Sigma-Aldrich). Cell homogenates were centrifuged (10.000 × *g*, 10 min, 4°C) to sediment intact cells and nuclei. The RIPA buffer-soluble protein content of the respective supernatants was quantified via the Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) following the manufacturer's instructions.

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting

For immunoblotting, protein samples were supplemented with 6 M urea protein loading buffer. After boiling (95°C) for 5 min, proteins (60 µg/slot) were separated in 12% or 15% polyacrylamide gels via electrophoresis (100 V, 1.5 h; tetra system, Bio-Rad). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) (300 mA, 2 h at 4°C). Samples were blocked in 3% BSA in TBS [50 mM Tris-Cl, pH 7.6; 150 mM NaCl containing 0.1% Tween (blocking solution); Sigma-Aldrich] for 1 h at room temperature (RT) and then incubated in primary antibodies (**Table 1**) diluted in blocking solution (overnight, 4°C). Detection of vinculin was

TABLE 1 | Primary and secondary antibodies used in the current study.

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies				
Vinculin	Santa Cruz	sc-73614	mouse	1:1000
Cyclin A2	Abcam	ab38	mouse	1:1000
Cyclin B1	Abcam	ab32053	rabbit	1:3000
PCNA	Abcam	ab18197	rabbit	1:1000
Lamin B1	Abcam	ab16048	rabbit	1:2000
Neospora caninum	in-house	—	dog	1:50
Secondary antibodies				
Antigen/conjugate	Company	Cat. number	Host/target	Dilution
Goat anti-mouse IgG peroxidase-conjugated	Pierce	31430	goat/mouse	1:40,000
Goat anti-rabbit IgG peroxidase-conjugated	Pierce	31460	goat/rabbit	1:40,000
Alexa Fluor 594	ThermoFisher	R37117	goat/rabbit	1:500
Alexa Fluor 594	Jackson Immuno	304–585-003	rabbit/dog	1:500

used as a loading control for sample normalization. Following three washes in TBS-Tween 0.1% buffer, blots were incubated with secondary antibody (**Table 1**) solutions (dilution in blocking solution, 30 min, RT). Following three further washes in TBS-Tween (0.1%) buffer, signal detection was accomplished by an enhanced chemiluminescence detection system (ECL® plus kit, GE Healthcare) and recorded using a ChemoCam Imager (Intas Science Imaging). Protein masses were controlled by a protein ladder (PageRuler Plus® Prestained Protein Ladder ~10–250 kDa, Thermo Fisher Scientific). Protein band intensities were quantified by the Fiji Gel Analyzer plugin (Schindelin et al., 2012).

Immunofluorescence Assays

BUVEC layers were fixed with paraformaldehyde (4%, 15 min, RT; Roth), washed thrice with PBS and incubated in blocking/permeabilization solution (PBS with 3% BSA, 0.1% Triton X-100; 1 h, RT). Thereafter, samples were incubated in primary antibodies (**Table 1**) diluted in blocking/permeabilization solution (overnight, 4°C, in a humidified chamber). After three washes in PBS, samples were incubated in secondary antibody solutions (**Table 1**; 30 min at RT and complete darkness). Cell nuclei were labeled with DAPI-supplemented mounting medium (Fluoromount G, ThermoFisher).

Detection of *N. caninum* Infection-Driven Paracrine Effects

To study potential *N. caninum* infection-driven paracrine effects on bystander cells, we incubated non-infected BUVEC isolates ($n = 6$) with filtered (0.2 µm filter) supernatants either originating from non-infected control BUVEC or from the same BUVEC isolates that had been infected with *N. caninum* for 32 h. After 24 h of supplementation, host cells were collected, fixed and analyzed by FACS reading for cell cycle phases (FxCycle Far® red staining; Invitrogen) or probed for lamin B1 and actin.

Flow Cytometry-Based Analysis of Cell Cycle Phases

Cellular DNA content was measured using the FxCycle Far red stain reagent (Invitrogen, F10348) according to the manufacturer's instructions. The samples were analyzed by a BD LSRFortessa™ cell analyzer (Becton-Dickinson, Heidelberg, Germany) applying 633/5 nm excitation and emission collected in a 660/20 band-pass. Cells were gated according to their size and granularity. Exclusively morphologically intact host cells were included in the analysis.

Image Acquisition and Image Reconstruction

Fluorescence images were acquired with a ReScan Confocal microscope instrumentation (RCM 1.1 Visible, Confocal. nl) equipped with a fixed 50 µm pinhole size and combined with a Nikon Ti2-A inverted microscope. The microscope was equipped with a motorized Z-stage (DI1500, Nikon). The RCM unit was connected to the Toptica CLE laser with the following excitations: 405/488/561/640 nm. Images were taken via an sCMOS camera (PCO edge) using a CFI Plan Apochromat ×60 lambda-immersion oil objective (NA 1.4/0.13; Nikon). The setup was operated by the NIS-Elements software (version 5.11). Images were acquired via z-stack optical series with a step size of 0.1 microns depth to cover all structures of interest within the analyzed host cells. The Z-series were displayed as maximum z-projections. Identical brightness and contrast conditions were applied for each data set within one experiment using Fiji software (Schindelin et al., 2012).

Further, images were edited by deconvolution software (3D deconvolution module, NIS-Element module, Nikon). The algorithm used for image deconvolution was selected depending on the structures to be shown in the pictures: Landweber for lamin B1 (20 iterations), Richardson-Lucy for phalloidin (20 iterations). Deconvoluted images were displayed as maximum z-projections, brightness, and contrast were adjusted using Fiji software (Schindelin et al., 2012). Deconvoluted z-stacks were submitted to the NIS-Element software volume viewer module, applying maximum intensity to z-projections.

Proliferation cell nuclear antigen (PCNA) localization analyses were performed by an automated selection of the nuclear area using the Fiji software, applying the following workflow: An Otsu threshold was subjected to the DAPI channel to obtain the total nuclear area. Particles larger than 800 pixels were selected and merged with the PCNA channel (nuclear selection is exemplary illustrated in **Figure 3** as white circles surrounding the nuclei). The number of host cells in each S-subphase was counted manually according to the instructions

given by Schönenberger et al. (2015). For nuclear size analysis, ROIs were measured using Fiji measure plugins following nuclear selection. Cell nuclei were segmented using Otsu thresholding as the binary image. Finally, morphological features (circularity, axes ratio, area and average intensity) were obtained using particle analysis in Fiji software. Actin-related average intensity per host cell was calculated using the pixel area enclosed in the nuclear masks.

Transmission Electron Microscopy Analysis

Three BUVEC isolates were grown until confluence in T-75 cm^2 culture flasks (Greiner), infected with freshly isolated *N. caninum* tachyzoites and cultured until 32 h p. i. Then cells were washed with PBS and fixed with 3 ml of fixing solution per flask (0.25% glutaraldehyde, 0.1 M cacodylate buffer and 4% PFA; Merck). After 2 min of treatment, the cells were gently scraped in larger pieces from the flask bottom to preserve the monolayer by using a rubberpolice (Greiner). After 24 h of fixation, samples were washed with 0.15 M Hepes buffer and stabilized with 1% osmic acid for 2 h. For contrasting, the samples were incubated overnight in half-saturated uranyl acetate solution (both Merck) and then washed with distilled water. Samples were dehydrated in an ascending ethanol series and finally embedded in Agar 100 resin (Agar scientific Ltd. United Kingdom). Ultrathin sections were cut using an ultramicrotome (Reichert Ultracut E, Leica) and examined in a transmission electron microscope (Zeiss EM 902). Digital images were captured with a slow-scan 2 K CCD camera (TRS, Tröndle, Moorenweis, Germany).

STATISTICAL ANALYSIS

All data were expressed as mean ± SD from three independent experiments. In all cases, an unpaired *t*-test (non-parametric) was performed to compare infected and non-infected/non-treated data sets. Significance was defined as $p \leq 0.05$. All graphs and statistical analyses were performed using GraphPad Prism® 9 software.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: ZV and AT. Data curation: ZV and AT. Formal analysis: ZV. Funding: AT. Acquisition: ZV. Investigation: ZV. Methodology: ZV. Writing—original draft preparation: ZV and AT. Writing—review and editing: ZV, AT, and CH. ZV, LR-B, and CL performed most of the experiments. ZV performed 3D holotomography, confocal microscopy, and figure preparation. MS performed nuclear parameter-based analyses, UG conducted TEM analysis, and LP-O and SH helped in FACS-based experiments. All authors revised and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Authors would like to acknowledge A. Wehrend (Clinic for Obstetrics, Gynecology, and Andrology of Large and Small Animals, Justus Liebig University Giessen, Giessen, Germany) for the continuous supply of bovine umbilical cords.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.946335/full#supplementary-material

Supplementary Figure S1 | FACS-based analysis showing the gating strategy of BUVEC cells and the representative histogram. In a first step, the total cell population was gated according to shape and granularity of the cells **(A)**. In a second step, the PI-positive population was selected **(B)** and the representative histogram displaying main peaks for the G0/1 (2N)-, S- and the G2/M-phase (4N) was generated **(C)**.

Supplementary Video S1 | Lamin B1 detection in *N. caninum*-infected cell layers (32 h p. i., 3D-reconstruction). PFA-fixed *N. caninum*-infected BUVEC were stained for Lamin B1 (green; the image corresponds to the one shown in **Figure 7A**). 3D reconstruction was performed by NIS element software built-in plugins. Background and signal levels were equally adjusted to illustrate nuclear membrane folding of the cells.

Supplementary Video S2 | F- actin detection in *N. caninum*-infected cells (32 h p. i., 3D-reconstruction). PFA-fixed *N. caninum*-infected BUVEC were stained for F-actin (Phalloidin: green; the image corresponds to the one shown in **Figure 7A**). 3D reconstruction was performed by NIS element software built-in plugins. Background and signal levels were equally adjusted to illustrate the actin cytoskeleton in both non-infected and *N. caninum*-infected cells.

Supplementary Video S3 | F- actin detection in *N. caninum*-infected cells (32 h p. i., 3D-reconstruction). PFA-fixed *N. caninum*-infected BUVEC were stained for F-actin (Phalloidin: green; the image corresponds to the one shown in **Figure 7A**). 3D reconstruction was performed by NIS element software built-in plugins. Background and signal levels were equally adjusted to illustrate the actin cytoskeleton in both non-infected and *N. caninum*-infected cells.

REFERENCES

Barr, B. C., Conrad, P. A., Breitmeyer, R., Sverlow, K., Anderson, M. L., Reynolds, J., et al. (1993). Congenital Neospora Infection in Calves Born from Cows that Had Previously Aborted Neospora-Infected Fetuses: Four Cases (1990-1992). *J. Am. Vet. Med. Assoc.* 202 (1), 113–117.

Barr, B. C., Rowe, J. D., Sverlow, K. W., BonDurant, R. H., Ardans, A. A., Oliver, M. N., et al. (1994). Experimental Reproduction of Bovine Fetal Neospora Infection and Death with a Bovine Neospora Isolate. *J. Vet. Diagn Invest* 6 (2), 207–215. doi:10.1177/104063879400600212

Brunet, A., Forsberg, F., Fan, Q., Sæther, T., and Collas, P. (2019). Nuclear Lamin B1 Interactions with Chromatin during the Circadian Cycle Are Uncoupled from Periodic Gene Expression. *Front. Genet.* 10, 917. doi:10.3389/fgene.2019.00917

Brunet, J., Pfaff, A. W., Abidi, A., Unoki, M., Nakamura, Y., Guinard, M., et al. (2008). Toxoplasma Gondii Exploits UHRF1 and Induces Host Cell Cycle Arrest at G2 to Enable its Proliferation. *Cell Microbiol.* 10 (4), 908–920. doi:10.1111/j.1462-5822.2007.01093.x

Coppens, I., Sinai, A. P., and Joiner, K. A. (2000). Toxoplasma Gondii Exploits Host Low-Density Lipoprotein Receptor-Mediated Endocytosis for Cholesterol Acquisition. *J. Cell Biol.* 149 (1), 167–180. doi:10.1083/jcb.149.1.167

Dobrowolski, J. M., and Sibley, L. D. (1996). Toxoplasma Invasion of Mammalian Cells Is Powered by the Actin Cytoskeleton of the Parasite. *Cell* 84 (6), 933–939. doi:10.1016/s0092-8674(00)81071-5

Dubey, J. P., Lindsay, D. S., Anderson, M. L., Davis, S. W., and Shen, S. K. (1992). Induced Transplacental Transmission of Neospora Caninum in Cattle. *J. Am. Vet. Med. Assoc.* 201 (5), 709–713.

Dubey, J. P., Schares, G., and Ortega-Mora, L. M. (2007). Epidemiology and Control of Neosporosis and Neospora Caninum. *Clin. Microbiol. Rev.* 20 (2), 323–367. doi:10.1128/cmr.00031-06

Fung, T. K., Ma, H. T., and Poon, R. Y. C. (2007). Specialized Roles of the Two Mitotic Cyclins in Somatic Cells: Cyclin A as an Activator of M Phase-Promoting Factor. *MBoC* 18 (5), 1861–1873. doi:10.1091/mbc.e06-12-1092

Furuno, N., den Elzen, N., and Pines, J. (1999). Human Cyclin A Is Required for Mitosis until Mid Prophase. *J. Cell Biol.* 147 (2), 295–306. doi:10.1083/jcb.147.2.295

García-Sánchez, M., Jiménez-Pelayo, L., Horcajo, P., Collantes-Fernández, E., Ortega-Mora, L. M., and Regidor-Cerrillo, J. (2020). Neospora Caninum Infection Induces an Isolate Virulence-dependent Pro-inflammatory Gene Expression Profile in Bovine Monocyte-Derived Macrophages. *Parasit. Vectors* 13 (1), 374.

Goebel, S., Gross, U., and Lüder, C. G. K. (2001). Inhibition of Host Cell Apoptosis by Toxoplasma Gondii Is Accompanied by Reduced Activation of the Caspase Cascade and Alterations of poly(ADP-Ribose) Polymerase Expression. *J. Cell Sci.* 114 (19), 3495–3505. doi:10.1242/jcs.114.19.3495

Goodswen, S. J., Kennedy, P. J., and Ellis, J. T. (2013). A Review of the Infection, Genetics, and Evolution of Neospora Caninum: from the Past to the Present. *Infect. Genet. Evol.* 13, 133–150. doi:10.1016/j.meegid.2012.08.012

Graewe, S., Rankin, K. E., Lehmann, C., Deschermeier, C., Hecht, L., Froehlke, U., et al. (2011). Hostile Takeover by Plasmodium: Reorganization of Parasite and Host Cell Membranes during Liver Stage Egress. *PLoS Pathog.* 7 (9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3164640/.

Hartwell, L. H., and Weinert, T. A. (1989). Checkpoints: Controls that Ensure the Order of Cell Cycle Events. *Science* 246 (4930), 629–634. doi:10.1126/science.2683079

Hemphill, A., Gottstein, B., and Kaufmann, H. (1996). Adhesion and Invasion of Bovine Endothelial Cells byNeospora Caninum. *Parasitology* 112 (2), 183–197. doi:10.1017/s0031182000084754

Hermosilla, C., Schröpfer, E., Stowasser, M., Eckstein-Ludwig, U., Behrendt, J. H., and Zahner, H. (2008). Cytoskeletal Changes in Eimeria Bovis-Infected Host Endothelial Cells during First Merogony. *Vet. Res. Commun.* 32 (7), 521–531. doi:10.1007/s11259-008-9054-x

Hermosilla, C., Zahner, H., and Taubert, A. (2006). Eimeria Bovis Modulates Adhesion Molecule Gene Transcription in and PMN Adhesion to Infected Bovine Endothelial Cells. *Int. J. Parasitol.* 36 (4), 423–431. doi:10.1016/j.ijpara.2006.01.001

Hetzer, M. W. (2010). The Nuclear Envelope. *Cold Spring Harb. Perspect. Biol.* 2 (3), a000539. doi:10.1101/cshperspect.a000539

Horcajo, P., Jiménez-Pelayo, L., García-Sánchez, M., Regidor-Cerrillo, J., Collantes-Fernández, E., Rozas, D., et al. (2017). Transcriptome Modulation of Bovine Trophoblast Cells *In Vitro* by Neospora Caninum. *Int. J. Parasitol.* 47 (12), 791–799. doi:10.1016/j.ijpara.2017.08.007

Jiménez-Pelayo, L., García-Sánchez, M., Regidor-Cerrillo, J., Horcajo, P., Collantes-Fernández, E., Gómez-Bautista, M., et al. (2019). Immune Response Profile of Caruncular and Trophoblast Cell Lines Infected by High- (Nc-Spain7) and Low-Virulence (Nc-Spain1H) Isolates of Neospora Caninum. *Parasit. Vectors* 12 (1), 218.

Kastan, M. B., and Bartek, J. (2004). Cell-cycle Checkpoints and Cancer. *Nature* 432 (7015), 316–323. doi:10.1038/nature03097

Khatau, S. B., Hale, C. M., Stewart-Hutchinson, P. J., Patel, M. S., Stewart, C. L., Searson, P. C., et al. (2009). A Perinuclear Actin Cap Regulates Nuclear Shape. *Proc. Natl. Acad. Sci. U.S.A.* 106 (45), 19017–19022. doi:10.1073/pnas.0908686106

Kim, D.-H., Chambliss, A. B., and Wirtz, D. (2013). The Multi-Faceted Role of the Actin Cap in Cellular Mechanosensation and Mechanotransduction. *Soft Matter* 9 (23), 5516–5523. doi:10.1039/c3sm50798j

Lang, M., Kann, M., Zahner, H., Taubert, A., and Hermosilla, C. (2009). Inhibition of Host Cell Apoptosis by Eimeria Bovis Sporozoites. *Vet. Parasitol.* 160 (1), 25–33. doi:10.1016/j.vetpar.2008.10.100

Lavine, M. D., and Arrizabalaga, G. (2009). Induction of Mitotic S-phase of Host and Neighboring Cells by Toxoplasma Gondii Enhances Parasite Invasion. *Mol. Biochem. Parasitol.* 164 (1), 95–99. doi:10.1016/j.molbiopara.2008.11.014

Lei, Y., Davey, M., and Ellis, J. T. (2005). Attachment and Invasion ofToxoplasma gondiiandNeospora Caninumto Epithelial and Fibroblast Cell Linesin Vitro. *Parasitology* 131 (5), 583–590. doi:10.1017/s0031182005008310

Maninova, M., Caslavsky, J., and Vomastek, T. (2017). The Assembly and Function of Perinuclear Actin Cap in Migrating Cells. *Protoplasma* 254 (3), 1207–1218. doi:10.1007/s00709-017-1077-0

Meier, J., Campbell, K. H., Ford, C. C., Stick, R., and Hutchison, C. J. (1991). The Role of Lamin LIII in Nuclear Assembly and DNA Replication, in Cell-free Extracts of Xenopus Eggs. *J. Cell Sci.* 98 (Pt 3), 271–279. doi:10.1242/jcs.98.3.271

Moir, R. D., Spann, T. P., Herrmann, H., and Goldman, R. D. (2000). Disruption of Nuclear Lamin Organization Blocks the Elongation Phase of DNA Replication. *J. Cell Biol.* 149 (6), 1179–1192. doi:10.1083/jcb.149.6.1179

Molestina, R. E., El-Guendy, N., and Sinai, A. P. (2008). Infection with Toxoplasma Gondii Results in Dysregulation of the Host Cell Cycle. *Cell Microbiol.* 10 (5), 1153–1165. doi:10.1111/j.1462-5822.2008.01117.x

Molestina, R. E., Payne, T. M., Coppens, I., and Sinai, A. P. (2003). Activation of NF-Kb byToxoplasma Gondiicorrelates with Increased Expression of Antiapoptotic Genes and Localization of Phosphorylated IκB to the Parasitophorous Vacuole Membrane. *J. Cell Sci.* 116 (21), 4359–4371. doi:10.1242/jcs.00683

Newport, J. W., Wilson, K. L., and Dunphy, W. G. (1990). A Lamin-independent Pathway for Nuclear Envelope Assembly. *J. Cell Biol.* 111 (6 Pt 1), 2247–2259. doi:10.1083/jcb.111.6.2247

Nishikawa, Y., Quittnat, F., Stedman, T. T., Voelker, D. R., Choi, J.-Y., Zahn, M., et al. (2005). Host Cell Lipids Control Cholesteryl Ester Synthesis and Storage in Intracellular Toxoplasma. *Cell Microbiol.* 7 (6), 849–867. doi:10.1111/j.1462-5822.2005.00518.x

Nolan, S. J., Romano, J. D., Luechtefeld, T., and Coppens, I. (2015). Neospora Caninum Recruits Host Cell Structures to its Parasitophorous Vacuole and Salvages Lipids from Organelles. *Eukaryot. Cell* 14 (5), 454–473. doi:10.1128/ec.00262-14

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A Is Required at Two Points in the Human Cell Cycle. *EMBO J.* 11 (3), 961–971. doi:10.1002/j.1460-2075.1992.tb05135.x

Petersen, B. O., Lukas, J., Sørensen, C. S., Bartek, J., and Helin, K. (1999). Phosphorylation of Mammalian CDC6 by Cyclin A/CDK2 Regulates its Subcellular Localization. *EMBO J.* 18 (2), 396–410. doi:10.1093/emboj/18.2.396

Regidor-Cerrillo, J., Xia, D., Jiménez-Pelayo, L., García-Sánchez, M., Collantes-Fernández, E., Randle, N., et al. (2020). Proteomic Characterization of Host-Pathogen Interactions during Bovine Trophoblast Cell Line Infection by Neospora Caninum. *Pathogens* 9 (9), 749. doi:10.3390/pathogens9090749

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an Open-Source Platform for Biological-Image Analysis. *Nat. Methods* 9 (7), 676–682. doi:10.1038/nmeth.2019

Schönenberger, F., Deutzmann, A., Ferrando-May, E., and Merhof, D. (2015). Discrimination of Cell Cycle Phases in PCNA-Immunolabeled Cells. *BMC Bioinforma.* 16. Available from: http://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-015-0618-9.

