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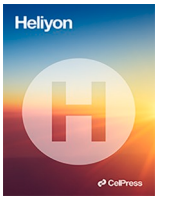

Review article

Toxoplasma gondii, a plea for a thorough investigation of its oncogenic potential

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ABSTRACT

It is estimated that 30 % of the world's population harbours the parasite Toxoplasma gondii, particularly in the brain. Beyond its implication in potentially severe opportunistic or congenital infections, this persistence has long been considered as without consequence. However, certain data in animals and humans suggest that this carriage may be linked to various neuropsychiatric or neurodegenerative disorders. The hypothesis of a potential cerebral oncogenicity of the parasite is also emerging.

In this personal view, we will present the epidemiological arguments in favour of an association between toxoplasmosis and cerebral malignancy, before considering the points that could underlie a potential causal link. More specifically, we will focus on the brain as the preferred location for T. gondii persistence and the propensity of this parasite to interfere with the apoptosis and cell cycle signalling pathways of their host cell.

1. Introduction

Toxoplasma gondii is an intracellular parasite capable of infecting various organs including the nervous system of most warm-blooded vertebrates, among them humans, following ingestion of infected meat or contaminated aliments. The prevalence of *T. gondii* infection is estimated as about one third of the human population worldwide. Genetically, *T. gondii* strains are mainly grouped into three genotypes or clonal lineages (namely type I, II, and III), alongside a high diversity of atypical strains. In addition to having a heterogeneous distribution throughout the world, they exhibit differential characteristics including variable virulence, cystogenic capabilities thus impacting the establishment of brain impairment, neuropathology as well as clinical manifestations [1]. Toxoplasmosis is associated with a great risk of severe encephalitis or disseminated disease for individuals with impaired immune defence. In addition, maternal infection during pregnancy exposes the foetus to the risk of congenital toxoplasmosis. It may lead to foetal lesions ranging from subclinical to life-threatening manifestations, such as miscarriages, stillbirths and neurological (hydrocephalus,

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meningoencephalitis) or ocular (chorioretinitis) abnormalities [2].

Until recently, infection of a non-pregnant immunocompetent host was considered as benign and leading to no clinical consequences [2]. However, the long-term persistence of the parasite may not be as innocuous as expected as the body of evidence obtained in animals in well-built studies suggests that it may lead to physiological and behavioural modifications. Indeed, in infected rodents, *T. gondii* infection has been associated with decreased fear of their predators, attraction to their urine, altered alertness and anxiety, and increased activity, which may facilitate predation of infected rodents [3,4]. The attenuation of fear of certain predators in *T. gondii*-infected rodents would perfectly illustrate the manipulation hypothesis, according to which a parasite could modify the host's behaviour for its own benefit [5]. However, a recent study showed that the infection induced an overall decrease in fear in mice, without specifically targeting the behaviour towards felines, and the degree of behavioural impairment was also correlated with intracerebral cyst load and central nervous system (CNS) inflammation [6].

Some authors suggest that humans could also be affected by these behavioural modifications since several clinical and experimental studies point that latent infection by *T. gondii* could potentially be associated with neurological, psychiatric and psychological disorders in the host, which have been examined in detail but remain largely controversial and still need clear and up to date and high quality clinical evaluation [7]. As regards psychiatric disorders, a meta-analysis extracted only 50 studies that met the inclusion criteria out of the 2866 identified in the literature, which illustrates the weaknesses of many studies on the subject [8]. This analysis found an association between *T. gondii* seropositivity and schizophrenia, bipolar disorder, obsessive-compulsive disorder, and addiction, albeit with relatively modest odds ratios (1·81; 1·52; 3·4; and 1·91 respectively) [8]. Concerning personality traits, some preliminary studies that however remain to be confirmed suggest an association between toxoplasmosis and aggressive behaviours and impulsivity [9,10], or even self-inflicted violence and suicide [11–14]. However, analytical biases plague many of these studies [15]. Interestingly these hypotheses are supported by morpho-anatomical and neurochemichal changes reported in infected brains [16,17].