Shimi, T., Pfleghaar, K., Kojimaichiro, S.-i., Pack, C.-G., Solovei, I., Goldman, A. E., et al. (2008). The A- and B-type Nuclear Lamin Networks: Microdomains Involved in Chromatin Organization and Transcription. *Genes Dev.* 22 (24), 3409–3421. doi:10.1101/gad.1735208

Shumaker, D. K., Solimando, L., Sengupta, K., Shimi, T., Adam, S. A., Grunwald, A., et al. (2008). The Highly Conserved Nuclear Lamin Ig-fold Binds to PCNA: its Role in DNA Replication. *J. Cell Biol.* 181 (2), 269–280. doi:10.1083/jcb.200708155

Spann, T. P., Goldman, A. E., Wang, C., Huang, S., and Goldman, R. D. (2002). Alteration of Nuclear Lamin Organization Inhibits RNA Polymerase II-dependent Transcription. *J. Cell Biol.* 156 (4), 603–608. doi:10.1083/jcb.200112047

Taubert, A., Zahner, H., and Hermosilla, C. (2006). Dynamics of Transcription of Immunomodulatory Genes in Endothelial Cells Infected with Different Coccidian Parasites. *Vet. Parasitol.* 142 (3), 214–222. doi:10.1016/j.vetpar.2006.07.021

Taubert, A., Wimmers, K., Ponsuksili, S., Jimenez, C. A., Zahner, H., and Hermosilla, C. (2010). Microarray-based Transcriptional Profiling ofEimeria Bovis-Infected Bovine Endothelial Host Cells. *Vet. Res.* 41 (5), 70. doi:10.1051/vetres/2010041

Velásquez, Z. D., Conejeros, I., Larrazabal, C., Kerner, K., Hermosilla, C., and Taubert, A. (2019). Toxoplasma Gondii-Induced Host Cellular Cell Cycle Dysregulation Is Linked to Chromosome Missegregation and Cytokinesis Failure in Primary Endothelial Host Cells. *Sci. Rep.* 9 (1), 12496. doi:10.1038/s41598-019-48961-0

Velásquez, Z. D., López-Osorio, S., Mazurek, S., Hermosilla, C., and Taubert, A. (2021). Eimeria Bovis Macromeront Formation Induces Glycolytic Responses and Mitochondrial Changes in Primary Host Endothelial Cells. *Front. Cell Infect. Microbiol.* 11, 703413.

Velásquez, Z. D., López-Osorio, S., Waiger, D., Manosalva, C., Pervizaj-Oruqaj, L., Herold, S., et al. (2020). Eimeria Bovis Infections Induce G1 Cell Cycle Arrest and a Senescence-like Phenotype in Endothelial Host Cells. *Parasitology* 26, 1–13.

Velásquez, Z. D., Lopez-Osorio, S., Pervizaj-Oruqaj, L., Herold, S., Hermosilla, C., and Taubert, A. (2020). Besnoitia Besnoiti-Driven Endothelial Host Cell Cycle Alteration. *Parasitol. Res.* 119, 2563–2577. doi:10.1007/s00436-020-06744-x

Vélez, J., Velasquez, Z., Silva, L. M. R., Gärtner, U., Failing, K., Daugschies, A., et al. (2021). Metabolic Signatures of Cryptosporidium Parvum-Infected HCT-8 Cells and Impact of Selected Metabolic Inhibitors on C. Parvum Infection under Physioxia and Hyperoxia. *Biol. (Basel)* 10 (1), 60.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

3. RESULTS AND DISCUSSION

The current work mainly focused on molecular and cellular mechanisms involved in *T. gondii*-driven cell cycle dysregulation in a specific host cell type, i. e. primary bovine endothelial cells (BUVEC). In former studies, species-specific effects driven by different coccidian parasites on the cell cycle of the identical host cell type (BUVEC) were already demonstrated. In this context, infections with *B. besnoiti* triggered a host cell cycle stasis at G1/S transition concomitant with a significant upregulation of cyclin E1 and of histone H3 (S10) phosphorylation in infected BUVEC (Velásquez *et al*., 2020). In the same host cell type, infections with *E. bovis* also induced a cell cycle arrest at G1/S transition and cyclin E1 upregulation but additionally caused premature senescence during first merogony (Velásquez *et al*., 2021b). In contrast, *T. gondii* fostered a G2/M-phase arrest in BUVEC, which was accompanied by an increased proportion of binucleated cells, thereby indicating cytokinesis failure (Velásquez *et al*., 2019). In line, Aurora B kinase, a key regulator of cytokinesis onset, was reduced in its abundance and changed in cellular distribution. Regarding to different phases of mitosis, several severe alterations like cell spindle malformation, chromosome missegregation and supernumerary centrosome formation were observed in *T. gondii*-infected BUVEC (Velásquez *et al*., 2019).

Referring to this cell cycle-related background, the current project aimed to deepen the knowledge on *T. gondii*-triggered cell cycle perturbation by especially focusing on the role of the host cell origin, cell type and parasite strain in addition to host cellular DNA damage responses. Moreover, to elucidate coccidian species-specific reactions, comparative analyses on the closely related apicomplexan parasite *N. caninum* were performed. By intention, all experimentation (except for the cell type-related studies) was conducted in the same primary endothelial cell type in order (*i*) to avoid immortalization-driven effects on cell cycle regulation, as commonly reported for permanent tumorous cell lines, (*ii*) to be as close as possible to the *in vivo* scenario, and (*iii*) to perform current studies in exactly the same cell type as previous reports on the *T. gondii* RH strain, thereby allowing for direct comparison of the data.

3.1. *T. gondii*-driven alteration of endothelial host cell cycling is mainly independent of the host cell type or cell donor species

T. gondii infections were described to foster host cell cycle arrest between the G1 and G2/M transition phase, depending on the experimental model (Brunet *et al*., 2008;

Molestina *et al*., 2008; Pierre-Louis *et al*., 2022; Velásquez *et al*., 2019). However, these studies were carried-out under different experimental conditions in terms of host cell type, MOI or time point of infection. Thus, some studies used immortalized or tumour cells, which are well known to differ substantially from primary cells in terms of cell cycle regulation. In the current work, different primary cell types (endothelial cells, epithelial cells, fibroblasts) of human (HUVEC, FHs74, HFF) and bovine (BSIEC, BCEC) origin were infected with the *T. gondii* RH strain and analyzed for cell cycling to assess the impact of the cell type and donor species and to exclude artefacts driven by tumorous cell characteristics. Using a FACS-based approach for DNA content measurement to assess cell cycle phases, almost all cell types showed an equal reaction pattern with a higher proportion of cells being arrested in S-phase (except for BCEC, in which *T. gondii* infection did not affect cell cycle progression), when compared to non-infected cells thereby proving that the overall parasite-driven effects on cell cycle arrest was cell type- and origin-independent (**Publication 1**). Of note, minor deviations in reaction patterns were observed since HUVEC and HFF additionally showed a decreased number of cells in G1-phase, and only HFF experienced a reduced percentage of cells in G2-phase. So far, it remains unclear why BCECs resisted to *T. gondii*-driven cell cycle alteration, especially since this cell type showed a similar growth behaviour and equal kinetics of intracellular *T. gondii* development like the other cell types. The finding of a *T. gondii*-driven S-phase arrest in principle matches with former data. Hence, Molestina *et al.* (2008) revealed an accumulation of cells in S-phase with a progressive decrease in G2/M transition for *T. gondii*-infected HFF cells, suggesting a delayed exit from S-phase. Likewise, Kim *et al.* (2016) demonstrated a decreased proliferation in a *T. gondii*-infected L6 rat myoblast cell line suggesting a tendency of the cells to remain in S- or G2/M-phase. However, current results contrast on findings in human trophoblast cells (BeWo) and human dermal fibroblasts (NHDF) since even though *T. gondii* infections stimulated G1/S transition, the cell cycle arrest occurred in the G2-phase concomitant with cyclin B1 downregulation (Brunet *et al*., 2008; Sabou *et al*., 2020). Comparable results were found in infected bovine endothelial cells (BUVEC) by a cell cycle arrest in G2/M transition (Velásquez *et al*., 2019).

When considering typical events in different cell cycle phases, it seems tempting to hypothesize that an S-phase arrest would be beneficial for *T. gondii* since the cell is then stuck in the main phase of DNA synthesis, from which the parasite could profit.

Of note, not only infected cells but also non-infected neighbouring cells showed enhanced signals of an S-phase marker (BrdU), suggesting that soluble factors from infected cells or the parasites themselves may affect the cell cycle status without the need of the parasite to physically invade or even contact the cell (Kim *et al*., 2016; Lavine and Arrizabalaga, 2009). In line, Grimwood *et al* (1996) demonstrated a better efficiency of *T. gondii* invasion and infection in cells experiencing S-phase, suggesting that cells in S-phase indeed bear advantages for *T. gondii* development. In this respect, Correa *et al*. (2007) hypothesized that S-phase arrest of non-infected neighbouring cells allows for an improved adhesion and invasion in new host cells once the parasite egresses, thereby reducing the exposure duration in the potentially adverse, extracellular environment. Moreover, Pierre-Louis *et al.* (2022) demonstrated that S-phase-arrested host cells fail to incorporate new DNA molecules on their own. It is well-documented that *T. gondii* lacks the capacity of own purine and pyrimidine *de novo* synthesis (Campagnaro *et al*., 2022; de Koning *et al*., 2005; Donaldson *et al*., 2014; Krug *et al*., 1989; Schwartzman and Pfefferkorn, 1981; Sullivan *et al*., 1999), consequently the parasite will profit from its S-phase-arrested host cell by enhancing the chance to scavenge the molecules required for its own division. In line, *T. gondii*-driven nucleotide scavenge was reported for different cell lines (Coppens *et al*., 2006a; Pratt *et al*., 2013; Romano *et al*., 2013). A further explanation for cell cycle arrest in S-phase is that during this phase also protein, amino sugar and pentose phosphate syntheses are upregulated, besides other metabolic pathways (e. g. glycolysis/gluconeogenesis, nucleotide sugar metabolism), all of which are fundamental for both, host cell maintenance and intracellular parasite proliferation (Regidor-Cerrillo *et al*., 2020; Velásquez *et al*., 2021a; Vélez *et al*., 2021). Considering that tachyzoite replication is an energy- and building block-demanding process, the access to any kind of these molecules would ease parasite proliferation on one hand, and, on the other hand, the transient or continuous deprivation of essential molecules could indeed induce an energy-lacking status in infected host cells, finally causing a block in DNA synthesis and/or mitotic process.

Obviously, S-phase stasis has the consequence that these cells fail to progress into G2-phase and mitosis. In general, cell cycle progression is tightly controlled by different checkpoints regulating the entry into the subsequent phase or the exit from the current cell cycle phase. Any irregularities, potentially occurring in each type of cell cycle phase, may block cell cycle progression. Cyclins and CDKs form part of these cell cycle

checkpoints. Given that cyclin B1 starts to accumulate at the end of G2-phase with a peak at the onset of M-phase and subsequent degradation, signifying the key event that allows cells to enter mitosis (Lindqvist *et al*., 2007; Vorlaufer and Peters, 1998), we here quantified its abundance in the different cell types. However, none of the currently used cell types showed any changes in cyclin B1 expression at 24 hours p. i. (**Publication 1**), thereby suggesting that mitosis checkpoint modulation seemed not involved in *T. gondii*-driven host cell arrest in S-phase. These findings are in agreement with Velásquez *et al.* (2019), where cyclin B1 levels also proved unaffected by *T. gondii* infection in BUVEC. However, contrasting results were found in *T. gondii*-infected HFF. Here, cyclin B1 gene expression was found increased at later time points of infection (32 h p. i.) despite an apparent block in cell progression to the G2/M stage, concomitant with a poor response of mitotic regulators like MAD2L1 and MAD2L2 (mitotic spindle assembly checkpoint proteins) (Molestina *et al*., 2008). The delayed but sustained effects in cyclin B1 levels in *T. gondii* infections seemed compatible with a delayed exit from S-phase and undefined progression through G2/M transition with an eventual perturbation in the mitotic process (Molestina *et al*., 2008). In contrast to HFF, cyclin B1 expression was notably downregulated in human trophoblast and human dermal fibroblast, suggesting that *T. gondii* infection fosters cyclin B1 dysregulation to maintain host cell stasis in the G2/M transition phase (Brunet *et al*., 2008; Sabou *et al*., 2020).

It has to be emphasized that an S-phase arrest of *T. gondii*-infected host cells never applied to all cells within an infected cell layer, but always affected only a sub-fraction of this cell population. Thus, *T. gondii* may in parallel drive additional cell cycle-related alterations in host cells besides S-phase stasis. In line, Velasquez *et al*. (2019) described *T. gondii* infections to induce mitotic errors in BUVEC leading to impaired cytokinesis. In the current study, aberrant mitosis was defined as mitotic spindle-related alterations resulting in chromosome missegregation, mislocated centrioles or supernumerary centrosome formation. Whilst the mitotic index was not affected in bovine cell types (BSIEC, BCEC), FHs74 and HFF revealed a parasite-driven enhancement of this cell cycle parameter (**Publication 1**). When estimating proper mitosis progression from prophase to telophase, aberrant mitosis was detected in all mitotic subphases. Thus, more than two centrosome poles as well as chromosome miscondensation was observed in prometa- or metaphase and supernumerary centrosomes were found in telophase, thereby confirming that *T. gondii* indeed drives mitotic failures, independent of cell donor origin or cell type (**Publication 1**). These

data perfectly matched with findings of Velásquez *et al*. (2019) describing similar parasite-mediated cell cycle defects in BUVEC. Interestingly, it is well-documented that *T. gondii* infection fosters microtubule rearrangement in host cells resulting in aberrant chromosome alignment (Coppens *et al*., 2006b). In non-infected cells and under physiological conditions, microtubules radiate from the host nucleus to form an ordered network throughout the cytoplasm to allow mitotic spindle organization, which aids chromosome separation during cell division (Naghavi and Walsh, 2017). Notably, in *T. gondii*-infected fibroblasts, microtubules formed circular, basket-like structures in close proximity to the PV, thereby re-modelling the host cell by giving rise to a larger space for parasite duplication, but also causing mitotic defects due to microtubule malarrangement (Walker *et al*., 2008). Moreover, *T. gondii* was reported to increase the number of γ-tubulin-based foci within the host cell, suggesting a disruption of mitotic spindles or decoupling MTOC duplication from mitosis (Walker *et al*., 2008). Overall, it seems that the recruitment of microtubules to the PV is not cell type-specific since this phenomenon occurs in several cell types like BCS-1 cells and Raw 264.7 macrophages-like cell line (Walker *et al*., 2008) and results in numerical and structural chromosome alterations, which finally lead to chromosome instability (Levine and Holland, 2018).

Whenever a cell fails to correctly pass through all mitotic phases, a block in cytokinesis will be the logical consequence to avoid the formation of daughter cells with faulty genomic information. As a final parameter of cell cycling, all cell types used in this study were analysed for proper daughter cell formation (**Publication 1**). Cytokinesis failure implies a defect in cytosol division at the end of mitosis, thereby blocking the final mother cell division into two daughter cells (Alberts *et al*., 2007). In case of cytokinesis inhibition, cells display two nuclei (binucleated phenotype). When estimating the number of nuclei in *T. gondii*-infected host cells, all cell types (BSIEC, BCEC, HUVEC, FHs74, HFF) uniformly showed the same reaction pattern and presented a significantly enhanced occurrence of the binucleated phenotype, thereby indicating that all cell types indeed experienced cytokinesis failure, rendering this parasite-driven alteration as cell type- and donor origin-independent (**Publication 1**). In line, Velásquez *et al*. (2019) also reported on cytokinesis failures in *T. gondii*-infected BUVEC. Moreover, Franco *et al*. (2016) demonstrated an enhanced proportion of *T. gondii*-infected murine RAW264 cells experiencing polyploidy (8n), probably due to cytokinesis impairment. So far, it remains entirely unknown if this

phenomenon results from an initial parasite-induced insult (i. e. by arresting the cell in S-phase), whether it is driven by intention by the parasite or if it simply signifies a collateral effect. However, mitosis and cytokinesis both require proper cytoskeleton function, especially of the tubulin system mediating mitotic spindle or midbody formation (Kline-Smith and Walczak, 2004). As already mentioned above, *T. gondii* is well-known to modulate the host cellular tubulin cytoskeleton by fostering tubulin accumulation around the PV and γ-tubulin foci formation, thereby inducing critical changes in microtubule dynamics and cytokinesis failure in infected cells (Walker et al., 2008). Therefore, current data may also be interpreted in the context of a *T. gondii* infection-driven impact on host cellular cytoskeletal dynamics, which, in final consequence, blocks proper host cell division as a general mechanism, irrespective of host cell origin and type.

3.2. Basic mechanisms of *T. gondii*-mediated cell cycle dysregulation do not differ with the parasite strain

It is well-documented that *T. gondii* owns a considerable genetic and phenotypic diversity, currently being represented by three main clonal lineages (I-III), which vary in pathogenicity and mortality (Fernández-Escobar *et al*., 2021). The *T. gondii* type I strain is considered as the most pathogenic strain and causes lethal infections in mice, whilst mortality and tachyzoite dissemination are significantly lower in infections with type II and III strains. Moreover, type III strains are even described as apathogenic in mice (Calero-Bernal *et al*., 2022; Dardé *et al*., 2014; Sibley and Boothroyd, 1992; Su *et al*., 2002). Representative strains for each lineage are *T. gondii* RH for haplotype I, Me49 for haplotype II and NED for haplotype III (Wu *et al*., 2022). Overall, the *T. gondii* RH haplotype is the most studied strain *in vitro*, while the NED strain is more commonly used in *in vivo* murine models (Croken *et al*., 2014; Wang and Sibley, 2019). Whilst mainly the classical haplotypes I-III with an estimated moderate to low virulence circulate in Europe, atypical and mixed haplotypes eventually bearing high pathogenicity are spread in other regions like South America (Dardé *et al*., 2014; Galal *et al*., 2022; Miller *et al*., 2023).

One aim of this study was to assess the potential influence of different *T. gondii* haplotypes on host cell cycle dysregulation. In addition to the RH strain used for experimentation in **Publication 1**, *T. gondii* Me49 (haplotype II) and NED (haplotype III) strains were here applied in parallel for strain-dependent analyses. To allow for

direct comparisons with the data on the RH strain (**Publication 1**), experiments were performed in the same primary bovine endothelial host cell type (BUVEC). By first assessing growth kinetics of Me49 and NED tachyzoites in BUVEC, a comparable developmental speed of all strains was confirmed, thereby excluding potential kinetic-driven differences in host cell cycling. Referring to RH strain-based host cell reactions (**Publication 1**), similar parameters of host cell cycling were here assessed using Me49 and NED tachyzoites for BUVEC infections: *i*) DNA content-based analysis of the cell cycle phases via FACS, *ii*) microscopic analysis of the mitotic rate and of mitotic spindle formation, *ii*) microscopic assessment of cytokinesis (by analysing the proportion of binucleated cells in infected cell layers), and *iv*) quantification of cyclin A2 and cyclin B1 expression in infected cell layers at 24 hours p. i. via Western blotting.

In line with RH-based data, an enhanced proportion of cells of both *T. gondii* Me49- and NED-infected BUVEC experienced a parasite infection-driven stasis in S-phase (**Publication 2**). Simultaneously, a significantly decreased proportion of cells were in G1-phase. When analysing the expression of cyclins A2 and B1, which signify key regulatory proteins of S-phase control and M-phase enter, respectively, only NED tachyzoite infections fostered a cyclin B1 upregulation whilst cyclin A2 expression was not affected by any strain (**Publication 2**), thereby differing from infected HFF, in which an increased cyclin A2 level was found by RT-PCR with a delayed response by Westernblot-based profiling, indicating a delayed exit from S-phase (Molestina *et al*., 2008). In line, the mitotic rate was found significantly reduced only for NED tachyzoite-infected BUVEC (**Publication 2**).

To verify whether mitotic failures may also occur in Me49 and NED strain-infected BUVEC and eventually be linked to inadequate mitotic spindle and centrosome formation (Levine and Holland, 2018) like in case of RH strain infections (**Publication 1**), mitosis spindle formation and different mitosis phases were here analysed. Indeed, approximately 10% of infected mitotic cells showed an altered chromosome arrangement (**Publication 2**). Moreover, some host cells with irregular chromosome bridges between the poles of the mitotic spindle were detected. Notably, these chromosome bridges are characteristic for tumour cells and are mainly observed in mid-late anaphase, eventually persisting throughout mitosis, but are atypical for early G1-phase (Pampalona *et al*., 2016; Rodriguez-Muñoz *et al*., 2021). Since bovine endothelial cells are of primary and non-tumoral origin, and therefore own a physiological cell cycle control, findings on chromosome bridges must have been due

to *T. gondii* infections. As an interesting additional finding, centrosomes seemed to be composed of more than one centrosomic structure. Of note, by definition, a centrosome consists of two centrioles (mother and daughter) surrounded by pericentriolar material (PCM). To confirm this observation, spatial centrosome signals were converted into a quantitative histogram, which indeed indicated a possible centriole overduplication at each centrosome (**Publication 2**). Since *T. gondii* has been reported to alter cell cycle-related host gene expression (e. g. Cdc25, Wee1, APC/C, p53) (Chang *et al*., 2015; Syn *et al*., 2017), and to modulate CDK-cyclin activities (Brunet *et al*., 2008; Molestina *et al*., 2008), aberrations in chromosome size, shape, number and position (also known as centrosome amplification) can theoretically be influenced by direct or indirect mechanisms: (*i*) cell-to-cell fusion, generating a single cell possessing twice the DNA amount and double the number of centrosomes (Mittal *et al*., 2021); (*ii*) a cytokinesis failure which means that cells have already performed DNA and centrosome duplication but remain as a single cell with doubled DNA and centrosomes (Karakaya *et al*., 2012); (*iii*) separase activity dysregulation triggering premature centriole disengagement driving early separation of the daughter centriole from the mother cell and resulting in the formation of multipolar mitotic spindle (Tsou and Stearns, 2006); and (*iv*) pericentriolar material fragmentation when significant PCM components aggregate in random places in the cytoplasm (Loncarek *et al*., 2008). Any of these phenomena may lead to supernumerary centrosome formation and irregular centriole duplication (Kalkan *et al*., 2022). Even more interesting, the centrosome plays an important role as a signalling centre during cell cycling, e. g. by facilitating G1/S and G2/M transition by recruiting key molecules to the centrosome (Ferguson and Maller, 2010; Matsumoto and Maller, 2004; Pascreau *et al*., 2011). However, in presence of cellular stress, e. g. caused by centrosome amplification or DNA damage, the centrosome promotes checkpoint responses, which directly or indirectly inhibit CDK-cyclin complexes thereby fostering cell cycle arrest *(Barr et al*., 2010; Hinchcliffe *et al*., 2001; Krämer *et al*., 2004; Lin *et al*., 2005; Tibelius *et al*., 2009). However, summarizing mitosis-related findings, only minor differences were detected in NED and Me49 strain infections when compared to previous data published on the *T. gondii* RH strain (**Publication 1**) (Velásquez *et al*., 2019) thereby indicating that basic mechanisms of cell cycle perturbation did not differ in between *T. gondii* strains.

In agreement with RH-related findings (**Publication 1**), Me49 and NED strains both induced a significantly enhanced proportion of binucleated cells in infected BUVEC

layers **(Publication 2)**, thereby indicating cytokinesis failures. Endothelial cells of vessels physiologically do not exhibit binucleated phenotypes without subsequent cytokinesis, thus evidencing that current findings are directly correlated with *T. gondii* infections. Hereby (**Publications 1 + 2**), this host cellular phenotype is now described for haplotypes I-III in BUVEC and can be considered as a strain-independent, specific hallmark of *T. gondii* tachyzoite replication.

As described before, *T. gondii* tachyzoites have evolved several clonal lineages. All of them bear the potential to infect both human and animals; however, types I and II are more prevalent in immunocompromised patients or pregnant women than in animals (Ayi *et al*., 2016; Khan *et al*., 2005; Wujcicka *et al*., 2014). Consequently, type III strains primarily infect animals as opposed to people (Howe and Sibley, 1995). In current findings, the type III strain (NED) signified the distinct strain, which slightly differed in reactions from the other ones in terms of cell cycle and mitosis control throughout the mitosis checkpoint (cyclin B1). Given that we here used bovine cells, this slight modification may suggest an improved adaptation of the NED strain to an intermediate host species. When considering *T. gondii*-driven cell cycle arrest, a recent work suggested that S-phase-arrested host cells fail to incorporate new DNA molecules (Pierre-Louis *et al*., 2022). However, S-phase stasis equally were reported for primary, immortalized and tumour cells, thereby reflecting a parasite strategy maintained throughout haplotype evolution (Brunet *et al*., 2008; Kim *et al*., 2016; Molestina *et al*., 2008; Pierre-Louis *et al*., 2022; Velásquez *et al*., 2019). Summarizing current data, host cell cycle modulation, chromosome missegregation and cytokinesis failure signify intrinsic mechanisms or consequences of *T. gondii* tachyzoite infections, which occur more or less independent of the parasite haplotype.

3.3. *T. gondii* infection fosters host cellular DNA damage and induces cellular attempts of DNA repair

Given that the current work revealed that all *T. gondii* strains uniformly induced chromosome missegregation and cytokinesis impairment, the question arose whether these finding were also linked to failures in genomic integrity surveillance. Genomic integrity represents an essential feature of cellular homeostasis and is a key prerequisite for delivering the correct genetic information to offspring cells during cell division. In general, chromosome missegregation and cytokinesis failures are linked to chromosomal instability (Holland and Cleveland, 2009), besides other alterations like

chromosomal aberrations, such as loss, gain or disarrangement of chromosomes (Bakhoum and Cantley, 2018). Likewise, chromosomal instability is well-known to foster DNA damage, thereby resulting in DNA strand breaks and cellular stress during DNA replication (Wilhelm *et al*., 2020).

To address parasite strain-dependent host cell DNA damage and chromosomal instability induction in the current work, *T. gondii* infections with RH, Me49 and NED strain tachyzoites were here analyzed in BUVEC. DNA strand integrity was first assessed by comet assays, indicating general DNA strand lesions in individual cells without distinguishing single- or double-strand breaks (Barghouth *et al*., 2022; Kawaguchi *et al*., 2010; Sykora *et al*., 2018). Here, *T. gondii* RH, Me49 and NED tachyzoite-infected BUVEC all showed a significantly increased proportion of cells experiencing DNA damage with the RH strain inducing by far the strongest effects (**Publication 3**). Furthermore, the nuclear expression of γ-H2A.X (a specific marker for double DNA strand breaks) indicating DNA damage foci was analysed in *T. gondii*-infected BUVEC. By this method, a parasite-driven induction of DNA damage was confirmed, since the proportion of cells expressing γ-H2A.X increased with parasite infection (**Publication 3**). However, these reactions appeared haplotype-dependent since a significant increase of tachyzoite-driven DNA damage foci was observed only for RH- and NED-infected cells, but not for Me49 infections (**Publication 3**). In line to current findings, Zhuang *et al.* (2020) recently reported on a double-strand DNA break induction in *T. gondii* RH tachyzoite-infected Hela, HEK293T and Vero cells. To verify a potential link between DNA damage and cytokinesis failure, DNA damage foci were here additionally analysed in *T. gondii*-infected binucleated cells. Again, a significantly increased proportion of binucleated cells experiencing DNA damage foci was only stated for RH- and NED-infected BUVEC (**Publication 3**) indicating that DNA damage might indeed be associated with parasite-driven cytokinesis failure and may vary with the parasite strain.

In general, chromosome miscondensation and segregation result in a failure of proper chromosome displacement and may also cause the formation of both, chromosome bridges and micronuclei, which are considered as hallmarks of genomic instability (Fenech *et al*., 2011; Siri *et al*., 2021; Ye *et al*., 2019). In the current work, a significant enhanced proportion of host cells with micronuclei was exclusively found for NED-

infected cells (**Publication 3**), thereby emphasizing strain-dependent parasite-driven insults on host cellular genomic stability.

DNA damage is a highly severe and life threatening insult for a cell. To counteract this defect and to avoid damaged DNA to be inherited by daughter cells, cells own several DNA repair mechanisms. Thus, cells under genotoxic stress activate a DNA damage response (DDR) to maintain genome integrity, which includes the homologous recombination (HR) and the non-homologous end joining (NHEJ) pathway (Campos and Clemente-Blanco, 2020; Petsalaki and Zachos, 2020). Interestingly, the HR pathway mainly operates in S- and G2-phase, whilst NHEJ-based repair is primarily activated in G1-phase (Li and Heyer, 2008; Watanabe and Lieber, 2022). In the current work, DNA damage response-related analyses focused on RH infections, which had induced the most prominent DNA damage effects. On a quantitative level, most proteins of the NHEJ pathway were either unaffected by RH infections (DNA ligase I, Artemis) or even downregulated (Ku70, DNA-PKcs), indicating that host cells fail or are even blocked to activate the NHEJ pathway in response to parasite-driven DNA damage (**Publication 3**). In contrast, several key proteins of the HR pathways were significantly upregulated in RH tachyzoite-infected BUVEC (**Publication 3**). The HR pathway splits into two branches (ATM- and ATR-related pathways), which are activated in dependence of the DNA damage type (Bakr *et al*., 2015; Williams and Zhang, 2021; Yan *et al*., 2014). Hence, the ATM-related pathway is mainly induced by DNA double-strand breaks, whilst the ATR-related pathways is activated by single-strand breaks or by stalled replication forks (Bakr *et al*., 2015; Blackford and Jackson, 2017; Karnitz and Zou, 2015). As expected, *T. gondii* RH infections exclusively induced an upregulation of ATM pathway-related proteins (ATM, Mre11, Rad50, pBRCA) in BUVEC. ATM pathway activation plays a pivotal role in host cells to maintain a balance between survival and apoptosis during *T. gondii* infection, probably for the benefit of parasite survival (Zhuang *et al*., 2020) (**Publication 3**). According to current data, infected BUVEC obviously tried to repair *T. gondii*-driven DNA damages via the ATM pathway, but most likely failed in their attempts. These findings were in agreement with Zhuang *et al*., (2020), who also reported that *T. gondii* infections elicit a DNA damage response by activating the ATM-dependent branch of the HR pathway in HeLa, Vero and HEK293 cells. Hence, this kind of DNA damage response may reflect a common, cell type-independent host cellular strategy.

3.4. Comparative analyses on *Neospora caninum* infections reveal both similarities and differences to *T. gondii*-driven cell cycle perturbation

N. caninum is an apicomplexan parasite closely related to *T. gondii*. To estimate whether the findings of the current work on *T. gondii* (**Publications 1-3**) would also apply to this parasite and, consequently, indicate a general, species-independent strategy of host cell manipulation, cell cycle-related experimentation was here extended to *N. caninum* infections, thereby using the same primary host cell type (BUVEC) to generate comparative data.

To avoid infection kinetic-based effects, *N. caninum* intracellular development was first thoroughly monitored over time in BUVEC, revealing that *N. caninum* proliferation took slightly longer than that of *T. gondii* (**Publication 4**). Therefore, 24 and 32 h p. i. were included as time points of analyses on host cell cycle progression.

In line to current findings on different *T. gondii* strains (**Publications 1-3**), FACS-based assessment of cell cycle phases revealed an increased proportion of *N. caninum*-infected host cells in S-phase at both 24 and 32 h p. i., being accompanied by a simultaneous decrease of cells in G0/G1-phase, thereby confirming a parasite-driven arrest of host cells in S-phase (**Publication 4**). These results contrast on findings in human brain microvascular endothelial cells (hBMECs), in which cell cycle arrest occurred in S- or G2-phase at 12 and 24 h p. i., respectively, along with reduced proliferation of infected cells without compromising the overall viability of infected cells (Elsheikha *et al*., 2020). In line, the proportion of mitotic cells in human lymphoma cells (K562) decreased by *N. caninum* infection (AlKurashi *et al*., 2011).

In the current study, we additionally analysed S-subphases via both the presence and the nuclear distribution of the S-phase marker PCNA (proliferation cell nuclear antigen) in *N. caninum*-infected BUVEC in comparison to non-infected control layers. At 24 h p. i., an infection-driven shift in S-subphases was stated, since an increased proportion of host cells were in late S-phase being accompanied by an almost entire lack of host cells in mid S-phase and a reduced proportion of BUVEC in early S-phase. This finding indicated that *N. caninum*-infected BUVEC were already arrested in early S-phase (**Publication 4**). At 32 h p. i., infected host cells still showed a reduced proportion of host cells in late S-phase, but more host cells had progressed into mid S-phase suggesting that arrest was not irrevocable (**Publication 4**). Overall, it seemed that

parasite-infected cells experienced difficulties in their progress to late S-phase, which may, in consequence, impair them to proceed to G2/M-phase.

Referring to cell cycle checkpoint regulation, *N. caninum*-infected BUVEC revealed by tendency an upregulation of cyclin A2 (which is typically increased throughout S-phase) at 24 hours p. i., followed by a significant downregulation of this key molecule later in infection (32 hours p. i.). Synchronously, cyclin B1 (starts to accumulate at the end of G2-phase and peaks at the G2/M border) was significantly upregulated at 24 hours p. i. (**Publication 4**), thereby confirming irregularities in S phase control and during S-to-G2/M-transition-phase. As mentioned before, cyclin A2 is a key regulator of both mitotic entry and DNA replication, following a coordinated pattern of expression and subcellular localization (Desdouets *et al*., 1995; Huet *et al*., 1996). Thus, the cyclin A/CDK2 complex downregulation correlates with S-phase arrest, blocking cells to enter into G2/M-phase (Bačević *et al*., 2017). Therefore, the finding of *N. caninum*-driven S-phase arrest correlates with a concomitant downregulation of cyclinA2.

As an additional and interesting finding, irregular nuclear morphologies were noticed microscopically in both *N. caninum*-infected and non-infected cells within the same monolayer. Of note, a general correlation between cell shape and nuclear size exists, that owns a central role in cellular and developmental processes and is maintained by mechanical forces mediated via cytoskeletal elements from in- and outside the cell (Khatau *et al*., 2009). To verify these morphological observations and to exclude fixation-based artefacts, the nuclear morphology of cells within an *N. caninum*-infected BUVEC layer was further studied in both living and PFA-fixed cells. Under both conditions, an increased proportion of cells revealed nuclear irregularities like invaginations, corners, strictures and dentings, or nuclear fragmentation (**Publication 4**). TEM analyses illustrated irregular and deformed nuclei at 24 and 32 h p. i., with invaginations and stretches of nuclear membrane disintegration in *N. caninum*-infected host cells (**Publication 4**). Quantitative estimations showed that the nuclear area was smaller in *N. caninum*-infected host cells at 32 h p. i. compared to non-infected controls, whilst nuclear circularity was not affected by infection (**Publication 4**). Moreover, the axes ratio was larger in *N. caninum*-infected cells when compared to non-infected cells, both findings indicating that *N. caninum* infection indeed affects the host cellular nuclear structure (**Publication 4**). To follow this assumption and to study nuclear elements in more detail, the nuclear lamina morphology was analysed at 32

hours p. i. applying lamin B1 staining. The nuclear envelope is formed by two concentric membranes with nuclear pore complexes and supported by an underlying lamina composed of a meshwork of nuclear intermediate filaments formed by type A and B lamins (Hetzer, 2010). Lamin B1 not only forms part of the nuclear lamina, but also directly interacts with chromatin (Brunet *et al*., 2019). In line with above mentioned observations, an increased proportion of cells within *N. caninum*-infected BUVEC layers revealed an altered nuclear Lamin B1 distribution, being characterized by inhomogeneous Lamin B1 abundance, illustrating several nuclear folding and invaginations and bubble-like nuclear protrusions (**Publication 4**). Quantitative analyses confirmed these findings by stating a significant increase of infected cells with inadequate Lamin B1 distribution patterns and misshaped nuclei in comparison to non-infected cell layers (**Publication 4**). Of note, most prominent Lamin B1-related effects were surprisingly found in non-infected cells within an *N. caninum*-infected cell layer. However, *N. caninum*-driven paracrine effects on nuclear Lamin B1 were not confirmed, since treatments of non-infected cell layers with *N. caninum* infection conditioned medium failed to drive all, lamin B1-defect cells, changes in cyclin B1 and actin abundance, but indeed induced S-phase arrest with concomitant G0/G1-phase reduction (**Publication 4**).

To the best of our knowledge, a Lamin B1-based nuclear alteration has not been reported before for coccidian parasite-infected host cells. This prompted us to analyse the nucleoskeleton in more detail. In general, the shape of a cell nucleus is promoted by several cytoskeletal elements like actin, intermediate filaments and microtubules. Of special interest for nuclear shaping and function is the perinuclear actin cap, being composed of thick, parallel and highly contractile actinomyosin filaments being physically attached to the nucleus and to the nuclear lamina by the so-called linker of nucleoskeleton and cytoskeleton complex (LINC) (Khatau *et al*., 2009; Kim *et al*., 2013; Maninova *et al*., 2017). When detecting actin filaments in *N. caninum*-infected BUVEC, the perinuclear area was considerably affected since actin filaments typically transversing the nucleus and being anchored to the nuclear periphery in non-infected cells, were consistently absent in *N. caninum*-infected cells (**Publication 4**). In line, FACS-based quantification revealed a decrease in the total actin content of infected cells (**Publication 4**). These overall observations suggested that *N. caninum* intracellular development interferes with the host cellular actin cytoskeleton and most

likely leads to nuclear membrane destabilization, thereby contributing to abnormal nuclear shaping.

Overall, in the current study we showed that *N. caninum* infections indeed interfere with the host cell cycle progression by inducing S-phase arrest, nuclear lamin B1 maldistribution, perinuclear actin losses, and host cellular nuclear deformation. Importantly, some of these findings clearly differed from alterations driven by different strains of the closely related parasite *T. gondii* (**Publications 1-3**), thereby emphasizing that coccidia-driven cell cycle modulation is - at least in parts - a species-dependent process.

4. ZUSAMMENFASSUNG

Die Apikomplexa beinhalten eine große Gruppe protozoärer Parasiten mit obligat intrazellulärer Lebensweise, die weltweit wichtige Infektionen bei Mensch und Tier verursachen. Zur Familie der Sarcocystidae gehören Arten wie *Toxoplasma gondii*, ein bedeutender zoonotischer Erreger, und *Neospora caninum*, der Aborte bei Rindern und neurologische Störungen bei kaninen Wirten verursachen kann. Obwohl sich beide Parasiten in wichtigen Aspekten der Wirtsspezifität und des Lebenszyklus unterscheiden, modulieren sie gleichermaßen diverse funktionelle Kategorien der Wirtszellen, um ihre intrazelluläre Entwicklung zu ermöglichen. Die vorliegende Studie konzentrierte sich auf vergleichende Untersuchungen zur *T. gondii*- und *N. caninum*-vermittelten Dysregulation des wirtszellulären Zellzyklus.