Infections are now widely accepted as contributing factors in the development of cancers. If viruses are the main incriminated agents, the involvement of bacteria such as *Helicobacter pylori*, and even of parasites such as *Plasmodium falciparum* or *Schistosoma haematobium* is well established [18]. Furthermore, recent seroepidemiological studies suggests that chronic *T. gondii* infection might be associated with an increased risk of brain cancer. Although this hypothesis is not yet supported by direct evidence, when considering the impact of *T. gondii* infection in terms of cell biology, some mechanistic hypotheses that might underlie the involvement of *T. gondii* in tumour development can be inferred. Indeed, the parasite is not only actively present in the central nervous system during chronic infection, but as we will cover below, it is also able to interfere with various host cell signalling pathways and protective mechanisms against carcinogenesis such as apoptosis and cell cycle regulation, or to alter the levels of miRNAs involved in gene regulation.

1.1. Epidemiological presumption for a toxoplasmosis-cancer connection in humans?

Various epidemiological studies report an association between toxoplasmosis and cancer. Thomas et al. established from national brain cancer incidence and *T. gondii* seroprevalence data from 37 countries that *T. gondii* infection was associated with a 1·8-fold increase in brain malignant tumours incidence [19]. This was further suggested by a recent meta-analysis based on seven observational studies involving more than 2000 patients and 5000 control cases, which resulted in the estimation of an odds ratio of 1·96, in favour of an increased risk of glioma, meningioma and other types of brain tumours, associated with *T. gondii* infection [20]. However, these seroepidemiological surveys are characterized by a great heterogeneity in terms of included populations (age, geographical origin, type of cancer, environmental and genetic predisposing factors …), reference tumour definition as well as evaluation methods. The validity of clues provided by these human studies is severely limited by the difficulty to determine the individual infection status from a single blood sample, thus not allowing to establish the time elapsed since infection and the relative time course of infection vs. the disorder of interest, and the congenital vs. postnatal timing of infection, which is important because of likely differences in outcomes [21,22].

Another limit lies in the decrease of IgG over time with the risk of dropping below the detection limit while the parasite remains viable in the brain [23,24]. The use of highly specific IgG Western blot tests reduces the risk of misclassifying patients as exposed or unexposed [25,26]. Moreover, it is not possible to differentiate the mode of contamination (by cysts or oocysts) and it would seem that this has an impact on the anamnesis of the pathology [27]. Finally, as there is no method to date to specifically assess the presence of cysts in humans, it is not possible to confirm their lifelong persistence, even though this could influence the repercussions of the infection [23,28]. The development of serological tests based on cyst and bradyzoite specific antigens could help to address this issue [29].

1.2. Parasite persistence in the brain

T. gondii is successively encountered in its hosts as a tachyzoite stage during acute infection and as a latent encysted bradyzoite stage in chronic infection. The tachyzoite is capable of infecting all nucleated cells and disseminating throughout the organism by lymphatic and blood routes and can notably penetrate the CNS. *T. gondii* is indeed one of the rare microorganisms able to access this 'immune privileged' area, protected by the blood-brain barrier. If acute infection allows the dissemination of the parasite in different organs, some data and in particular bioluminescence experiments carried out in mice, suggest that the brain is the main organ involved in cyst formation [30]. Besides, although *T. gondii* is able to infect astrocytes, microglial cells and neurons in vitro, a study in a mouse model demonstrates that in the CNS, neurons are the main target of the parasite in vivo. The infection of mice expressing GFP under the dependence of Cre recombinase, by type II (Prugniaud) and type III (CEP) strains of *T. gondii* which injects the latter into its host cells,

has shown that the parasite interacts preferentially with neurons [31].

The differentiation of the tachyzoite into a bradyzoite is concomitant not only with a change in the gene expression profile but also with a slowing down of the cell cycle [32]. Thus, the cyst and its bradyzoite content has long been considered a homogeneous and dormant entity. A study, however, has brought forth the picture of a chronic infection that is far more active than expected. Indeed, in 2015, Watts et al. demonstrated that cysts were dynamic entities, showing a general pattern of expansion during the chronic phase, and in which not only occurred sporadic replications but also more clustered or even synchronized phenomena, previously thought to be the prerogative of the tachyzoite [33].

On the other hand, far from being inert structures, cysts trigger a humoral but also cellular immune response. Indeed, a humoral response directed against a cyst-envelope antigen specific to the bradyzoite has recently been demonstrated [29]. Furthermore, the presence in the infected CNS of a population of macrophages activated by the chitin of the cystic wall and able to lyse the cysts, also demonstrates that the cysts are not totally invisible to the immune system [34].