Grundsätzlich beeinflusst *T. gondii* den Wirtszellzyklus signifikant, unabhängig von MOI, Infektionszeitpunkt und dem verwendeten Zellmodell. Während frühere Studien meist immortalisierte oder Tumorzellen, die typischerweise einen veränderten Zellzyklus aufweisen, analysierten, wurden hier primäre Zelllinien als Wirtzellen vergleichend untersucht, und dabei sowohl unterschiedliche Zelltypen (Endothelzellen, Epithelzellen und Fibroblasten) als auch Herkunft (Mensch und Rind) berücksichtigt. Insgesamt zeigte sich, dass *T. gondii*-Infektionen in zelltyp- und herkunfts-unabhängiger Weise zu einer wirtszellulären Stase in der S-Phase führten. Bemerkenswerterweise blieb dabei die Expression des Zyklin B1 als wichtigen Regulator des Mitose-Eintritts bei allen Zelltypen unverändert; entsprechend scheint der Mitose-Kontrollpunkt nicht an Zellzyklusarretierung beteiligt zu sein. Neben Interphase-assoziierten Alterationen induzierten *T. gondii*-Infektionen Defekte in allen Mitosephasen, wie z. B. Fehlkondensierung der Chromosomen und Bildung überzähliger Zentrosomen. Darüber hinaus zeigten alle verwendeten Zelltypen einen erhöhten Anteil an Doppelkernigkeit, was auf eine gestörte Zytokinese hinwies.

Da unterschiedliche *T. gondii*-Genotypen im Feld mit einer variierenden Pathogenität assoziiert sind, wurde weiterhin untersucht, ob Ausmaß oder Effekte der *T. gondii*-vermittelten Wirtszellzyklusdysregulierung haplotypabhängig sind. Dazu wurden die Effekte der *T. gondii*-Stämme Me49 (Haplotyp II) und NED (Haplotyp III) im direkten Vergleich mit dem RH-Stamm (Haplotyp I) in primären Wirtszellen unter identischen Bedingungen untersucht. In Übereinstimmung mit dem RH-Stamm induzierte sowohl der Me49- als auch der NED-Stamm eine Arretierung der Wirtszelle in der S-Phase,

allerdings konnte nur für NED-Infektionen eine Verminderung des Zyklin B1 als wichtigen Regulator konstatiert werden. Darüber hinaus war die Mitoserate in NED-infizierten Zellen signifikant reduziert und es traten irreguläre Chromosomenanordnungen und -brücken in der Mitose auf. In Analogie zum RH-Stamm führten beide Stämme zu einem gesteigerten Anteil doppelkerniger Wirtszellen und somit ebenfalls zur Beeinträchtigung der Zytokinese. In der vorliegenden Arbeit wird dieser Phänotyp erstmals für alle Haplotypen gleichermaßen beschrieben, so dass ein *T. gondii*-intrinsischer Mechanismus abgeleitet werden kann.

Chromosomenfehlverteilungen und Beeinträchtigungen der Zytokinese sind Schlüsselmerkmale der Chromosomeninstabilität, die meist mit DNA-Schäden in Zellen einhergeht. Tatsächlich induzierten in der vorliegenden Arbeit sowohl der RH- als auch die Me49- und NED-Stämme DNA-Doppelstrangbrüche in Wirtszellen, wobei RH-Infektionen die stärksten Schäden verursachten. Dies bestätigte sich auch für zytokinesedefekte, doppelkernige Wirtszellen nach Infektionen mit RH- und NED-Tachyzoiten. Interessanterweise wiesen NED-infizierte Zellen zudem ein erhöhtes Maß an Mikronuklei auf, was auf eine stammspezifische Beeinträchtigung der genomischen Stabilität von Wirtszellen hinweisen könnte.

Bei genotoxischem Stress aktivieren Zellen die sog. *DNA Damage Response*, um die Integrität des Genoms zu erhalten. Diese DNA-Reparaturmechanismen beinhalten die Aktivierung der Signalwege der *homologous recombination* (HR) oder des *non-homologous end joining* (NHEJ). Die hiesigen Untersuchungen bestätigten eine (RH) infektionsinduzierte Aktivierung des HR-Signalwegs in primären Wirtszellen, die mit einer Hochregulierung von im ATM-Stoffwechselweg eingebundenen Proteinen verbunden war, was typisch für DNA-Doppelstrangbrüche ist.

In einem letzten Arbeitspaket wurden die zellzyklus-assoziierte Analysen auf einen weiteren apikomplexen Parasiten, *N. caninum,* ausgedehnt, um eventuelle artspezifische Mechanismen herauszustellen. Tatsächlich führte die *N. caninum*-Infektion zu einer Stase des Zellzyklus in der späten S-Phase und zu einer Hochregulierung der Zykline A2 und B1 24 Stunden p. i. (ersteres war 32 Stunden p. i. wieder vermindert in seiner Expression), was Unregelmäßigkeiten im Zeitraum von S-Phase zum G2/M-Übergang bestätigte. Interessanterweise wurden bei dieser Infektion irreguläre Kernmorphologien, wie beispielsweise nukleäre Invaginationen, Abschnitte mit degradierter Kernmembran oder auch eine insgesamt verringerte Kernausdehnung

beobachtet, was eine speziesspezifisch induzierte Alteration der Kernstruktur der Wirtszelle vermuten ließ. Weiterführende Analysen zum nukleären Protein Lamin B1 ergaben eine mit *N. caninum*-Infektion assoziierte, inhomogene Lamin B1-Verteilung, Kernfaltungen und -einstülpungen - Phänomene, die hier erstmals für Kokzidieninfektionen beschrieben wurden. Auch das perinukleäre Zytoskelett war verändert in infizierten Zellen, da bestimmte Aktinfilamente, die physiologischerweise an der Kernperipherie verankert sind und transversal über den Nukleus ziehen (sog. *Actin cap*), in infizierten Zellen fehlten. Insgesamt wurde ein vermindertes Gesamtvorkommen von Aktin in *N. caninum*-infizierten Zellen gemessen, was eine parasiteninduzierte Beeinträchtigung des perinukleären Aktinzytoskeletts mit nachfolgender Destabilisierung der Kernmembran und abnormen Formgebung des Kerns vermuten ließ. Da solche Beobachtungen nicht auf *T. gondii*-infizierte Zellen zutrafen, unterstreichen hiesige Daten eine artspezifische Modulation der Wirtszellen über Kokzidien.

5. SUMMARY

Apicomplexan parasites are a large group of protists with an obligate intracellular lifestyle, impacting human and veterinary health worldwide. The Sarcocystidae family contains notable species like *Toxoplasma gondii*, a critical zoonotic pathogen, and *Neospora caninum*, cause of abortion in cattle and of neurological disorders in canid hosts. Although both parasites differ in their host specificity and in distinct aspects of their life cycle, they also share common characteristics in their sophisticated ability to modulate host cell functions. Here, we focused on molecular and cellular mechanisms involved in parasite infection-driven host cell cycle dysregulation.

T. gondii is well recognized to significantly affect host cell cycle progression, regardless of MOI, infection times and, importantly, the cellular model used in experimentation. While many former studies used immortalized or tumour cells, potentially exhibiting dysregulated cell cycling, current analyses addressed *T. gondii* infections in primary cells by analyzing different cell types (fibroblasts, endothelial and epithelial cells) and donor species (human, bovine). Here, we revealed a *T. gondii*-driven cell type- and origin-independent S-phase arrest. Notably, cyclin B1, a critical regulator of mitosis entry, remained unchanged across all cell types, indicating that mitosis checkpoint modulation is not involved in host cellular S-phase stasis. Beyond interphase effects, *T. gondii* infection led to aberrant mitosis in all mitotic subphases, characterized by chromosome miscondensation and supernumerary centrosome formation. Moreover, all cell types showed an increased proportion of binucleated phenotypes, indicating impaired cytokinesis, which also occurred independently of cell origin or type.

Given that different *T. gondii* genotypes show varying pathogenicity in the field, host cell cycle regulation may also be influenced by haplotypes. To explore this, different strain infections (Me49, NED) were comparatively analysed in primary host cells. In line with RH, Me49 and NED strains also induced host S-phase arrest. Further analyses on key regulatory proteins of S-phase control and M-phase enter revealed a cyclin B1 downregulation only for NED infections. Additionally, the mitotic rate was reduced by NED infections, concomitant with altered chromosome arrangement and irregular chromosome bridges within the mitotic spindle. Moreover, *T. gondii* Me49 and NED strains also led to an enhanced proportion of binucleated host cells, indicative of cytokinesis failure. Thereby, this cellular phenotype was here described for the first

time for all haplotype infections, demonstrating cytokinesis impairment as intrinsic, haplotype-independent mechanism of *T. gondii*.

Chromosome missegregation and cytokinesis impairment are key features of chromosome instability being associated with DNA damage in cells. As determined in the current work, RH, Me49 and NED strains indeed all induced DNA double-strand breaks with the RH strain driving - by far - the most pronounced effects. Moreover, referring to cytokinesis failure, a significant proportion of both RH- and NED-infected binucleated host cells showed DNA damage foci. Interestingly, NED-infected cells exhibited an increased proportion of micronuclei, thereby highlighting parasite strain-specific insults on host cellular genomic stability.

Under genotoxic stress, cells activate the DNA damage response to maintain genome integrity. Repair mechanisms for this type of damage include the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways, which were here profiled for RH strain infections. As expected, the HR pathway was activated by an upregulation of ATM pathway-related proteins, which classically are induced by DNA double-strand breaks.

Finally, current cell cycle-related analyses were extended to *N. caninum* infections in the same host cell type to elucidate eventual species-specific strategies. *N. caninum* caused late S-phase arrest concomitant with cyclin A2 and cyclin B1 upregulation at 24 h p. i. (followed by cyclin A2 decrease at 32 h), confirming irregularities from S- to G2/M transition-phase. Interestingly, irregular nuclear morphologies were observed in *N. caninum*-infected cells, illustrated as invaginations and stretches of nuclear membrane disintegration and quantified as smaller nuclear areas, indicating that the host cellular nuclear structure was affected by *N. caninum*. Further analyses on the nuclear protein lamin B1 revealed an increased proportion of cells with inhomogeneous lamin B1 patterns, several nuclear folding and invaginations, phenomena reported for the first time for coccidian infections. Moreover, the perinuclear area was altered since actin filaments normally being anchored to the nuclear periphery and transversing the nucleus (actin cap) were absent in infected cells alongside with a decreased total cellular actin abundance, highlighting that *N. caninum* infection indeed interferes with the host actin cytoskeleton leading to nuclear membrane destabilization and abnormal shaping. Overall, these findings contrast with alterations induced by different *T. gondii* strains, emphasizing species-specific events in host cell modulation.

6. REFERENCES

Abbas, T., Dutta, A., 2009. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 9, 400–414. https://doi.org/10.1038/nrc2657

Abbastabar, M., Kheyrollah, M., Azizian, K., Bagherlou, N., Tehrani, S.S., Maniati, M., Karimian, A., 2018. Multiple functions of p27 in cell cycle, apoptosis, epigenetic modification and transcriptional regulation for the control of cell growth: A double-edged sword protein. DNA Repair 69, 63–72. https://doi.org/10.1016/j.dnarep.2018.07.008

Ajzenberg, D., Yera, H., Marty, P., Paris, L., Dalle, F., Menotti, J., Aubert, D., Franck, J., Bessières, M., Quinio, D., Pelloux, H., Delhaes, L., Desbois, N., Thulliez, P., Robert-Gangneux, F., Kauffmann-Lacroix, C., Pujol, S., Rabodonirina, M., Bougnoux, M., Cuisenier, B., Duhamel, C., Duong, T.H., Filisetti, D., Flori, P., Gay-Andrieu, F., Pratlong, F., Nevez, G., Totet, A., Carme, B., Bonnabau, H., Dardé, M., Villena, I., 2009. Genotype of 88 *Toxoplasma gondii* Isolates Associated with Toxoplasmosis in Immunocompromised Patients and Correlation with Clinical Findings. J INFECT DIS 199, 1155–1167. https://doi.org/10.1086/597477

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2007. The Cell Cycle, in: Molecular Biology of the Cell. W.W. Norton & Company.

Albuquerque, S.S., Carret, C., Grosso, A.R., Tarun, A.S., Peng, X., Kappe, S.H., Prudêncio, M., Mota, M.M., 2009. Host cell transcriptional profiling during malaria liver stage infection reveals a coordinated and sequential set of biological events. BMC Genomics 10, 270. https://doi.org/10.1186/1471-2164-10-270

AlKurashi, M., Eastick, F.A., Kuchipudi, S.V., Rauch, C., Madouasse, A., Zhu, X.-Q., Elsheikha, H.M., 2011. Influence of culture medium pH on internalization, growth and phenotypic plasticity of *Neospora caninum*. Veterinary Parasitology 177, 267–274. https://doi.org/10.1016/j.vetpar.2010.11.053

Alvarado-Esquivel, C., Rico-Almochantaf, Y. del R., Hernández-Tinoco, J., Quiñones-Canales, G., Sánchez-Anguiano, L.F., Torres-González, J., Schott, B., Liesenfeld, O., Dunay, I.R., 2017. Toxoplasma Gondii Exposure and Neurological Disorders: An Age- and Gender-Matched Case-Control Pilot Study. Eur J Microbiol Immunol (Bp) 7, 303–309. https://doi.org/10.1556/1886.2017.00033

Anvarbatcha, R., Kunnathodi, F., Islam, M., 2023. Induction of G0/G1 phase cell cycle arrest and apoptosis by thymol through ROS generation and caspase-9/-3 activation in breast and colorectal cancer cell lines. J Cancer Res Ther 19, 1915–1924. https://doi.org/10.4103/jcrt.jcrt_308_22

Attias, M., Teixeira, D.E., Benchimol, M., Vommaro, R.C., Crepaldi, P.H., De Souza, W., 2020. The life-cycle of Toxoplasma gondii reviewed using animations. Parasites & Vectors 13, 588. https://doi.org/10.1186/s13071-020-04445-z

Awasthi, P., Foiani, M., Kumar, A., 2015. ATM and ATR signaling at a glance. Journal of Cell Science 128, 4255–4262. https://doi.org/10.1242/jcs.169730

Ayi, I., Kwofie, K.D., Blay, E.A., Osei, J.H.N., Frempong, K.K., Koku, R., Ghansah, A., Lartey, M., Suzuki, T., Boakye, D.A., Koram, K.A., Ohta, N., 2016. Clonal types of *Toxoplasma gondii* among immune compromised and immune competent individuals in Accra, Ghana. Parasitology International 65, 238–244. https://doi.org/10.1016/j.parint.2016.01.004

Bačević, K., Lossaint, G., Achour, T.N., Georget, V., Fisher, D., Dulić, V., 2017. Cdk2 strengthens the intra-S checkpoint and counteracts cell cycle exit induced by DNA damage. Sci Rep 7, 13429. https://doi.org/10.1038/s41598-017-12868-5

Bakhoum, S.F., Cantley, L.C., 2018. The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. Cell 174, 1347–1360. https://doi.org/10.1016/j.cell.2018.08.027

Bakr, A., Oing, C., Köcher, S., Borgmann, K., Dornreiter, I., Petersen, C., Dikomey, E., Mansour, W.Y., 2015. Involvement of ATM in homologous recombination after end resection and RAD51 nucleofilament formation. Nucleic Acids Res 43, 3154–3166. https://doi.org/10.1093/nar/gkv160

Barber, J.S., Trees, A.J., 1998. Naturally occurring vertical transmission of *Neospora caninum* in dogs. International Journal for Parasitology, 10th International Congress of Protozoology 28, 57–64. https://doi.org/10.1016/S0020-7519(97)00171-9

Barford, D., 2011. Structural insights into anaphase-promoting complex function and mechanism. Philos Trans R Soc Lond B Biol Sci 366, 3605–3624. https://doi.org/10.1098/rstb.2011.0069

Barghouth, P.G., Rojas, S., O'Dell, L.R., Betancourt, A.M., Oviedo, N.J., 2022. Analysis of DNA Double-Stranded Breaks Using the Comet AssayComet assays in PlanariansPlanarians, in: Blanchoud, S., Galliot, B. (Eds.), Whole-

Body Regeneration: Methods and Protocols. Springer US, New York, NY, pp. 479–491. https://doi.org/10.1007/978-1-0716-2172-1_25

Barnum, K.J., O'Connell, M.J., 2014. Cell Cycle Regulation by Checkpoints. Methods Mol Biol 1170, 29–40. https://doi.org/10.1007/978-1-4939-0888-2_2

Barr, A.R., Kilmartin, J.V., Gergely, F., 2010. CDK5RAP2 functions in centrosome to spindle pole attachment and DNA damage response. J Cell Biol 189, 23–39. https://doi.org/10.1083/jcb.200912163

Bartolomé del Pino, L.E., Zanón-Moreno, V., 2024. Systematic Review on the Relationship between Toxoplasmosis and Mental Disorders. Actas Esp Psiquiatr 52, 149–160. https://doi.org/10.62641/aep.v52i2.1658

Behnke, M.S., Radke, J.B., Smith, A.T., Sullivan, W.J., White, M.W., 2008. The transcription of bradyzoite genes in Toxoplasma gondii is controlled by autonomous promoter elements. Mol Microbiol 68, 1502–1518. https://doi.org/10.1111/j.1365-2958.2008.06249.x

Berenreiterová, M., Flegr, J., Kuběna, A.A., Němec, P., 2011. The distribution of Toxoplasma gondii cysts in the brain of a mouse with latent toxoplasmosis: implications for the behavioral manipulation hypothesis. PLoS One 6, e28925. https://doi.org/10.1371/journal.pone.0028925

Bergeron, N., Fecteau, G., Paré, J., Martineau, R., Villeneuve, A., 2000. Vertical and horizontal transmission of Neospora caninum in dairy herds in Québec. Can Vet J 41, 464–467.

Bernsteen, L., Gregory, C.R., Aronson, L.R., Lirtzman, R.A., Brummer, D.G., 1999. Acute toxoplasmosis following renal transplantation in three cats and a dog. J Am Vet Med Assoc 215, 1123–1126.

Bertoli, C., Skotheim, J.M., de Bruin, R.A.M., 2013. Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol 14, 518–528. https://doi.org/10.1038/nrm3629

Besteiro, S., 2019. The role of host autophagy machinery in controlling Toxoplasma infection. Virulence 10, 438–447. https://doi.org/10.1080/21505594.2018.1518102

Besteiro, S., n.d. Toxoplasma control of host apoptosis: the art of not biting too hard the hand that feeds you. Microb Cell 2, 178–181. https://doi.org/10.15698/mic2015.06.209

Black, M.W., Boothroyd, J.C., 2000. Lytic cycle of Toxoplasma gondii. Microbiol Mol Biol Rev 64, 607–623. https://doi.org/10.1128/MMBR.64.3.607-623.2000

Blackford, A.N., Jackson, S.P., 2017. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Molecular Cell 66, 801–817. https://doi.org/10.1016/j.molcel.2017.05.015

Blader, I.J., Coleman, B.I., Chen, C.-T., Gubbels, M.-J., 2015. Lytic Cycle of Toxoplasma gondii: 15 Years Later. Annual Review of Microbiology 69, 463–485. https://doi.org/10.1146/annurev-micro-091014-104100

Blader, I.J., Saeij, J.P., 2009. Communication between Toxoplasma gondii and its host: impact on parasite growth, development, immune evasion, and virulence. APMIS 117, 458–476. https://doi.org/10.1111/j.1600-0463.2009.02453.x

Bottari, D., Troje, N.F., Ley, P., Hense, M., Kekunnaya, R., Röder, B., 2015. The neural development of the biological motion processing system does not rely on early visual input. Cortex 71, 359–367. https://doi.org/10.1016/j.cortex.2015.07.029

Brennan, A., Donahoe, S.L., Beatty, J.A., Belov, K., Lindsay, S., Briscoe, K.A., Šlapeta, J., Barrs, V.R., 2016. Comparison of genotypes of Toxoplasma gondii in domestic cats from Australia with latent infection or clinical toxoplasmosis. Vet Parasitol 228, 13–16. https://doi.org/10.1016/j.vetpar.2016.06.008

Brownlee, L., Sellon, R.K., 2001. Diagnosis of naturally occurring toxoplasmosis by bronchoalveolar lavage in a cat. J Am Anim Hosp Assoc 37, 251–255. https://doi.org/10.5326/15473317-37-3-251

Brunet, A., Forsberg, F., Fan, Q., Sæther, T., Collas, P., 2019. Nuclear Lamin B1 Interactions With Chromatin During the Circadian Cycle Are Uncoupled From Periodic Gene Expression. Front Genet 10, 917. https://doi.org/10.3389/fgene.2019.00917

Brunet, J., Pfaff, A.W., Abidi, A., Unoki, M., Nakamura, Y., Guinard, M., Klein, J.-P., Candolfi, E., Mousli, M., 2008. Toxoplasma gondii exploits UHRF1 and induces host cell cycle arrest at G2 to enable its proliferation. Cell Microbiol 10, 908–920. https://doi.org/10.1111/j.1462-5822.2007.01093.x

Burton, D.G.A., Krizhanovsky, V., 2014. Physiological and pathological consequences of cellular senescence. Cell Mol Life Sci 71, 4373–4386. https://doi.org/10.1007/s00018-014-1691-3

Buxton, D., McAllister, M.M., Dubey, J.P., 2002. The comparative pathogenesis of neosporosis. Trends in Parasitology 18, 546–552. https://doi.org/10.1016/S1471-4922(02)02414-5

Calero-Bernal, R., Fernández-Escobar, M., Katzer, F., Su, C., Ortega-Mora, L.M., 2022. Unifying Virulence Evaluation in Toxoplasma gondii: A Timely Task. Frontiers in Cellular and Infection Microbiology 12.

Campagnaro, G.D., Elati, H.A.A., Balaska, S., Martin Abril, M.E., Natto, M.J., Hulpia, F., Lee, K., Sheiner, L., Van Calenbergh, S., de Koning, H.P., 2022. A Toxoplasma gondii Oxopurine Transporter Binds Nucleobases and Nucleosides Using Different Binding Modes. Int J Mol Sci 23, 710. https://doi.org/10.3390/ijms23020710

Campisi, J., d'Adda di Fagagna, F., 2007. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8, 729–740. https://doi.org/10.1038/nrm2233

Campos, A., Clemente-Blanco, A., 2020. Cell Cycle and DNA Repair Regulation in the Damage Response: Protein Phosphatases Take Over the Reins. Int J Mol Sci 21, 446. https://doi.org/10.3390/ijms21020446

Carme, B., Bissuel, F., Ajzenberg, D., Bouyne, R., Aznar, C., Demar, M., Bichat, S., Louvel, D., Bourbigot, A.M., Peneau, C., Neron, P., Dardé, M.L., 2002. Severe Acquired Toxoplasmosis in Immunocompetent Adult Patients in French Guiana. J Clin Microbiol 40, 4037–4044. https://doi.org/10.1128/JCM.40.11.4037-4044.2002

Castaño, P., Fuertes, M., Ferre, I., Fernández, M., Ferreras, M. del C., Moreno-Gonzalo, J., González-Lanza, C., Katzer, F., Regidor-Cerrillo, J., Ortega-Mora, L.M., Pérez, V., Benavides, J., 2014. Placental thrombosis in acute phase abortions during experimental Toxoplasma gondii infection in sheep. Vet Res 45, 9. https://doi.org/10.1186/1297-9716-45-9

Chakraborty, S., Roy, S., Mistry, H.U., Murthy, S., George, N., Bhandari, V., Sharma, P., 2017. Potential Sabotage of Host Cell Physiology by Apicomplexan Parasites for Their Survival Benefits. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.01261

Chang, S., Shan, X., Li, X., Fan, W., Zhang, S.Q., Zhang, J., Jiang, N., Ma, D., Mao, Z., 2015. Toxoplasma gondii Rhoptry Protein ROP16 Mediates Partially SH-SY5Y Cells Apoptosis and Cell Cycle Arrest by Directing Ser15/37

Phosphorylation of p53. Int J Biol Sci 11, 1215–1225. https://doi.org/10.7150/ijbs.10516

Chen, J., Zhang, C., Yang, Z., Wu, W., Zou, W., Xin, Z., Zheng, S., Liu, R., Yang, L., Peng, H., 2024. Intestinal microbiota imbalance resulted by anti-Toxoplasma gondii immune responses aggravate gut and brain injury. Parasites & Vectors 17, 284. https://doi.org/10.1186/s13071-024-06349-8

Cheng, A., Zhang, H., Chen, B., Zheng, S., Wang, H., Shi, Y., You, S., Li, M., Jiang, L., 2022. Modulation of autophagy as a therapeutic strategy for Toxoplasma gondii infection. Front Cell Infect Microbiol 12, 902428. https://doi.org/10.3389/fcimb.2022.902428

Cheung, T.H., Rando, T.A., 2013. Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol 14, 10.1038/nrm3591. https://doi.org/10.1038/nrm3591

Chu, L., Zhang, Z., Mukhina, M., Zickler, D., Kleckner, N., 2022. Sister chromatids separate during anaphase in a three-stage program as directed by interaxis bridges. Proceedings of the National Academy of Sciences 119, e2123363119. https://doi.org/10.1073/pnas.2123363119

Cohen, T.M., Blois, S., Vince, A.R., 2016. Fatal extraintestinal toxoplasmosis in a young male cat with enlarged mesenteric lymph nodes. Can Vet J 57, 483–486.

Coppens, I., Dunn, J.D., Romano, J.D., Pypaert, M., Zhang, H., Boothroyd, J.C., Joiner, K.A., 2006a. Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125, 261–274. https://doi.org/10.1016/j.cell.2006.01.056

Coppens, I., Dunn, J.D., Romano, J.D., Pypaert, M., Zhang, H., Boothroyd, J.C., Joiner, K.A., 2006b. Toxoplasma gondii Sequesters Lysosomes from Mammalian Hosts in the Vacuolar Space. Cell 125, 261–274. https://doi.org/10.1016/j.cell.2006.01.056

Coppens, I., Romano, J.D., 2018. Hostile intruder: Toxoplasma holds host organelles captive. PLOS Pathogens 14, e1006893. https://doi.org/10.1371/journal.ppat.1006893

Correia, A., Ferreirinha, P., Botelho, S., Belinha, A., Leitão, C., Caramalho, Í., Teixeira, L., González-Fernandéz, Á., Appelberg, R., Vilanova, M., 2015. Predominant role of interferon-γ in the host protective effect of CD8+ T cells against Neospora caninum infection. Sci Rep 5, 14913. https://doi.org/10.1038/srep14913

Coverley, D., Laman, H., Laskey, R.A., 2002. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat Cell Biol 4, 523–528. https://doi.org/10.1038/ncb813

Croken, M.M., Ma, Y., Markillie, L.M., Taylor, R.C., Orr, G., Weiss, L.M., Kim, K., 2014. Distinct Strains of Toxoplasma gondii Feature Divergent Transcriptomes Regardless of Developmental Stage. PLoS ONE 9, e111297. https://doi.org/10.1371/journal.pone.0111297

Dardé, M.-L., Ajzenberg, D., Su, C., 2014. Chapter 3 - Molecular Epidemiology and Population Structure of Toxoplasma gondii, in: Weiss, L.M., Kim, K. (Eds.), Toxoplasma Gondii (Second Edition). Academic Press, Boston, pp. 61–97. https://doi.org/10.1016/B978-0-12-396481-6.00003-9

Daugschies, A., Najdrowski, M., 2005. Eimeriosis in cattle: current understanding. J Vet Med B Infect Dis Vet Public Health 52, 417–427. https://doi.org/10.1111/j.1439-0450.2005.00894.x

de Barros, R.A.M., Torrecilhas, A.C., Marciano, M.A.M., Mazuz, M.L., Pereira-Chioccola, V.L., Fux, B., 2022. Toxoplasmosis in Human and Animals Around the World. Diagnosis and Perspectives in the One Health Approach. Acta Trop 231, 106432. https://doi.org/10.1016/j.actatropica.2022.106432

de Koning, H.P., Bridges, D.J., Burchmore, R.J.S., 2005. Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. FEMS Microbiol Rev 29, 987–1020. https://doi.org/10.1016/j.femsre.2005.03.004

de Oliveira, J.M.B., Silva, B.P. e, Ribeiro-Andrade, M., Porto, W.J.N., de Melo, R.P.B., Junior, J.W.P., da Fonseca Oliveira, A.A., Mota, R.A., 2022. Toxoplasma gondii infection in goats: serological, pathological, and clinical monitoring during gestation. Parasitol Res 121, 3147–3153. https://doi.org/10.1007/s00436-022-07633-1

De Tommasi, A.S., Morini, M., Turba, M.E., Otranto, D., Bettini, G., 2014. Hyperplastic cholangitis in a naturally Toxoplasma gondii-infected cat. Veterinary Quarterly 34, 229–231. https://doi.org/10.1080/01652176.2014.978510

del Campo, J., Heger, T.J., Rodríguez-Martínez, R., Worden, A.Z., Richards, T.A., Massana, R., Keeling, P.J., 2019. Assessing the Diversity and Distribution of Apicomplexans in Host and Free-Living Environments Using High-Throughput Amplicon Data and a Phylogenetically Informed Reference Framework. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02373

Desdouets, C., Matesic, G., Molina, C.A., Foulkes, N.S., Sassone-Corsi, P., Brechot, C., Sobczak-Thepot, J., 1995. Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. Mol Cell Biol 15, 3301–3309.

Diallo, M.A., Sausset, A., Gnahoui-David, A., Ribeiro E Silva, A., Brionne, A., Le Vern, Y., Bussière, F.I., Tottey, J., Lacroix-Lamandé, S., Laurent, F., Silvestre, A., 2019. Eimeria tenella ROP kinase EtROP1 induces G0/G1 cell cycle arrest and inhibits host cell apoptosis. Cellular Microbiology 21, e13027. https://doi.org/10.1111/cmi.13027

Dincel, G.C., Atmaca, H.T., 2015. Nitric oxide production increases during Toxoplasma gondii encephalitis in mice. Exp Parasitol 156, 104–112. https://doi.org/10.1016/j.exppara.2015.06.009

Dobbelaere, D., Fernandez, P., Machado, J., Botteron, C., Heussler, V., 1999. Interference by the intracellular parasite Theileria parva with T-cell signal transduction pathways induces transformation and protection against apoptosis. Vet Immunol Immunopathol 72, 95–100. https://doi.org/10.1016/s0165-2427(99)00121-x

Dobbelaere, D., Heussler, V., 1999. Transformation of leukocytes by Theileria parva and T. annulata. Annu Rev Microbiol 53, 1–42. https://doi.org/10.1146/annurev.micro.53.1.1

Dobbelaere, D.A.E., Fernandez, P.C., Heussler, V.T., 2000. Theileria parva: taking control of host cell proliferation and survival mechanisms. Cellular Microbiology 2, 91–99. https://doi.org/10.1046/j.1462-5822.2000.00045.x

Donahoe, S.L., Lindsay, S.A., Krockenberger, M., Phalen, D., Šlapeta, J., 2015. A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. International Journal for Parasitology: Parasites and Wildlife 4, 216–238. https://doi.org/10.1016/j.ijppaw.2015.04.002

Donaldson, T.M., Cassera, M.B., Ho, M.-C., Zhan, C., Merino, E.F., Evans, G.B., Tyler, P.C., Almo, S.C., Schramm, V.L., Kim, K., 2014. Inhibition and Structure of Toxoplasma gondii Purine Nucleoside Phosphorylase. Eukaryot Cell 13, 572–579. https://doi.org/10.1128/EC.00308-13

DU, K., LU, F., XIE, C., DING, H., SHEN, Y., GAO, Y., LU, S., ZHUO, X., 2022. Toxoplasma gondii infection induces cell apoptosis via multiple pathways

revealed by transcriptome analysis. J Zhejiang Univ Sci B 23, 315–327. https://doi.org/10.1631/jzus.B2100877

Dubey, J.P., 2009a. History of the discovery of the life cycle of Toxoplasma gondii. International Journal for Parasitology, Toxoplasma Centennial Issue 39, 877–882. https://doi.org/10.1016/j.ijpara.2009.01.005

Dubey, J.P., 2009b. Toxoplasmosis in sheep--the last 20 years. Vet Parasitol 163, 1–14. https://doi.org/10.1016/j.vetpar.2009.02.026

Dubey, J.P., 2009c. Toxoplasmosis in sheep—The last 20 years. Veterinary Parasitology 163, 1–14. https://doi.org/10.1016/j.vetpar.2009.02.026

Dubey, J.P., 1999. Recent advances in Neospora and neosporosis. Vet Parasitol 84, 349–367. https://doi.org/10.1016/s0304-4017(99)00044-8

Dubey, J.P., Carpenter, J.L., 1993. Neonatal toxoplasmosis in littermate cats. J Am Vet Med Assoc 203, 1546–1549.

Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of Toxoplasma gondii Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts. Clin Microbiol Rev 11, 267–299.

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and Neospora caninum. Clin Microbiol Rev 20, 323–367. https://doi.org/10.1128/CMR.00031-06

Dvorak, J.A., Crane, M.St.J., 1981. Vertebrate Cell Cycle Modulates Infection by Protozoan Parasites. Science 214, 1034–1036. https://doi.org/10.1126/science.7029713

Elsheikha, H.M., Alkurashi, M., Palfreman, S., Castellanos, M., Kong, K., Ning, E., Elsaied, N.A., Geraki, K., MacNaughtan, W., 2020. Impact of Neospora caninum Infection on the Bioenergetics and Transcriptome of Cerebrovascular Endothelial Cells. Pathogens 9, 710. https://doi.org/10.3390/pathogens9090710

Engeland, K., 2022a. Cell cycle regulation: p53-p21-RB signaling. Cell Death Differ 29, 946–960. https://doi.org/10.1038/s41418-022-00988-z

Engeland, K., 2022b. Cell cycle regulation: p53-p21-RB signaling. Cell Death Differ 29, 946–960. https://doi.org/10.1038/s41418-022-00988-z

Evans, N.A., Walker, J.M., Manchester, A.C., Bach, J.F., 2017. Acute respiratory distress syndrome and septic shock in a cat with disseminated toxoplasmosis.

J Vet Emerg Crit Care (San Antonio) 27, 472–478. https://doi.org/10.1111/vec.12621

Ewald, S., Nasuhidehnavi, A., Feng, T.-Y., Lesani, M., McCall, L.-I., 2024. The intersection of host in vivo metabolism and immune responses to infection with kinetoplastid and apicomplexan parasites. Microbiology and Molecular Biology Reviews 88, e00164-22. https://doi.org/10.1128/mmbr.00164-22

Fabiani, S., Pinto, B., Bonuccelli, U., Bruschi, F., 2015. Neurobiological studies on the relationship between toxoplasmosis and neuropsychiatric diseases. J Neurol Sci 351, 3–8. https://doi.org/10.1016/j.jns.2015.02.028

Feitoza, L., Costa, L., Guerra, M., 2017. Condensation patterns of prophase/prometaphase chromosome are correlated with H4K5 histone acetylation and genomic DNA contents in plants. PLoS One 12, e0183341. https://doi.org/10.1371/journal.pone.0183341

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D., Thomas, P., 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26, 125–132. https://doi.org/10.1093/mutage/geq052

Fereig, R.M., Nishikawa, Y., 2020. From Signaling Pathways to Distinct Immune Responses: Key Factors for Establishing or Combating Neospora caninum Infection in Different Susceptible Hosts. Pathogens 9, 384. https://doi.org/10.3390/pathogens9050384

Ferguson, D.J.P., 2009. Toxoplasma gondii: 1908-2008, homage to Nicolle, Manceaux and Splendore. Mem. Inst. Oswaldo Cruz 104, 133–148. https://doi.org/10.1590/S0074-02762009000200003

Ferguson, D.J.P., Dubremetz, J.-F., 2020. The ultrastructure of *Toxoplasma gondii*, in: Weiss, L.M., Kim, K. (Eds.), Toxoplasma Gondii (Third Edition). Academic Press, pp. 21–61. https://doi.org/10.1016/B978-0-12-815041-2.00002-5

Ferguson, R.L., Maller, J.L., 2010. Centrosomal Localization of Cyclin E-Cdk2 is Required for Initiation of DNA Synthesis. Curr Biol 20, 856–860. https://doi.org/10.1016/j.cub.2010.03.028

Fernández-Escobar, M., Calero-Bernal, R., Regidor-Cerrillo, J., Vallejo, R., Benavides, J., Collantes-Fernández, E., Ortega-Mora, L.M., 2021. In vivo and in vitro models show unexpected degrees of virulence among Toxoplasma gondii type

II and III isolates from sheep. Vet Res 52, 82. https://doi.org/10.1186/s13567-021-00953-7

Fernández-Escobar, M., Schares, G., Maksimov, P., Joeres, M., Ortega-Mora, L.M., Calero-Bernal, R., 2022. Toxoplasma gondii Genotyping: A Closer Look Into Europe. Frontiers in Cellular and Infection Microbiology 12.

Flegr, J., 2015. Neurological and Neuropsychiatric Consequences of Chronic Toxoplasma Infection. Curr Clin Micro Rpt 2, 163–172. https://doi.org/10.1007/s40588-015-0024-0

Franco, M., Panas, M.W., Marino, N.D., Lee, M.-C.W., Buchholz, K.R., Kelly, F.D., Bednarski, J.J., Sleckman, B.P., Pourmand, N., Boothroyd, J.C., 2016. A Novel Secreted Protein, MYR1, Is Central to Toxoplasma's Manipulation of Host Cells. mBio 7, 10.1128/mbio.02231-15. https://doi.org/10.1128/mbio.02231-15

Frénal, K., Dubremetz, J.-F., Lebrun, M., Soldati-Favre, D., 2017. Gliding motility powers invasion and egress in Apicomplexa. Nat Rev Microbiol 15, 645–660. https://doi.org/10.1038/nrmicro.2017.86

Fritsch, M., Günther, S.D., Schwarzer, R., Albert, M.-C., Schorn, F., Werthenbach, J.P., Schiffmann, L.M., Stair, N., Stocks, H., Seeger, J.M., Lamkanfi, M., Krönke, M., Pasparakis, M., Kashkar, H., 2019. Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis. Nature 575, 683–687. https://doi.org/10.1038/s41586-019-1770-6

Fukada, S., Uezumi, A., Ikemoto, M., Masuda, S., Segawa, M., Tanimura, N., Yamamoto, H., Miyagoe-Suzuki, Y., Takeda, S., 2007. Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells 25, 2448–2459. https://doi.org/10.1634/stemcells.2007-0019

Fuks, J.M., Arrighi, R.B.G., Weidner, J.M., Kumar Mendu, S., Jin, Z., Wallin, R.P.A., Rethi, B., Birnir, B., Barragan, A., 2012. GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by Toxoplasma gondii. PLoS Pathog 8, e1003051. https://doi.org/10.1371/journal.ppat.1003051

Galal, L., Ariey, F., Gouilh, M.A., Dardé, M.-L., Hamidović, A., Letourneur, F., Prugnolle, F., Mercier, A., 2022. A unique Toxoplasma gondii haplotype accompanied the global expansion of cats. Nat Commun 13, 5778. https://doi.org/10.1038/s41467-022-33556-7

Galley, Y., Hagens, G., Glaser, I., Davis, W., Eichhorn, M., Dobbelaere, D., 1997. Jun NH2-terminal kinase is constitutively activated in T cells transformed by the intracellular parasite Theileria parva. Proc Natl Acad Sci U S A 94, 5119–5124.

García-Sánchez, M., Jiménez-Pelayo, L., Horcajo, P., Collantes-Fernández, E., Ortega-Mora, L.M., Regidor-Cerrillo, J., 2020. Neospora caninum infection induces an isolate virulence-dependent pro-inflammatory gene expression profile in bovine monocyte-derived macrophages. Parasit Vectors 13, 374. https://doi.org/10.1186/s13071-020-04239-3

Gatkowska, J., Wieczorek, M., Dziadek, B., Dzitko, K., Dlugonska, H., 2013. Sex-dependent neurotransmitter level changes in brains of Toxoplasma gondii infected mice. Exp Parasitol 133, 1–7. https://doi.org/10.1016/j.exppara.2012.10.005

Giglia-Mari, G., Zotter, A., Vermeulen, W., 2011. DNA Damage Response. Cold Spring Harb Perspect Biol 3, a000745. https://doi.org/10.1101/cshperspect.a000745

Gondim, L.F.P., McAllister, M.M., Anderson-Sprecher, R.C., Björkman, C., Lock, T.F., Firkins, L.D., Gao, L., Fischer, W.R., 2004. Transplacental transmission and abortion in cows administered Neospora caninum oocysts. J Parasitol 90, 1394–1400. https://doi.org/10.1645/GE-359R

Goodarzi, A.A., Block, W.D., Lees-Miller, S.P., 2003. The role of ATM and ATR in DNA damage-induced cell cycle control. Prog Cell Cycle Res 5, 393–411.