Thus, the CNS hosts the active persistence of the parasite. If this preferential localisation alone cannot explain the epidemiological association between brain cancer and toxoplasmosis, it is to be put in relation with the various molecular mechanisms deployed by *T. gondii* to interact with its host cell, which could as a corollary be involved in a malignant process (Fig. 1).

1.2.1. T. gondii and apoptosis signalling pathway

Apoptosis represents one of the natural protections against the development of cancer. This complex mechanism is triggered by either intrinsic or extrinsic stimuli and involves a cascade of cellular events resulting in the activation of caspases which are potent effectors of this programmed cell death (Fig. 2). While alteration of apoptosis is a fundamental aspect of malignant transformation, several studies indicate that *T. gondii* is able to interfere with this process in different ways depending on the cell type, parasite strain or

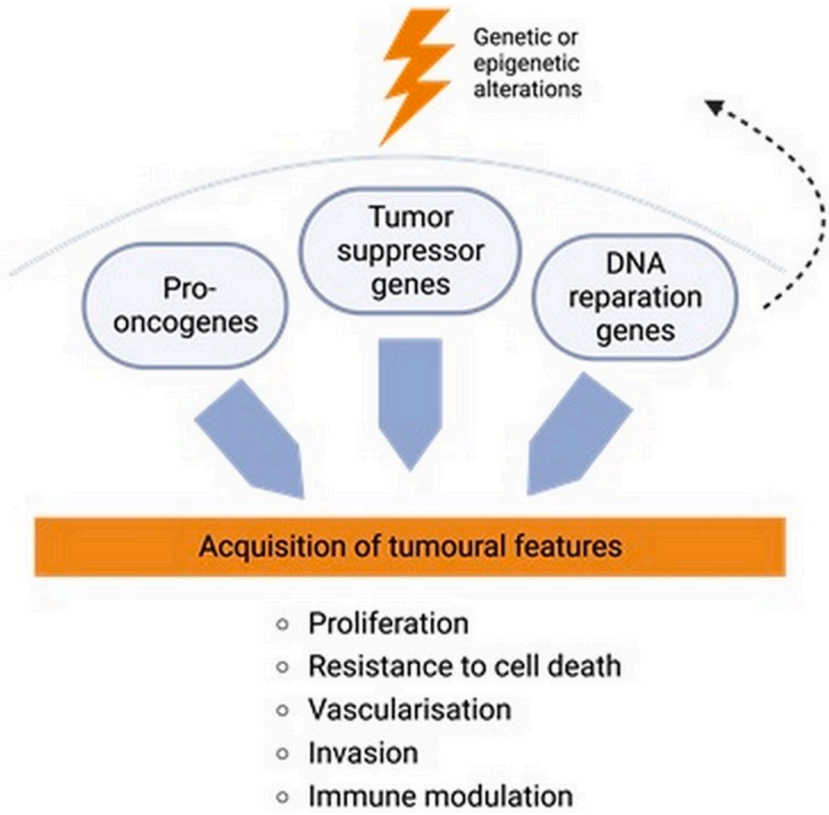

Fig. 1. Schematic representation of the tumoral process.
The tumoral process is characterized by the progressive acquisition by normal cells of characteristics that allow them to become cancerous. It is favoured by the occurrence of genetic or epigenetic alterations affecting 3 types of genes: (i) Pro-oncogenes which play a major role in cell proliferation, differentiation and survival, and which uncontrolled activation leads to the formation of tumoral cells, (ii) Tumour suppressor genes whose main function is to counter cell proliferation to allow DNA repair, and cell apoptosis in case it has failed. The mutation of these genes leads to their inactivation thus signalling non-proliferation. For example, p53 protein is coded by a gene of the tumour suppressor family, which is found mutated in approximately 50 % of human cancers. (iii) The alteration of genes maintaining the human genome integrity code for repair systems of damaged DNA (BRCA1, rad50, MLH-1) leads to an increased susceptibility to cancers because of genetic instability. Among the characteristics of cancer cells are the ability to resist cell death and proliferate, to promote vascularization and invasion, or to resist destruction by immunity. Created with Biorender.

infection stage [35].