Green, D.R., 2022. The Death Receptor Pathway of Apoptosis. Cold Spring Harb Perspect Biol 14, a041053. https://doi.org/10.1101/cshperspect.a041053

Grigg, M.E., Bonnefoy, S., Hehl, A.B., Suzuki, Y., Boothroyd, J.C., 2001. Success and virulence in Toxoplasma as the result of sexual recombination between two distinct ancestries. Science 294, 161–165. https://doi.org/10.1126/science.1061888

Grigg, M.E., Suzuki, Y., 2003. Sexual recombination and clonal evolution of virulence in *Toxoplasma*. Microbes and Infection 5, 685–690. https://doi.org/10.1016/S1286-4579(03)00088-1

Grimwood, J., Mineo, J.R., Kasper, L.H., 1996. Attachment of Toxoplasma gondii to host cells is host cell cycle dependent. Infect Immun 64, 4099–4104.

Gu, Y., Rosenblatt, J., Morgan, D.O., 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J 11, 3995–4005.

Gubbels, M.-J., White, M., Szatanek, T., 2008. The cell cycle and Toxoplasma gondii cell division: Tightly knit or loosely stitched? International Journal for Parasitology 38, 1343–1358. https://doi.org/10.1016/j.ijpara.2008.06.004

Gulinello, M., Acquarone, M., Kim, J.H., Spray, D.C., Barbosa, H.S., Sellers, R., Tanowitz, H.B., Weiss, L.M., 2010. Acquired infection with Toxoplasma gondii in adult mice results in sensorimotor deficits but normal cognitive behavior despite widespread brain pathology. Microbes Infect 12, 528–537. https://doi.org/10.1016/j.micinf.2010.03.009

Guo, Y., Kim, C., Mao, Y., 2013. New Insights into the Mechanism for Chromosome Alignment in Metaphase. Int Rev Cell Mol Biol 303, 237–262. https://doi.org/10.1016/B978-0-12-407697-6.00006-4

Gutierrez, R., O'Connor, T.R., 2021. DNA direct reversal repair and alkylating agent drug resistance. Cancer Drug Resist 4, 414–423. https://doi.org/10.20517/cdr.2020.113

Hakem, R., 2008. DNA-damage repair; the good, the bad, and the ugly. EMBO J 27, 589–605. https://doi.org/10.1038/emboj.2008.15

Hanson, K.K., March, S., Ng, S., Bhatia, S.N., Mota, M.M., 2015. In vitro alterations do not reflect a requirement for host cell cycle progression during Plasmodium liver stage infection. Eukaryot Cell 14, 96–103. https://doi.org/10.1128/EC.00166-14

Hellmuth, S., Gómez-H, L., Pendás, A.M., Stemmann, O., 2020. Securin-independent regulation of separase by checkpoint-induced shugoshin–MAD2. Nature 580, 536–541. https://doi.org/10.1038/s41586-020-2182-3

Hemphill, A., Gottstein, B., 2000. A European perspective on Neospora caninum. Int J Parasitol 30, 877–924. https://doi.org/10.1016/s0020-7519(00)00072-2

Hetzer, M.W., 2010. The Nuclear Envelope. Cold Spring Harb Perspect Biol 2, a000539. https://doi.org/10.1101/cshperspect.a000539

Hill, D., Dubey, J.P., 2002. Toxoplasma gondii: transmission, diagnosis and prevention. Clinical Microbiology and Infection 8, 634–640. https://doi.org/10.1046/j.1469-0691.2002.00485.x

Hinchcliffe, E.H., Miller, F.J., Cham, M., Khodjakov, A., Sluder, G., 2001. Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. Science 291, 1547–1550. https://doi.org/10.1126/science.1056866

Hofer, U., 2019. The cat is out the bag about Toxoplasma host range. Nat Rev Microbiol 17, 646–647. https://doi.org/10.1038/s41579-019-0266-6

Hoffmann, A.R., Cadieu, J., Kiupel, M., Lim, A., Bolin, S.R., Mansell, J., 2012. Cutaneous toxoplasmosis in two dogs. J Vet Diagn Invest 24, 636–640. https://doi.org/10.1177/1040638712440995

Holec-Gąsior, L., Sołowińska, K., 2023. Detection of Toxoplasma gondii Infection in Small Ruminants: Old Problems, and Current Solutions. Animals (Basel) 13, 2696. https://doi.org/10.3390/ani13172696

Holland, A.J., Cleveland, D.W., 2009. Boveri revisited: Chromosomal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol 10, 478–487. https://doi.org/10.1038/nrm2718

Horcajo, P., Regidor-Cerrillo, J., Aguado-Martínez, A., Hemphill, A., Ortega-Mora, L.M., 2016. Vaccines for bovine neosporosis: current status and key aspects for development. Parasite Immunology 38, 709–723. https://doi.org/10.1111/pim.12342

House, P.K., Vyas, A., Sapolsky, R., 2011. Predator Cat Odors Activate Sexual Arousal Pathways in Brains of Toxoplasma gondii Infected Rats. PLoS One 6, e23277. https://doi.org/10.1371/journal.pone.0023277

Howe, D.K., Sibley, L.D., 1995. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 172, 1561–1566. https://doi.org/10.1093/infdis/172.6.1561

Huet, X., Rech, J., Plet, A., Vié, A., Blanchard, J.M., 1996. Cyclin A expression is under negative transcriptional control during the cell cycle. Mol Cell Biol 16, 3789–3798.

Hunter, C.A., Sibley, L.D., 2012. Modulation of innate immunity by Toxoplasma gondii virulence effectors. Nat Rev Microbiol 10, 766–778. https://doi.org/10.1038/nrmicro2858

Ikemoto, S., 2010. Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory. Neurosci Biobehav Rev 35, 129–150. https://doi.org/10.1016/j.neubiorev.2010.02.001

Innes, E.A., 2010. A brief history and overview of Toxoplasma gondii. Zoonoses Public Health 57, 1–7. https://doi.org/10.1111/j.1863-2378.2009.01276.x

Innes, E.A., 2007. The host-parasite relationship in pregnant cattle infected with Neospora caninum. Parasitology 134, 1903–1910. https://doi.org/10.1017/S0031182007000194

Innes, E.A., Andrianarivo, A.G., Björkman, C., Williams, D.J.L., Conrad, P.A., 2002. Immune responses to *Neospora caninum* and prospects for vaccination. Trends in Parasitology 18, 497–504. https://doi.org/10.1016/S1471-4922(02)02372-3

Innes, E.A., Wright, S., Bartley, P., Maley, S., Macaldowie, C., Esteban-Redondo, I., Buxton, D., 2005. The host–parasite relationship in bovine neosporosis. Veterinary Immunology and Immunopathology, 7th International Veterinary Immunology Symposium 108, 29–36. https://doi.org/10.1016/j.vetimm.2005.07.004

Janouškovec, J., Paskerova, G.G., Miroliubova, T.S., Mikhailov, K.V., Birley, T., Aleoshin, V.V., Simdyanov, T.G., 2019. Apicomplexan-like parasites are polyphyletic and widely but selectively dependent on cryptic plastid organelles. Elife 8, e49662. https://doi.org/10.7554/eLife.49662

Jiménez-Pelayo, L., García-Sánchez, M., Regidor-Cerrillo, J., Horcajo, P., Collantes-Fernández, E., Gómez-Bautista, M., Hambruch, N., Pfarrer, C., Ortega-Mora, L.M., 2019. Immune response profile of caruncular and trophoblast cell lines infected by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of Neospora caninum. Parasit Vectors 12, 218. https://doi.org/10.1186/s13071-019-3466-z

Jordan, K.A., Hunter, C.A., 2010. Regulation of CD8+ T Cell Responses to Infection With Parasitic Protozoa. Exp Parasitol 126, 318–325. https://doi.org/10.1016/j.exppara.2010.05.008

Kalkan, B.M., Ozcan, S.C., Quintyne, N.J., Reed, S.L., Acilan, C., 2022. Keep Calm and Carry on with Extra Centrosomes. Cancers (Basel) 14, 442. https://doi.org/10.3390/cancers14020442

Kalogeropoulos, D., Sakkas, H., Mohammed, B., Vartholomatos, G., Malamos, K., Sreekantam, S., Kanavaros, P., Kalogeropoulos, C., 2022. Ocular toxoplasmosis: a review of the current diagnostic and therapeutic approaches. Int Ophthalmol 42, 295–321. https://doi.org/10.1007/s10792-021-01994-9

Kamerkar, S., Davis, P.H., 2012. Toxoplasma on the Brain: Understanding Host-Pathogen Interactions in Chronic CNS Infection. J Parasitol Res 2012, 589295. https://doi.org/10.1155/2012/589295

Kannan, G., Crawford, J.A., Yang, C., Gressitt, K.L., Ihenatu, C., Krasnova, I.N., Cadet, J., Yolken, R.H., Severance, E.G., Pletnikov, M.V., 2016. Anti-NMDA Receptor Autoantibodies and Associated Neurobehavioral Pathology in Mice are

Dependent on Age of First Exposure to Toxoplasma gondii. Neurobiol Dis 91, 307–314. https://doi.org/10.1016/j.nbd.2016.03.005

Karakaya, K., Herbst, F., Ball, C., Glimm, H., Krämer, A., Löffler, H., 2012. Overexpression of EVI1 interferes with cytokinesis and leads to accumulation of cells with supernumerary centrosomes in G0/1 phase. Cell Cycle 11, 3492–3503. https://doi.org/10.4161/cc.21801

Karnitz, L.M., Zou, L., 2015. Molecular Pathways: Targeting ATR in Cancer Therapy. Clinical Cancer Research 21, 4780–4785. https://doi.org/10.1158/1078-0432.CCR-15-0479

Kato, K., 2018. How does Toxoplama gondii invade host cells? J Vet Med Sci 80, 1702–1706. https://doi.org/10.1292/jvms.18-0344

Kaushansky, A., Ye, A.S., Austin, L.S., Mikolajczak, S.A., Vaughan, A.M., Camargo, N., Metzger, P.G., Douglass, A.N., MacBeath, G., Kappe, S.H.I., 2013. Interrogation of infected hepatocyte signaling reveals that suppression of host p53 is critical for Plasmodium liver stage infection. Cell Rep 3, 630–637. https://doi.org/10.1016/j.celrep.2013.02.010

Kawaguchi, S., Nakamura, T., Yamamoto, A., Honda, G., Sasaki, Y.F., 2010. Is the Comet Assay a Sensitive Procedure for Detecting Genotoxicity? J Nucleic Acids 2010, 541050. https://doi.org/10.4061/2010/541050

Khan, A., Fux, B., Su, C., Dubey, J.P., Darde, M.L., Ajioka, J.W., Rosenthal, B.M., Sibley, L.D., 2007. Recent transcontinental sweep of Toxoplasma gondii driven by a single monomorphic chromosome. Proc Natl Acad Sci U S A 104, 14872–14877. https://doi.org/10.1073/pnas.0702356104

Khan, A., Shaik, J.S., Sikorski, P., Dubey, J.P., Grigg, M.E., 2020. Neosporosis: An Overview of Its Molecular Epidemiology and Pathogenesis. Engineering 6, 10–19. https://doi.org/10.1016/j.eng.2019.02.010

Khan, A., Su, C., German, M., Storch, G.A., Clifford, D.B., Sibley, L.D., 2005. Genotyping of Toxoplasma gondii Strains from Immunocompromised Patients Reveals High Prevalence of Type I Strains. J Clin Microbiol 43, 5881–5887. https://doi.org/10.1128/JCM.43.12.5881-5887.2005

Khatau, S.B., Hale, C.M., Stewart-Hutchinson, P.J., Patel, M.S., Stewart, C.L., Searson, P.C., Hodzic, D., Wirtz, D., 2009. A perinuclear actin cap regulates nuclear shape. Proc Natl Acad Sci U S A 106, 19017–19022. https://doi.org/10.1073/pnas.0908686106

Khelifa, A.S., Guillen Sanchez, C., Lesage, K.M., Huot, L., Mouveaux, T., Pericard, P., Barois, N., Touzet, H., Marot, G., Roger, E., Gissot, M., 2021. TgAP2IX-5 is a key transcriptional regulator of the asexual cell cycle division in Toxoplasma gondii. Nat Commun 12, 116. https://doi.org/10.1038/s41467-020-20216-x

Kim, D.-H., Chambliss, A.B., Wirtz, D., 2013. The multi-faceted role of the actin cap in cellular mechanosensation and mechanotransduction. Soft Matter 9, 5516–5523. https://doi.org/10.1039/C3SM50798J

Kim, M.J., Jung, B.-K., Cho, J., Song, H., Pyo, K.-H., Lee, J.M., Kim, M.-K., Chai, J.-Y., 2016. Exosomes Secreted by *Toxoplasma gondii*-Infected L6 Cells: Their Effects on Host Cell Proliferation and Cell Cycle Changes. Korean J Parasitol 54, 147–154. https://doi.org/10.3347/kjp.2016.54.2.147

King, J.S., Jenkins, D.J., Ellis, J.T., Fleming, P., Windsor, P.A., Šlapeta, J., 2011. Implications of wild dog ecology on the sylvatic and domestic life cycle of Neospora caninum in Australia. Vet J 188, 24–33. https://doi.org/10.1016/j.tvjl.2010.03.002

Kline-Smith, S.L., Walczak, C.E., 2004. Mitotic Spindle Assembly and Chromosome Segregation: Refocusing on Microtubule Dynamics. Molecular Cell 15, 317–327. https://doi.org/10.1016/j.molcel.2004.07.012

Knowler, C., Wheeler, S.J., 1995. Neospora caninum infection in three dogs. J Small Anim Pract 36, 172–177. https://doi.org/10.1111/j.1748-5827.1995.tb02875.x

Kõivomägi, M., Valk, E., Venta, R., Iofik, A., Lepiku, M., Balog, E.R.M., Rubin, S.M., Morgan, D.O., Loog, M., 2011. Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase. Nature 480, 128–131. https://doi.org/10.1038/nature10560

Konradt, C., Ueno, N., Christian, D.A., Delong, J.H., Pritchard, G.H., Herz, J., Bzik, D.J., Koshy, A.A., McGavern, D.B., Lodoen, M.B., Hunter, C.A., 2016. Endothelial cells are a replicative niche for entry of Toxoplasma gondii to the central nervous system. Nat Microbiol 1, 16001. https://doi.org/10.1038/nmicrobiol.2016.1

Kousholt, A.N., Menzel, T., Sørensen, C.S., 2012. Pathways for Genome Integrity in G2 Phase of the Cell Cycle. Biomolecules 2, 579–607. https://doi.org/10.3390/biom2040579

Krämer, A., Mailand, N., Lukas, C., Syljuåsen, R.G., Wilkinson, C.J., Nigg, E.A., Bartek, J., Lukas, J., 2004. Centrosome-associated Chk1 prevents premature activation

of cyclin-B-Cdk1 kinase. Nat Cell Biol 6, 884–891. https://doi.org/10.1038/ncb1165

Krokan, H.E., Bjørås, M., 2013. Base Excision Repair. Cold Spring Harb Perspect Biol 5, a012583. https://doi.org/10.1101/cshperspect.a012583

Krug, E.C., Marr, J.J., Berens, R.L., 1989. Purine Metabolism in Toxoplasma gondii. Journal of Biological Chemistry 264, 10601–10607. https://doi.org/10.1016/S0021-9258(18)81663-5

Kul, O., Atmaca, H.T., Anteplioglu, T., Ocal, N., Canpolat, S., 2015. Neospora caninum: the First Demonstration of the Enteroepithelial Stages in the Intestines of a Naturally Infected Dog. J Comp Pathol 153, 9–13. https://doi.org/10.1016/j.jcpa.2015.03.005

Kuo, L.J., Yang, L.-X., 2008. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. In Vivo 22, 305–309.

Lavine, M.D., Arrizabalaga, G., 2009. Induction of mitotic S-phase of host and neighboring cells by Toxoplasma gondii enhances parasite invasion. Molecular and Biochemical Parasitology 164, 95–99. https://doi.org/10.1016/j.molbiopara.2008.11.014

Levine, M.S., Holland, A.J., 2018. The impact of mitotic errors on cell proliferation and tumorigenesis. Genes Dev 32, 620–638. https://doi.org/10.1101/gad.314351.118

Li, G.-M., 2008. Mechanisms and functions of DNA mismatch repair. Cell Res 18, 85–98. https://doi.org/10.1038/cr.2007.115

Li, L., Guan, Y., Chen, X., Yang, J., Cheng, Y., 2021. DNA Repair Pathways in Cancer Therapy and Resistance. Front. Pharmacol. 11. https://doi.org/10.3389/fphar.2020.629266

Li, M., Sang, X., Zhang, X., Li, X., Feng, Y., Yang, N., Jiang, T., 2023. A Metabolomic and Transcriptomic Study Revealed the Mechanisms of Lumefantrine Inhibition of Toxoplasma gondii. Int J Mol Sci 24, 4902. https://doi.org/10.3390/ijms24054902

Li, X., Heyer, W.-D., 2008. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 18, 99–113. https://doi.org/10.1038/cr.2008.1

Lieber, M.R., 2010. The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End Joining Pathway. Annu Rev Biochem 79, 181–211. https://doi.org/10.1146/annurev.biochem.052308.093131

Lin, S.-Y., Rai, R., Li, K., Xu, Z.-X., Elledge, S.J., 2005. BRIT1/MCPH1 is a DNA damage responsive protein that regulates the Brca1–Chk1 pathway, implicating checkpoint dysfunction in microcephaly. Proc Natl Acad Sci U S A 102, 15105–15109. https://doi.org/10.1073/pnas.0507722102

Lindqvist, A., Zon, W. van, Rosenthal, C.K., Wolthuis, R.M.F., 2007. Cyclin B1–Cdk1 Activation Continues after Centrosome Separation to Control Mitotic Progression. PLOS Biology 5, e123. https://doi.org/10.1371/journal.pbio.0050123

Lindsay, D.S., Collins, M.V., Mitchell, S.M., Wetch, C.N., Rosypal, A.C., Flick, G.J., Zajac, A.M., Lindquist, A., Dubey, J.P., 2004. Survival of Toxoplasma gondii oocysts in Eastern oysters (Crassostrea virginica). J Parasitol 90, 1054–1057. https://doi.org/10.1645/GE-296R

Lindsay, D.S., Dubey, J.P., 2020. Neosporosis, Toxoplasmosis, and Sarcocystosis in Ruminants: An Update. Vet Clin North Am Food Anim Pract 36, 205–222. https://doi.org/10.1016/j.cvfa.2019.11.004

Loncarek, J., Hergert, P., Magidson, V., Khodjakov, A., 2008. Control of daughter centriole formation by the pericentriolar material. Nat Cell Biol 10, 322–328. https://doi.org/10.1038/ncb1694

Lu, D., Hsiao, J.Y., Davey, N.E., Van Voorhis, V.A., Foster, S.A., Tang, C., Morgan, D.O., 2014. Multiple mechanisms determine the order of APC/C substrate degradation in mitosis. J Cell Biol 207, 23–39. https://doi.org/10.1083/jcb.201402041

Luo, S., Tong, L., 2021a. Structure and function of the separase-securin complex. Subcell Biochem 96, 217–232. https://doi.org/10.1007/978-3-030-58971-4_4

Luo, S., Tong, L., 2021b. Structure and function of the separase-securin complex. Subcell Biochem 96, 217–232. https://doi.org/10.1007/978-3-030-58971-4_4

Mangot, A.G., 2016. Psychiatric aspects of toxoplasmosis: an Indian perspective. J Parasit Dis 40, 1636–1639. https://doi.org/10.1007/s12639-015-0684-y

Maninova, M., Caslavsky, J., Vomastek, T., 2017. The assembly and function of perinuclear actin cap in migrating cells. Protoplasma 254, 1207–1218. https://doi.org/10.1007/s00709-017-1077-0

Maréchal, A., Zou, L., 2013. DNA Damage Sensing by the ATM and ATR Kinases. Cold Spring Harb Perspect Biol 5, a012716. https://doi.org/10.1101/cshperspect.a012716

Marshall, C., 1999. How do small GTPase signal transduction pathways regulate cell cycle entry? Current Opinion in Cell Biology 11, 732–736. https://doi.org/10.1016/S0955-0674(99)00044-7

Martínez-Ocampo, F., 2018. Genomics of Apicomplexa, in: Farm Animals Diseases, Recent Omic Trends and New Strategies of Treatment. IntechOpen. https://doi.org/10.5772/intechopen.72633

Martorelli Di Genova, B., Knoll, L.J., 2020. Comparisons of the Sexual Cycles for the Coccidian Parasites Eimeria and Toxoplasma. Front Cell Infect Microbiol 10, 604897. https://doi.org/10.3389/fcimb.2020.604897

Masala, G., Porcu, R., Daga, C., Denti, S., Canu, G., Patta, C., Tola, S., 2007. Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J Vet Diagn Invest 19, 96–98. https://doi.org/10.1177/104063870701900116

Masala, G., Porcu, R., Madau, L., Tanda, A., Ibba, B., Satta, G., Tola, S., 2003. Survey of ovine and caprine toxoplasmosis by IFAT and PCR assays in Sardinia, Italy. Veterinary Parasitology 117, 15–21. https://doi.org/10.1016/j.vetpar.2003.07.012

Mathur, V., Kolísko, M., Hehenberger, E., Irwin, N.A.T., Leander, B.S., Kristmundsson, Á., Freeman, M.A., Keeling, P.J., 2019. Multiple Independent Origins of Apicomplexan-Like Parasites. Curr Biol 29, 2936-2941.e5. https://doi.org/10.1016/j.cub.2019.07.019

Matsumoto, Y., Maller, J.L., 2004. A centrosomal localization signal in cyclin E required for Cdk2-independent S phase entry. Science 306, 885–888. https://doi.org/10.1126/science.1103544

Matsuura, S., Matsumoto, Y., Morishima, K., Izumi, H., Matsumoto, H., Ito, E., Tsutsui, K., Kobayashi, J., Tauchi, H., Kajiwara, Y., Hama, S., Kurisu, K., Tahara, H., Oshimura, M., Komatsu, K., Ikeuchi, T., Kajii, T., 2006. Monoallelic BUB1B mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome. Am J Med Genet A 140, 358–367. https://doi.org/10.1002/ajmg.a.31069

McAllister, Milton M, Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Rapid communication: Dogs are definitive hosts of Neospora caninum. International Journal for Parasitology 28, 1473–1479. https://doi.org/10.1016/S0020-7519(98)00138-6

McAllister, M. M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of Neospora caninum. Int J Parasitol 28, 1473–1478.