Indeed, apoptosis of the host cell is a means of limiting the proliferation of intracellular parasites. Inhibiting it therefore appears to be an effective defence mechanism of the parasite to survive in its host. Thus, different studies have shown that *T. gondii* infection induces resistance of different cell lines to several inducers of apoptosis such as IL2 deprivation, gamma or UV irradiation, calcium ionophore beauvericin, actinomycin D or staurosporine treatment [36–39]. Various inhibition pathways have been identified. It was thus evidenced that the activation of caspases 3, 8 and 9 was inhibited in murine fibroblasts infected by the virulent type I RH strain, and that this blockade of apoptosis was dependent on NF-κB via an increase in the transcription of antiapoptotic molecules such as Bfl-1 and Bcl-xL, or the inhibitors of apoptosis IAP1 and IAP2 [40,41]. Similarly, the involvement of NF-κB has also been demonstrated in the decrease of Bax expression and the inactivation of caspase 3 leading to the inhibition of apoptosis in *T. gondii* infected mouse spleen cells [42]. Other signalling pathways have also been implicated, including the phosphoinositide 3-kinase (PI 3-kinase) and PKB/Akt pathway [38,43]. Inhibition of caspase 3 and 7 activation by viable type II NTE strain tachyzoites has also been reported in cytosolic extracts of human-derived Jurkat leukemic T cells [44]. Several studies also suggest interference of *T. gondii* with the intrinsic pathway of apoptosis. Thus, in human-derived HL-60 and U937 cells infected with tachyzoites of the NTE strain, pro-apoptotic stimuli induce a decreased release of cytochrome C associated with an increase in Mcl-1, and a decrease in the activation of caspases 3 and 9 [45]. Likewise, the human leukemic cell line THP-1 infected with the RH strain shows reduced activation of caspases 3 and 9, translocation of Bax and release of cytochrome *c* after pro-apoptotic treatment [43]. On the other hand, *T. gondii* also appears to be able to interact with this pathway by inhibiting apoptosome formation and caspase 9 activation [46]. The inhibition of apoptosis also occurs on the extrinsic pathway since *T. gondii* can prevent the activation of caspase 8 by the death receptor Fas/CD95 [47,48]. Yamada et al. also demonstrated the inhibition of cytotoxic lymphocyte-mediated apoptosis via granzyme B by the RH strain [49]. A mechanism of