McConnell, J.F., Sparkes, A.H., Blunden, A.S., Neath, P.J., Sansom, J., 2007. Eosinophilic fibrosing gastritis and toxoplasmosis in a cat. J Feline Med Surg 9, 82–88. https://doi.org/10.1016/j.jfms.2006.11.005

McFarland, R., Wang, Z.T., Jouroukhin, Y., Li, Y., Mychko, O., Coppens, I., Xiao, J., Jones-Brando, L., Yolken, R.H., Sibley, L.D., Pletnikov, M.V., 2018. AAH2 gene is not required for dopamine-dependent neurochemical and behavioral abnormalities produced by Toxoplasma infection in mouse. Behav Brain Res 347, 193–200. https://doi.org/10.1016/j.bbr.2018.03.023

McIntosh, J.R., 2016. Mitosis. Cold Spring Harb Perspect Biol 8, a023218. https://doi.org/10.1101/cshperspect.a023218

Menard, K.L., Bu, L., Denkers, E.Y., 2021. Transcriptomics analysis of Toxoplasma gondii-infected mouse macrophages reveals coding and noncoding signatures in the presence and absence of MyD88. BMC Genomics 22, 130. https://doi.org/10.1186/s12864-021-07437-0

Miller, M.A., Newberry, C.A., Sinnott, D.M., Batac, F.I., Greenwald, K., Reed, A., Young, C., Harris, M.D., Packham, A.E., Shapiro, K., 2023. Newly detected, virulent Toxoplasma gondii COUG strain causing fatal steatitis and toxoplasmosis in southern sea otters (Enhydra lutris nereis). Frontiers in Marine Science 10.

Mittal, K., Kaur, J., Jaczko, M., Wei, G., Toss, M.S., Rakha, E.A., Janssen, E.A.M., Søiland, H., Kucuk, O., Reid, M.D., Gupta, M.V., Aneja, R., 2021. Centrosome amplification: a quantifiable cancer cell trait with prognostic value in solid malignancies. Cancer metastasis reviews 40, 319. https://doi.org/10.1007/s10555-020-09937-z

Molestina, R.E., El-Guendy, N., Sinai, A.P., 2008. Infection with Toxoplasma gondii results in dysregulation of the host cell cycle. Cellular Microbiology 10, 1153–1165. https://doi.org/10.1111/j.1462-5822.2008.01117.x

Molestina, R.E., Sinai, A.P., 2005. Host and parasite-derived IKK activities direct distinct temporal phases of NF-kappaB activation and target gene expression following Toxoplasma gondii infection. J Cell Sci 118, 5785–5796. https://doi.org/10.1242/jcs.02709

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 363, 1965–1976. https://doi.org/10.1016/S0140-6736(04)16412-X

Morgan, D.O., 1996. The dynamics of cyclin dependent kinase structure. Current Opinion in Cell Biology 8, 767–772. https://doi.org/10.1016/S0955-0674(96)80076-7

Müller, N., Schwarz, M.J., 2007. The immune-mediated alteration of serotonin and glutamate: towards an integrated view of depression. Mol Psychiatry 12, 988–1000. https://doi.org/10.1038/sj.mp.4002006

Naghavi, M.H., Walsh, D., 2017. Microtubule Regulation and Function during Virus Infection. J Virol 91, e00538-17. https://doi.org/10.1128/JVI.00538-17

Nalpas, N.C., Magee, D.A., Conlon, K.M., Browne, J.A., Healy, C., McLoughlin, K.E., Rue-Albrecht, K., McGettigan, P.A., Killick, K.E., Gormley, E., Gordon, S.V., MacHugh, D.E., 2015. RNA sequencing provides exquisite insight into the manipulation of the alveolar macrophage by tubercle bacilli. Sci Rep 5, 13629. https://doi.org/10.1038/srep13629

Nayeri, T., Sarvi, S., Daryani, A., 2024. Effective factors in the pathogenesis of Toxoplasma gondii. Heliyon 10. https://doi.org/10.1016/j.heliyon.2024.e31558

Nelson, M.M., Jones, A.R., Carmen, J.C., Sinai, A.P., Burchmore, R., Wastling, J.M., 2008. Modulation of the host cell proteome by the intracellular apicomplexan parasite Toxoplasma gondii. Infect Immun 76, 828–844. https://doi.org/10.1128/IAI.01115-07

Ngô, H.M., Zhou, Ying, Lorenzi, H., Wang, K., Kim, T.-K., Zhou, Yong, El Bissati, K., Mui, E., Fraczek, L., Rajagopala, S.V., Roberts, C.W., Henriquez, F.L., Montpetit, A., Blackwell, J.M., Jamieson, S.E., Wheeler, K., Begeman, I.J., Naranjo-Galvis, C., Alliey-Rodriguez, N., Davis, R.G., Soroceanu, L., Cobbs, C., Steindler, D.A., Boyer, K., Noble, A.G., Swisher, C.N., Heydemann, P.T., Rabiah, P., Withers, S., Soteropoulos, P., Hood, L., McLeod, R., 2017. Toxoplasma Modulates Signature Pathways of Human Epilepsy, Neurodegeneration & Cancer. Sci Rep 7, 11496. https://doi.org/10.1038/s41598-017-10675-6

Nicolle, C., Manceaux, L., Nicolle, C., Manceaux, L., 1909. Sur un Protozoaire nouveau du Gondi (Toxoplasma n. gen.).

Niedelman, W., Sprokholt, J.K., Clough, B., Frickel, E.-M., Saeij, J.P.J., 2013. Cell death of gamma interferon-stimulated human fibroblasts upon Toxoplasma

gondii infection induces early parasite egress and limits parasite replication. Infect Immun 81, 4341–4349. https://doi.org/10.1128/IAI.00416-13

Nishikawa, Y., Mishima, M., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H., Mikami, T., 2001. Interferon-gamma-induced apoptosis in host cells infected with Neospora caninum. Parasitology 123, 25–31. https://doi.org/10.1017/s0031182001008095

Nolan, S.J., Romano, J.D., Luechtefeld, T., Coppens, I., 2015. Neospora caninum Recruits Host Cell Structures to Its Parasitophorous Vacuole and Salvages Lipids from Organelles. Eukaryotic Cell 14, 454–473. https://doi.org/10.1128/ec.00262-14

Ojo, K.K., Reid, M.C., Kallur Siddaramaiah, L., Müller, J., Winzer, P., Zhang, Z., Keyloun, K.R., Vidadala, R.S.R., Merritt, E.A., Hol, W.G.J., Maly, D.J., Fan, E., Van Voorhis, W.C., Hemphill, A., 2014. Neospora caninum calcium-dependent protein kinase 1 is an effective drug target for neosporosis therapy. PLoS One 9, e92929. https://doi.org/10.1371/journal.pone.0092929

Oliveira, V. da C., Boechat, V.C., Mendes Junior, A.A.V., Madeira, M. de F., Ferreira, L.C., Figueiredo, F.B., Campos, M.P., de Carvalho Rodrigues, F. das C., Carvalhaes de Oliveira, R. de V., Amendoeira, M.R.R., Menezes, R.C., 2017. Occurrence of Leishmania infantum in the central nervous system of naturally infected dogs: Parasite load, viability, co-infections and histological alterations. PLoS One 12, e0175588. https://doi.org/10.1371/journal.pone.0175588

Ong, J.Y., Torres, J.Z., 2019. Dissecting the mechanisms of cell division. J Biol Chem 294, 11382–11390. https://doi.org/10.1074/jbc.AW119.008149

Opitz, C., Soldati, D., 2002. “The glideosome”: a dynamic complex powering gliding motion and host cell invasion by Toxoplasma gondii. Mol Microbiol 45, 597–604. https://doi.org/10.1046/j.1365-2958.2002.03056.x

Orr, B., Maiato, H., 2019. No chromosome left behind: The importance of metaphase alignment for mitotic fidelity. Journal of Cell Biology 218, 1086–1088. https://doi.org/10.1083/jcb.201902041

Ortega-Mora, L.M., Ferre, I., del-Pozo, I., Caetano-da-Silva, A., Collantes-Fernández, E., Regidor-Cerrillo, J., Ugarte-Garagalza, C., Aduriz, G., 2003. Detection of Neospora caninum in semen of bulls. Vet Parasitol 117, 301–308. https://doi.org/10.1016/j.vetpar.2003.09.015

Owen, M.R., Clarkson, M.J., Trees, A.J., 1998. Diagnosis of toxoplasma abortion in ewes by polymerase chain reaction. Vet Rec 142, 445–448. https://doi.org/10.1136/vr.142.17.445

Ozaki, T., Nakagawara, A., 2011. Role of p53 in Cell Death and Human Cancers. Cancers (Basel) 3, 994–1013. https://doi.org/10.3390/cancers3010994

Pampalona, J., Roscioli, E., Silkworth, W.T., Bowden, B., Genescà, A., Tusell, L., Cimini, D., 2016. Chromosome Bridges Maintain Kinetochore-Microtubule Attachment throughout Mitosis and Rarely Break during Anaphase. PLOS ONE 11, e0147420. https://doi.org/10.1371/journal.pone.0147420

Panas, M.W., Naor, A., Cygan, A.M., Boothroyd, J.C., 2019. Toxoplasma Controls Host Cyclin E Expression through the Use of a Novel MYR1-Dependent Effector Protein, HCE1. mBio 10, e00674-19. https://doi.org/10.1128/mBio.00674-19

Pascreau, G., Churchill, M.E., Maller, J.L., 2011. Centrosomal localization of cyclins E and A. Cell Cycle 10, 199–205. https://doi.org/10.4161/cc.10.2.14444

Patitucci, A.N., Alley, M.R., Jones, B.R., Charleston, W.A., 1997. Protozoal encephalomyelitis of dogs involving Neospora caninum and Toxoplasma gondii in New Zealand. N Z Vet J 45, 231–235. https://doi.org/10.1080/00480169.1997.36035

Pereyra, R., Mansilla, F.C., Petersen, M.I., Suarez, V., Capozzo, A.V., 2019. Evidence of reduced vertical transmission of *Neospora caninum* associated with higher IgG1 than IgG2 serum levels and presence of IFN-γ in non-aborting chronically infected cattle under natural condition. Veterinary Immunology and Immunopathology 208, 53–57. https://doi.org/10.1016/j.vetimm.2019.01.001

Peterson, J.L., Willard, M.D., Lees, G.E., Lappin, M.R., Dieringer, T., Floyd, E., 1991. Toxoplasmosis in two cats with inflammatory intestinal disease. J Am Vet Med Assoc 199, 473–476.

Petsalaki, E., Zachos, G., 2020. DNA damage response proteins regulating mitotic cell division: double agents preserving genome stability. FEBS J 287, 1700–1721. https://doi.org/10.1111/febs.15240

Pierre-Louis, E., Etheridge, M.G., de Paula Baptista, R., Khan, A., Chasen, N.M., Etheridge, R.D., 2022. Disruption of Toxoplasma gondii-Induced Host Cell DNA Replication Is Dependent on Contact Inhibition and Host Cell Type. mSphere 7, e0016022. https://doi.org/10.1128/msphere.00160-22

Pietenpol, J.A., Stewart, Z.A., 2002. Cell cycle checkpoint signaling:: Cell cycle arrest versus apoptosis. Toxicology 181–182, 475–481. https://doi.org/10.1016/S0300-483X(02)00460-2

Pratt, S., Wansadhipathi-Kannangara, N.K., Bruce, C.R., Mina, J.G., Shams-Eldin, H., Casas, J., Hanada, K., Schwarz, R.T., Sonda, S., Denny, P.W., 2013. Sphingolipid synthesis and scavenging in the intracellular apicomplexan parasite, *Toxoplasma gondii*. Molecular and Biochemical Parasitology 187, 43–51. https://doi.org/10.1016/j.molbiopara.2012.11.007

Qian, S., Wei, Z., Yang, W., Huang, J., Yang, Y., Wang, J., 2022. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. Front Oncol 12, 985363. https://doi.org/10.3389/fonc.2022.985363

Raaijmakers, J.A., Van Heesbeen, R.G.H.P., Blomen, V.A., Janssen, L.M.E., Van Diemen, F., Brummelkamp, T.R., Medema, R.H., 2018. BUB1 Is Essential for the Viability of Human Cells in which the Spindle Assembly Checkpoint Is Compromised. Cell Reports 22, 1424–1438. https://doi.org/10.1016/j.celrep.2018.01.034

Radke, J.R., Behnke, M.S., Mackey, A.J., Radke, J.B., Roos, D.S., White, M.W., 2005. The transcriptome of Toxoplasma gondii. BMC Biol 3, 26. https://doi.org/10.1186/1741-7007-3-26

Regidor-Cerrillo, J., Xia, D., Jiménez-Pelayo, L., García-Sánchez, M., Collantes-Fernández, E., Randle, N., Wastling, J., Ortega-Mora, L.-M., Horcajo, P., 2020. Proteomic Characterization of Host-Pathogen Interactions during Bovine Trophoblast Cell Line Infection by Neospora caninum. Pathogens 9, 749. https://doi.org/10.3390/pathogens9090749

Remmerie, A., Scott, C.L., 2018. Macrophages and lipid metabolism. Cell Immunol 330, 27–42. https://doi.org/10.1016/j.cellimm.2018.01.020

Riemann, H.P., Meyer, M.E., Theis, J.H., Kelso, G., Behymer, D.E., 1975. Toxoplasmosis in an infant fed unpasteurized goat milk. J Pediatr 87, 573–576. https://doi.org/10.1016/s0022-3476(75)80825-0

Robben, P.M., Mordue, D.G., Truscott, S.M., Takeda, K., Akira, S., Sibley, L.D., 2004. Production of IL-12 by macrophages infected with Toxoplasma gondii depends on the parasite genotype. J Immunol 172, 3686–3694. https://doi.org/10.4049/jimmunol.172.6.3686

Rodier, F., Campisi, J., 2011. Four faces of cellular senescence. J Cell Biol 192, 547–556. https://doi.org/10.1083/jcb.201009094

Rodriguez-Muñoz, M., Serrat, M., Soler, D., Genescà, A., Anglada, T., 2021. Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells. Front Cell Dev Biol 9, 745195. https://doi.org/10.3389/fcell.2021.745195

Rojas-Pirela, M., Medina, L., Rojas, M.V., Liempi, A.I., Castillo, C., Pérez-Pérez, E., Guerrero-Muñoz, J., Araneda, S., Kemmerling, U., 2021. Congenital Transmission of Apicomplexan Parasites: A Review. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.751648

Romano, J.D., Sonda, S., Bergbower, E., Smith, M.E., Coppens, I., 2013. Toxoplasma gondii salvages sphingolipids from the host Golgi through the rerouting of selected Rab vesicles to the parasitophorous vacuole. Mol Biol Cell 24, 1974–1995. https://doi.org/10.1091/mbc.E12-11-0827

Rueckert, S., Devetak, D., 2017. Gregarines (Apicomplexa, Gregarinasina) in psocids (Insecta, Psocoptera) including a new species description and their potential use as pest control agents. European Journal of Protistology 60, 60–67. https://doi.org/10.1016/j.ejop.2017.05.007

S. Al-Malki, E., 2021. Toxoplasmosis: stages of the protozoan life cycle and risk assessment in humans and animals for an enhanced awareness and an improved socio-economic status. Saudi J Biol Sci 28, 962–969. https://doi.org/10.1016/j.sjbs.2020.11.007

Sabou, M., Doderer-Lang, C., Leyer, C., Konjic, A., Kubina, S., Lennon, S., Rohr, O., Viville, S., Cianférani, S., Candolfi, E., Pfaff, A.W., Brunet, J., 2020. Toxoplasma gondii ROP16 kinase silences the cyclin B1 gene promoter by hijacking host cell UHRF1-dependent epigenetic pathways. Cell Mol Life Sci 77, 2141–2156. https://doi.org/10.1007/s00018-019-03267-2

Sacks, J.J., Roberto, R.R., Brooks, N.F., 1982. Toxoplasmosis infection associated with raw goat's milk. JAMA 248, 1728–1732. https://doi.org/10.1001/jama.1982.03330140038029

Saey, V., Martlé, V., Van Ham, L., Chiers, K., 2010. Neuritis of the cauda equina in a dog. J Small Anim Pract 51, 549–552. https://doi.org/10.1111/j.1748-5827.2010.00976.x

Sajiki, K., Tahara, Y., Uehara, L., Sasaki, T., Pluskal, T., Yanagida, M., 2018. Genetic regulation of mitotic competence in G0 quiescent cells. Sci Adv 4, eaat5685. https://doi.org/10.1126/sciadv.aat5685

Sale, J.E., Lehmann, A.R., Woodgate, R., 2012. Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat Rev Mol Cell Biol 13, 141–152. https://doi.org/10.1038/nrm3289

Sana, M., Rashid, M., Rashid, I., Akbar, H., Gomez-Marin, J.E., Dimier-Poisson, I., 2022. Immune response against toxoplasmosis—some recent updates RH: Toxoplasma gondii immune response. Int J Immunopathol Pharmacol 36, 03946320221078436. https://doi.org/10.1177/03946320221078436

Sánchez-Sánchez, R., Vázquez, P., Ferre, I., Ortega-Mora, L.M., 2018. Treatment of Toxoplasmosis and Neosporosis in Farm Ruminants: State of Knowledge and Future Trends. Curr Top Med Chem 18, 1304–1323. https://doi.org/10.2174/1568026618666181002113617

Satyanarayana, A., Kaldis, P., 2009. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene 28, 2925–2939. https://doi.org/10.1038/onc.2009.170

Schlüter, D., Barragan, A., 2019. Advances and Challenges in Understanding Cerebral Toxoplasmosis. Front Immunol 10, 242. https://doi.org/10.3389/fimmu.2019.00242

Schwartzman, J.D., Pfefferkorn, E.R., 1981. Pyrimidine Synthesis by Intracellular Toxoplasma gondii. The Journal of Parasitology 67, 150–158. https://doi.org/10.2307/3280627

Seeber, F., Steinfelder, S., 2016. Recent advances in understanding apicomplexan parasites. F1000Research 5. https://doi.org/10.12688/f1000research.7924.1

Shapiro, K., Bahia-Oliveira, L., Dixon, B., Dumètre, A., de Wit, L.A., VanWormer, E., Villena, I., 2019. Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food. Food Waterborne Parasitol 15, e00049. https://doi.org/10.1016/j.fawpar.2019.e00049

Shunmugam, S., Arnold, C.-S., Dass, S., Katris, N.J., Botté, C.Y., 2022. The flexibility of Apicomplexa parasites in lipid metabolism. PLoS Pathog 18, e1010313. https://doi.org/10.1371/journal.ppat.1010313

Sibley, L.D., Ajioka, J.W., 2008. Population Structure of *Toxoplasma gondii*: Clonal Expansion Driven by Infrequent Recombination and Selective Sweeps. Annu.