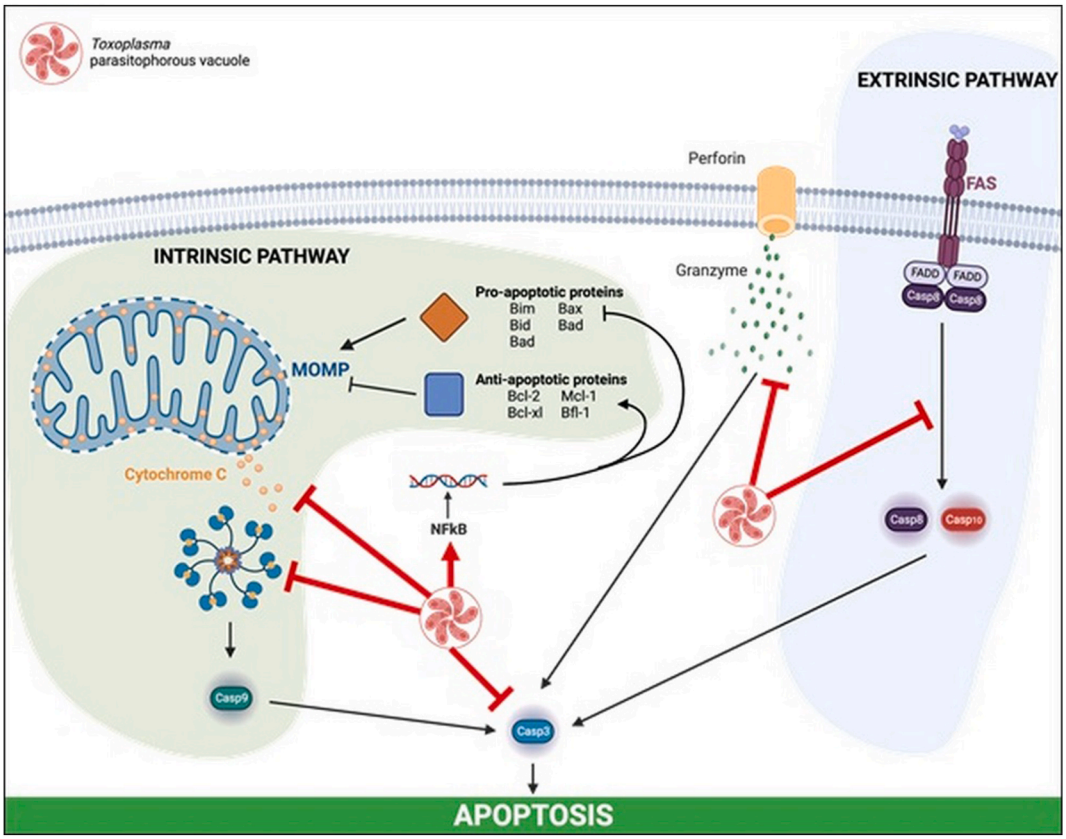

Fig. 2. Schematic representation of the mechanisms of inhibition of apoptosis by *Toxoplasma gondii*.
Apoptosis can be triggered by intrinsic or extrinsic stimuli. The extrinsic pathway requires the binding of extracellular ligands to their membrane receptors such as Fas, which induces the activation of the initiating caspases 8 and 10. These caspases in turn activate effector caspases such as caspase 3. The intrinsic pathway is regulated by the balance between pro (Bim, Bid, Bad, Bax and Bak) and anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1 or Bfl-1) proteins of the Bcl-2 family and involves mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome C which binds to the cytoplasmic protein APAF-1 to form the apoptosome that activates the initiator caspase 9. In parallel, T and NK cells can also induce apoptosis through the perforin/granzyme pathway. The influence of *Toxoplasma gondii* can be exerted, depending on the cell type, the parasite strain and infection stage, (i) on the intrinsic pathway via the decrease in cytochrome *c* release, apoptosome formation and caspase 9 activation, or via the NF-κB pathway and modification of the balance between pro- and anti-apoptotic proteins, (ii) on the extrinsic pathway via inhibition of caspase 8 activation, (iii) or on the perforin/granzyme pathway. Created with Biorender.

inhibition of apoptosis through overexpression of miR-17-92 miRNA targeting the pro-apoptotic effector Bim has also been identified in macrophages infected with the virulent atypical (China1 genotype) strain TgCtwh3, isolated from felids in China [50].

Overall, *T. gondii* appears to have evolved strategies to inhibit pro-apoptotic pathways in the host cell, either directly or indirectly. However, the situation is more nuanced as several studies indicate that *T. gondii* can also be an inducer of apoptosis in certain contexts. This can also be understood from the parasite's perspective since inducing apoptosis of specific defence cells may promote parasite survival [51]. Thus, infection with the RH strain has been implicated in the induction of apoptosis of $CD4^+$, $CD8^+$ or NK and splenic LT [52]. The parasite effectors rhoptry protein 18 (ROP18) and dense granule protein 15 (GRA15) were also involved in the induction of apoptosis through the ER stress-mediated apoptosis pathway [53,54]. On the other hand, unlike what has been described above, the balance between pro-apoptotic and anti-apoptotic factors such as Bcl2 or Mcl-1 may be in favour of apoptosis in infected cells [55,56]. Hence, *T. gondii* appears to exert a skilfully orchestrated effect on host cell apoptosis. The contribution of this ambivalent action to the establishment of a favourable environment for the initiation of a tumour process is a question worth asking.

1.3. Cell cycle impairment

The cell cycle is the series of events that leads to division into two daughter cells. It consists in consecutive phases mainly interphase and mitosis with checkpoints allowing the cell to monitor and regulate its cycle, by verifying processes and repairing DNA damages [57]. It is regulated mainly by two classes of regulatory molecules, cyclins and cyclin-dependent kinases, as well as p53 (Fig. 3). *T. gondii* is able to influence the progression of the cell cycle of the host cell and to promote its transition to the S phase, followed however by an arrest in the G2/M phase [58–60]. Recently, separate studies have also revealed the ability of the parasite protein TEEGR to be exported to the host cell nucleus to induce cyclin E [61–63]. In addition, GRA16, another parasite effector protein, is able to positively modulate the expression of genes involved in cell cycle progression, as well as p53, via its interaction with the HAUSP protein [64]. Furthermore, it has been shown that *T. gondii* GRA16 is responsible for the overexpression of c-myc, a well-known proto-oncogene [65]. In summary, *T. gondii* infection may play an ambiguous role in the progression of the host cell cycle by promoting its initiation while inhibiting its continuation to the final stage of cell division.

1.4. Other mechanisms: miRNAs and chronic inflammation

miRNAs are small noncoding RNAs that play a key part in the regulation of gene expression. Interestingly, *T. gondii* infection specifically alters the levels of different miRNAs involved in the regulation of different processes such as apoptosis, inflammatory response, or the cell cycle. For example, miR-17-92 involved in cell apoptosis and cytokine production and miR-146a and miR-155 both involved in immune response are over expressed in *T. gondii* infected brain cells [66,67].

Chronic inflammation is known to be correlated with several tumoral processes e.g., *H. pylori* and gastric cancer or hepatitis B and hepatocellular carcinoma. Furthermore, chronic inflammatory diseases such as Crohn's disease and ulcerative colitis are found to be

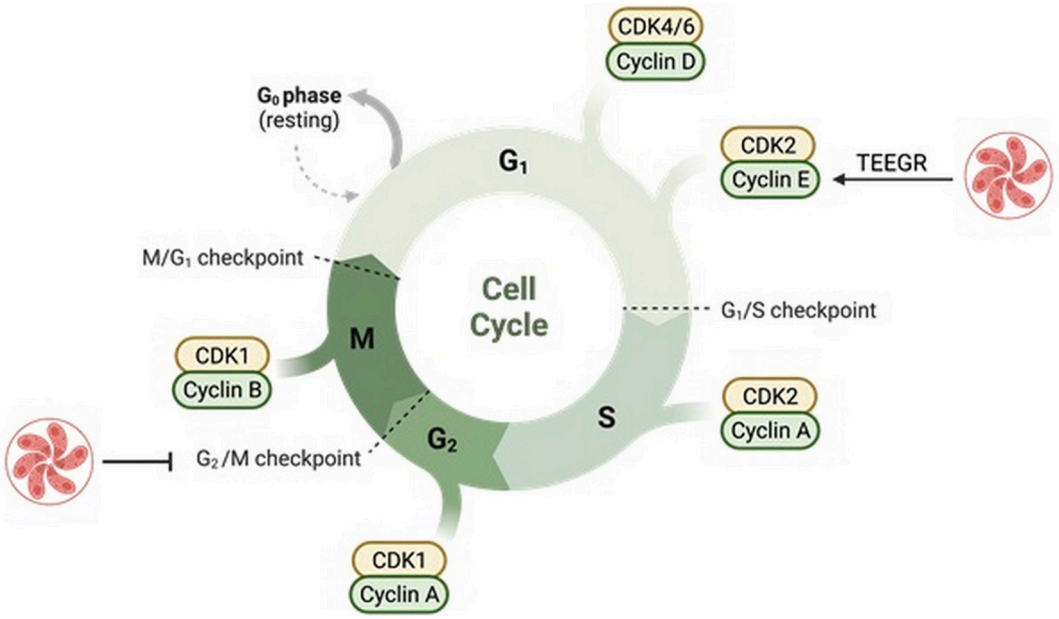

Fig. 3. Influence of *Toxoplasma gondii* on the cell cycle of the host cell.
The cell cycle consists of four successive phases G1, S (DNA replication), G2 and M (mitosis). Its progression is finely regulated by the action of cyclins and their associated cyclin-dependent kinases. T. gondii exerts a positive effect on host cell cycle initiation via induction of cyclin E by TEEGR, but this stimulation is followed by a blockage of the transition from G2 to M phase. Created with Biorender.

linked to mutations in p53 at identical frequencies as in cancers [68]. Basal inflammation triggered by Th1 oriented immune response to *T. gondii* chronic infection is the result of the production of pro-inflammatory cytokines (IFN-γ, IL-12, IL-1, IL-6 and TNF-α) and reactive oxygen species, thus inducing DNA lesions. On the other side, inflammation induced by *T. gondii* has been shown to demonstrate immunotherapeutic control on tumour progression in a murine model. This is in line with several studies reporting anti-neoplastic activity of *T. gondii*, resulting from its natural ability to stimulate immune responses, particularly IFN-γ [69].