Rev. Microbiol. 62, 329–351. https://doi.org/10.1146/annurev.micro.62.081307.162925

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of Toxoplasma gondii comprise a single clonal lineage. Nature 359, 82–85. https://doi.org/10.1038/359082a0

Silva, R.C., Machado, G.P., 2016. Canine neosporosis: perspectives on pathogenesis and management. Vet Med (Auckl) 7, 59–70. https://doi.org/10.2147/VMRR.S76969

Siri, S.O., Martino, J., Gottifredi, V., 2021. Structural Chromosome Instability: Types, Origins, Consequences, and Therapeutic Opportunities. Cancers (Basel) 13, 3056. https://doi.org/10.3390/cancers13123056

Sironi, L., Mapelli, M., Knapp, S., Antoni, A.D., Jeang, K.-T., Musacchio, A., 2002. Crystal structure of the tetrameric Mad1–Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. EMBO J 21, 2496–2506. https://doi.org/10.1093/emboj/21.10.2496

Sokol-Borrelli, S.L., Reilly, S.M., Holmes, M.J., Orchanian, S.B., Massmann, M.D., Sharp, K.G., Cabo, L.F., Alrubaye, H.S., Martorelli Di Genova, B., Lodoen, M.B., Sullivan, W.J., Boyle, J.P., 2023. A transcriptional network required for bradyzoite development in Toxoplasma gondii is dispensable for recrudescent disease. Nat Commun 14, 6078. https://doi.org/10.1038/s41467-023-40948-w

Stark, G.R., Taylor, W.R., 2004. Analyzing the G2/M checkpoint. Methods Mol Biol 280, 51–82. https://doi.org/10.1385/1-59259-788-2:051

Stelzer, S., Basso, W., Benavides Silván, J., Ortega-Mora, L.M., Maksimov, P., Gethmann, J., Conraths, F.J., Schares, G., 2019. Toxoplasma gondii infection and toxoplasmosis in farm animals: Risk factors and economic impact. Food Waterborne Parasitol 15, e00037. https://doi.org/10.1016/j.fawpar.2019.e00037

Su, C., Howe, D.K., Dubey, J.P., Ajioka, J.W., Sibley, L.D., 2002. Identification of quantitative trait loci controlling acute virulence in Toxoplasma gondii. Proceedings of the National Academy of Sciences 99, 10753–10758. https://doi.org/10.1073/pnas.172117099

Su, D., Zhu, S., Hou, Z., Hao, F., Xu, K., Xu, F., Zhu, Y., Liu, D., Xu, J., Tao, J., 2023. Toxoplasma gondii infection regulates apoptosis of host cells via miR-185/ARAF axis. Parasites & Vectors 16, 371. https://doi.org/10.1186/s13071-023-05991-y

Su, T.T., 2006. Cellular responses to DNA damage: one signal, multiple choices. Annu Rev Genet 40, 187–208. https://doi.org/10.1146/annurev.genet.40.110405.090428

Subba Rao, K., 2007. Mechanisms of disease: DNA repair defects and neurological disease. Nat Clin Pract Neurol 3, 162–172. https://doi.org/10.1038/ncpneuro0448

Suijkerbuijk, S.J.E., van Osch, M.H.J., Bos, F.L., Hanks, S., Rahman, N., Kops, G.J.P.L., 2010. Molecular Causes for BUBR1 Dysfunction in the Human Cancer Predisposition Syndrome Mosaic Variegated Aneuploidy. Cancer Res 70, 4891–4900. https://doi.org/10.1158/0008-5472.CAN-09-4319

Sullivan, W.J., Chiang, C.-W., Wilson, C.M., Naguib, F.N.M., el Kouni, M.H., Donald, R.G.K., Roos, D.S., 1999. Insertional tagging of at least two loci associated with resistance to adenine arabinoside in *Toxoplasma gondii,* and cloning of the adenosine kinase locus. Molecular and Biochemical Parasitology 103, 1–14. https://doi.org/10.1016/S0166-6851(99)00114-0

Sun, H., Li, J., Wang, L., Yin, K., Xu, C., Liu, G., Xiao, T., Huang, B., Wei, Q., Gong, M., Cao, J., 2021. Comparative Proteomics Analysis for Elucidating the Interaction Between Host Cells and Toxoplasma gondii. Front. Cell. Infect. Microbiol. 11. https://doi.org/10.3389/fcimb.2021.643001

Suryadinata, R., Sadowski, M., Sarcevic, B., 2010. Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. Bioscience Reports 30, 243–255. https://doi.org/10.1042/BSR20090171

Swinger, R.L., Schmidt, K.A., Dubielzig, R.R., 2009. Keratoconjunctivitis associated with Toxoplasma gondii in a dog. Vet Ophthalmol 12, 56–60. https://doi.org/10.1111/j.1463-5224.2009.00675.x

Sykora, P., Witt, K.L., Revanna, P., Smith-Roe, S.L., Dismukes, J., Lloyd, D.G., Engelward, B.P., Sobol, R.W., 2018. Next generation high throughput DNA damage detection platform for genotoxic compound screening. Sci Rep 8, 2771. https://doi.org/10.1038/s41598-018-20995-w

Syn, G., Anderson, D., Blackwell, J.M., Jamieson, S.E., 2017. Toxoplasma gondii Infection Is Associated with Mitochondrial Dysfunction in-Vitro. Front Cell Infect Microbiol 7, 512. https://doi.org/10.3389/fcimb.2017.00512

Tang, F., Wang, Yinan, Zhao, T., Yuan, J., Kellum, A.H., Wang, Yinsheng, 2024. DNA polymerase κ participates in early S-phase DNA replication in human cells.

Proceedings of the National Academy of Sciences 121, e2405473121. https://doi.org/10.1073/pnas.2405473121

Taubert, A., Zahner, H., Hermosilla, C., 2006. Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites. Veterinary Parasitology 142, 214–222. https://doi.org/10.1016/j.vetpar.2006.07.021

Teimouri, A., Goudarzi, F., Goudarzi, K., Alimi, R., Sahebi, K., Foroozand, H., Keshavarz, H., 2022. Toxoplasma gondii Infection in Immunocompromised Patients in Iran (2013–2022): A Systematic Review and Meta-Analysis. Iran J Parasitol 17, 443–457. https://doi.org/10.18502/ijpa.v17i4.11271

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. Toxoplasma gondii: from animals to humans. Int J Parasitol 30, 1217–1258.

Thoms, K.-M., Kuschal, C., Emmert, S., 2007. Lessons learned from DNA repair defective syndromes. Exp Dermatol 16, 532–544. https://doi.org/10.1111/j.1600-0625.2007.00559.x

Thurmond, M.C., Hietala, S.K., 1996. Culling associated with Neospora caninum infection in dairy cows. Am J Vet Res 57, 1559–1562.

Tibelius, A., Marhold, J., Zentgraf, H., Heilig, C.E., Neitzel, H., Ducommun, B., Rauch, A., Ho, A.D., Bartek, J., Krämer, A., 2009. Microcephalin and pericentrin regulate mitotic entry via centrosome-associated Chk1. J Cell Biol 185, 1149–1157. https://doi.org/10.1083/jcb.200810159

Tonin, A.A., Da Silva, A.S., Thomé, G.R., Sangoi, M.B., Oliveira, L.S., Flores, M.M., Schetinger, M.R.C., Fighera, R.A., Moresco, R.N., Camillo, G., Vogel, F.S.F., Lopes, S.T.A., 2014. Influence of toxoplasmosis on acetylcholinesterase activity, nitric oxide levels and cellular lesion on the brain of mice. Pathol Res Pract 210, 526–532. https://doi.org/10.1016/j.prp.2014.04.025

Tsou, M.-F.B., Stearns, T., 2006. Mechanism limiting centrosome duplication to once per cell cycle. Nature 442, 947–951. https://doi.org/10.1038/nature04985

Tubbs, A., Nussenzweig, A., 2017. Endogenous DNA Damage as a Source of Genomic Instability in Cancer. Cell 168, 644–656. https://doi.org/10.1016/j.cell.2017.01.002

Unzaga, J.M., Moré, G., Bacigalupe, D., Rambeaud, M., Pardini, L., Dellarupe, A., De Felice, L., Gos, M.L., Venturini, M.C., 2014. Toxoplasma gondii and Neospora

caninum infections in goat abortions from Argentina. Parasitol Int 63, 865–867. https://doi.org/10.1016/j.parint.2014.07.009

Uribe-Querol, E., Rosales, C., 2017. Control of Phagocytosis by Microbial Pathogens. Front Immunol 8, 1368. https://doi.org/10.3389/fimmu.2017.01368

van Velsen, C.M., 2021. Neosporosis in bulls: potential for venereal transmission, and effect on semen quality and production. N Z Vet J 69, 193–200. https://doi.org/10.1080/00480169.2020.1854883

Velásquez, Z.D., Conejeros, I., Larrazabal, C., Kerner, K., Hermosilla, C., Taubert, A., 2019. Toxoplasma gondii-induced host cellular cell cycle dysregulation is linked to chromosome missegregation and cytokinesis failure in primary endothelial host cells. Sci Rep 9, 12496. https://doi.org/10.1038/s41598-019-48961-0

Velásquez, Z.D., López-Osorio, S., Mazurek, S., Hermosilla, C., Taubert, A., 2021a. Eimeria bovis Macromeront Formation Induces Glycolytic Responses and Mitochondrial Changes in Primary Host Endothelial Cells. Frontiers in Cellular and Infection Microbiology 11.

Velásquez, Z.D., Lopez-Osorio, S., Pervizaj-Oruqaj, L., Herold, S., Hermosilla, C., Taubert, A., 2020. Besnoitia besnoiti–driven endothelial host cell cycle alteration. Parasitol Res 119, 2563–2577. https://doi.org/10.1007/s00436-020-06744-x

Velásquez, Z.D., López-Osorio, S., Waiger, D., Manosalva, C., Pervizaj-Oruqaj, L., Herold, S., Hermosilla, C., Taubert, A., 2021b. Eimeria bovis infections induce G1 cell cycle arrest and a senescence-like phenotype in endothelial host cells. Parasitology 148, 341–353. https://doi.org/10.1017/S0031182020002097

Vélez, J., Velasquez, Z., Silva, L.M.R., Gärtner, U., Failing, K., Daugschies, A., Mazurek, S., Hermosilla, C., Taubert, A., 2021. Metabolic Signatures of Cryptosporidium parvum-Infected HCT-8 Cells and Impact of Selected Metabolic Inhibitors on C. parvum Infection under Physioxia and Hyperoxia. Biology (Basel) 10, 60. https://doi.org/10.3390/biology10010060

Villa, L., Allievi, C., Di Cerbo, A.R., Zanzani, S.A., Sommariva, F., Zanini, L., Mortarino, M., Manfredi, M.T., 2024. *Neospora caninum* antibodies in bulk tank milk from dairy cattle herds in Italy in relation to reproductive and productive parameters and spatial analysis. Acta Tropica 254, 107194. https://doi.org/10.1016/j.actatropica.2024.107194

Virus, M.A., Ehrhorn, E.G., Lui, L.M., Davis, P.H., 2021. Neurological and Neurobehavioral Disorders Associated with Toxoplasma gondii Infection in Humans. Journal of Parasitology Research 2021, 6634807. https://doi.org/10.1155/2021/6634807

Vorlaufer, E., Peters, J.-M., 1998. Regulation of the Cyclin B Degradation System by an Inhibitor of Mitotic Proteolysis. Mol Biol Cell 9, 1817–1831.

Votýpka, J., Modrý, D., Oborník, M., Šlapeta, J., Lukeš, J., 2017. Apicomplexa, in: Archibald, J.M., Simpson, A.G.B., Slamovits, C.H. (Eds.), Handbook of the Protists. Springer International Publishing, Cham, pp. 567–624. https://doi.org/10.1007/978-3-319-28149-0_20

Vyas, A., Sapolsky, R., 2010. Manipulation of host behaviour by Toxoplasma gondii: what is the minimum a proposed proximate mechanism should explain? Folia Parasitol (Praha) 57, 88–94. https://doi.org/10.14411/fp.2010.011

Walker, M.E., Hjort, E.E., Smith, S.S., Tripathi, A., Hornick, J.E., Hinchcliffe, E.H., Archer, W., Hager, K.M., 2008. Toxoplasma gondii actively remodels the microtubule network in host cells. Microbes Infect 10, 1440–1449. https://doi.org/10.1016/j.micinf.2008.08.014

Wang, Q., Sibley, L.D., 2019. Assays for monitoring Toxoplasma gondii infectivity in the laboratory mouse. https://doi.org/10.1101/584474

Wang, X.W., Zhan, Q., Coursen, J.D., Khan, M.A., Kontny, H.U., Yu, L., Hollander, M.C., O'Connor, P.M., Fornace, A.J., Harris, C.C., 1999. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A 96, 3706–3711.

Wang, Y., Weiss, L.M., Orlofsky, A., 2009. Host Cell Autophagy Is Induced by Toxoplasma gondii and Contributes to Parasite Growth. J Biol Chem 284, 1694–1701. https://doi.org/10.1074/jbc.M807890200

Wang, Z., 2021. Regulation of Cell Cycle Progression by Growth Factor-Induced Cell Signaling. Cells 10, 3327. https://doi.org/10.3390/cells10123327

Watanabe, G., Lieber, M.R., 2022. Dynamics of the Artemis and DNA-PKcs Complex in the Repair of Double-Strand Breaks. J Mol Biol 434, 167858. https://doi.org/10.1016/j.jmb.2022.167858

Webster, J.P., McConkey, G.A., 2010. Toxoplasma gondii-altered host behaviour: clues as to mechanism of action. Folia Parasitol (Praha) 57, 95–104. https://doi.org/10.14411/fp.2010.012

Westendorp, B., Mokry, M., Groot Koerkamp, M.J.A., Holstege, F.C.P., Cuppen, E., de Bruin, A., 2012. E2F7 represses a network of oscillating cell cycle genes to control S-phase progression. Nucleic Acids Res 40, 3511–3523. https://doi.org/10.1093/nar/gkr1203

White, M.D., Angara, R.K., Dias, L.T., Shinde, D.D., Thomas, V.C., Augusto, L., 2024. Selective host autophagy is induced during the intracellular parasite Toxoplasma gondii infection controlling amino acid levels. mSphere 9, e00369-24. https://doi.org/10.1128/msphere.00369-24

White, M.W., Suvorova, E.S., 2018. Apicomplexa Cell Cycles: Something Old, Borrowed, Lost, and New. Trends in Parasitology 34, 759–771. https://doi.org/10.1016/j.pt.2018.07.006

Wilhelm, T., Said, M., Naim, V., 2020. DNA Replication Stress and Chromosomal Instability: Dangerous Liaisons. Genes (Basel) 11, 642. https://doi.org/10.3390/genes11060642

Williams, R.M., Zhang, X., 2021. Roles of ATM and ATR in DNA double strand breaks and replication stress. Progress in Biophysics and Molecular Biology 161, 27–38. https://doi.org/10.1016/j.pbiomolbio.2020.11.005

Wolfer, J., Grahn, B., 1996. Diagnostic ophthalmology. Case report of anterior uveitis and endophthalmitis. Can Vet J 37, 506–507.

Wu, M., Liu, S., Chen, Y., Liu, D., An, R., Cai, H., Wang, J., Zhou, N., Obed, C., Han, M., Shen, J., Chen, L., Du, J., 2022. Live-attenuated ME49Δcdpk3 strain of Toxoplasma gondii protects against acute and chronic toxoplasmosis. npj Vaccines 7, 1–10. https://doi.org/10.1038/s41541-022-00518-5

Wujcicka, W., Wilczyński, J., Nowakowska, D., 2014. Do the placental barrier, parasite genotype and Toll-like receptor polymorphisms contribute to the course of primary infection with various Toxoplasma gondii genotypes in pregnant women? Eur J Clin Microbiol Infect Dis 33, 703–709. https://doi.org/10.1007/s10096-013-2017-3

Yan, S., Sorrell, M., Berman, Z., 2014. Functional interplay between ATM/ATR-mediated DNA damage response and DNA repair pathways in oxidative stress. Cell Mol Life Sci 71, 3951–3967. https://doi.org/10.1007/s00018-014-1666-4

Ye, C.J., Sharpe, Z., Alemara, S., Mackenzie, S., Liu, G., Abdallah, B., Horne, S., Regan, S., Heng, H.H., 2019. Micronuclei and Genome Chaos: Changing the

System Inheritance. Genes (Basel) 10, 366. https://doi.org/10.3390/genes10050366

Yim, H., Erikson, R.L., 2011. Regulation of the final stage of mitosis by components of the pre-replicative complex and a polo kinase. Cell Cycle 10, 1374–1377. https://doi.org/10.4161/cc.10.9.15489

Zhu, W., Li, J., Pappoe, F., Shen, J., Yu, L., 2019. Strategies Developed by Toxoplasma gondii to Survive in the Host. Front Microbiol 10, 899. https://doi.org/10.3389/fmicb.2019.00899

Zhuang, H., Yao, C., Zhao, X., Chen, X., Yang, Yimin, Huang, S., Pan, L., Du, A., Yang, Yi, 2020. DNA double-strand breaks in the Toxoplasma gondii-infected cells by the action of reactive oxygen species. Parasit Vectors 13, 490. https://doi.org/10.1186/s13071-020-04324-7

7. EHRENWÖRTLICHE ERKLÄRUNG

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf welches direct Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

8. FUNDING

This project was funded by the Institute of Parasitology of the Justus Liebig University Gießen, Germany.

9. ACKNOWLEDGMENTS

At the end of this important stage of my life, I would like to express my deepest gratitude to all those who have accompanied me during this process and in some way smoothed the path I have travelled.

First of all, I would like to thank my parents María T. Barón and Ciro A. Rojas for always having the right word at the right time, for being an example of struggle, work, discipline and perseverance, for encouraging me to improve myself every day and especially for instilling in me that it is not about how much we do but about how much love we put into what we do.

To my sisters Liseth M. Rojas Barón and Adriana L. Rojas Barón for being an inexhaustible source of inspiration, the engine that drives me forward every day.

I would like to sincerely thank Prof. Taubert for having given me the opportunity to be part of her team, for her trust in me and my work, and for supervising this research.

To Prof. Hermosilla for his innumerable expressions of emotion towards the results I was progressively obtaining and for his encouragement to continue working.

To Dr. Zahady Velásquez for having dedicated much of her time to teaching me all the necessary tools for the development of my work, and who has promote each objective achieved with her knowledge. Also to Dr. Iván Conejeros for his willingness to solve problems.

To all my dear colleagues Constanza Salinas, Gabriel Espinosa, Jobst Fischer, Thomas Nowicki, Mireille Ampama, Alena Dusch, to think of them is to speak directly of emotional and professional support. Also to Lisa Segeritz, Daniela Grob and Camilo Larrazabal, who from a distance were an important part of this stage.

To all the technical assistants, especially to Christin Ritter, Hannah Salecker, Emma Belach, who were always receptive during the daily laboratory work.

To Ms. Maria and Ms. Sandra, for their valuable work. In general, we assume that the way it works here works everywhere, but it doesn't. Many thanks for making our work spaces always clean and tidy.

édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978 3 8359 7225 4

Photo cover: © Author