Furthermore, transcriptomic analyses reported that *T. gondii* was able to modulate certain signalling pathways that are commonly impaired during carcinogenesis. Interestingly, the network analysis predicted an activation in development of solid cancer [70].

2. Conclusion

Altogether, these results point out that *T. gondii*, an eukaryotic intracellular pathogen, could be associated with the development of malignant processes by impairing the signalling pathways of the host cell, as a side effect of its means to bypass the host's immune system. Basically, this point is not surprising, since eukaryotic protozoans share many pathways and structures with metazoans such as humans. The main limitation of studies dealing with the inhibition of apoptosis is the cell model chosen. Indeed, the studies use cultures of murine fibroblasts, human tumour cells or immune cells. It would be interesting to observe whether these changes are also found in astrocyte or neuronal cultures. One group used mouse astrocytes but infected with highly virulent RH strains of *T. gondii* [71]. Interestingly, however, *T. gondii* not only modulates the function of the cells it infects but can also manipulate uninfected cells via the injection of effector proteins into them [72].

Similarly, the vast majority of the literature investigates the changes brought by the tachyzoite stage. The use of bradyzoites or even cysts directly injected into cells may be relevant. However, it can be assumed that the tachyzoite form initiates an anti-apoptotic state during infection so that the conversion to bradyzoite takes place in an environment favourable to cell survival for long-term residence. Parasite-orchestrated blockade of apoptosis is a complex phenomenon that requires further research.

Secondly, as regards to the cell cycle disruption, studies on non-brain cells do not allow to draw parallels with the genesis of brain tumours. Furthermore, the effects of infection on the cell cycle are mostly studied using the type I RH strain which have probably a different mechanism of action than non-virulent type II strains, involved in most human cases. In addition, the infected cells are only observed over a period of 24–36 h, which does not reflect the influence of the parasite over the long term. It should also be kept in mind that the parasite excretes proteins capable of migrating into the nucleus and regulating the tumour suppressor gene p53, the dysfunction of which is involved in most cancers.

Thirdly, infection of HFF cells by *T. gondii* causes an increase in miRNAs derived from miR-17-92 [66]. A similar elevation of this type of miRNA is found in glioblastoma cells [73]. This is supported by the fact that these miRNAs have anti-apoptotic and proliferative effects. However, it is conceivable that the increase in miRNAs by the parasite varies according to the cell type infected. The measurement of miR-17-92 miRNAs in infected brain cells is an interesting path of study.

These pathophysiological mechanisms thus suggest the existence of a missing link between parasitic infection and the development of brain tumours. However, considering the heterogeneity of fundamental studies, this underlines the importance of the host cell type, its microenvironment and moreover of the parasite's strain and stage responsible for the infection as well as the time to infection. We believe that it is of utmost importance to delve deeper in the fundamental aspects of *T. gondii* infection and decipher its interactions with the host cell. The mechanisms behind should be elucidated in order to apprehend the specificities of the signalling pathways impaired in this context.

Currently, approximately 20 % of the global cancer burden is considered to be related to infectious agents, but the challenges of identifying infectious agents as causative factors for human cancers are such that it is likely that additional infectious aetiologies remain to be discovered [18]. We believe that fundamental research guided by clinical research could help evaluate the impact of T. gondii on brain tumours considering the lack of properly designed clinical studies, as regards to methodological, oncological as well as parasitological criteria. The definite identification of a causal link between toxoplasmosis and brain cancer could have favourable outcomes in terms of public health, through prevention and even management of this type of tumour.

Data availability statement: *No data was used for the research described in the article.*

CRediT authorship contribution statement

D. Dupont: Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing. **M.G. Robert:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **M.P. Brenier-Pinchart:** Validation, Writing – review & editing. **A. Lefevre:** Writing – original draft. **M. Wallon:** Conceptualization, Resources, Supervision, Writing – review & editing. **H. Pelloux:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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