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Review Article

Control of human toxoplasmosis

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ABSTRACT

Toxoplasmosis is caused by *Toxoplasma gondii,* an apicomplexan parasite that is able to infect any nucleated cell in any warm-blooded animal. *Toxoplasma gondii* infects around 2 billion people and, whilst only a small percentage of infected people will suffer serious disease, the prevalence of the parasite makes it one of the most damaging zoonotic diseases in the world. Toxoplasmosis is a disease with multiple manifestations: it can cause a fatal encephalitis in immunosuppressed people; if first contracted during pregnancy, it can cause miscarriage or congenital defects in the neonate; and it can cause serious ocular disease, even in immunocompetent people. The disease has a complex epidemiology, being transmitted by ingestion of oocysts that are shed in the faeces of definitive feline hosts and contaminate water, soil and crops, or by consumption of intracellular cysts in undercooked meat from intermediate hosts. In this review we examine current and future approaches to control toxoplasmosis, which encompass a variety of measures that target different components of the life cycle of *T. gondii*. These include: education programs about the parasite and avoidance of contact with infectious stages; biosecurity and sanitation to ensure food and water safety; chemo- and immunotherapeutics to control active infections and disease; prophylactic options to prevent acquisition of infection by livestock and cyst formation in meat; and vaccines to prevent shedding of oocysts by definitive feline hosts.

1. Introduction

Human toxoplasmosis is a global zoonotic disease with a complex epidemiology and multiple manifestations. It is caused by *Toxoplasma gondii,* a single-celled, eukaryotic parasitic organism within the supergroup Alveolata, Phylum Apicomplexa, Class Conoidasida, Subclass Coccidiasina, Order Eucoccidiorida, Suborder Eimeriorina and Family Sarcocystidae. *Toxoplasma gondii* is the only species in the genus, *Toxoplasma,* but it is closely related to *Neospora caninum* and *Hammondia hammondi,* with which it shares morphological, molecular and life cycle traits. A key to understanding the epidemiology and symptomology of toxoplasmosis lies in an appreciation of the complexity and elegance of its life cycle (see Ferguson, 2009, for a history and detailed illustrated description); this is also key to control strategies.

Unlike other coccidians, most of which are host-specific, *T. gondii* is seemingly able to infect any nucleated cell in any warm-blooded animal. However, it is only in the intermediate asexual phase of its life cycle that *T. gondii* is so promiscuous; in the sexual phase, only felids can serve as definitive hosts due to a unique deficiency, amongst mammals, in intestinal delta-6-saturase activity in cats (Martorelli Di Genova et al., 2019). Additionally, there is a distinct difference in expression profiles of parasite proteins involved in host cell invasion and modulation of intermediate versus definitive host cells (Behnke et al., 2014; Hehl et al., 2015); thus, tachyzoites (the rapidly-dividing asexual stage in intermediate hosts) express a much wider array of proteins that facilitate host cell invasion and modulation of the host response to infection than do merozoites (the rapidly dividing asexual stage that is found only in the definitive host intestine), potentially explaining the capacity of tachyzoites to infect a wide array of cells.

In intermediate hosts, *T. gondii* is found within microscopic cysts in any part of the body but, most especially, in long-lived cells of the musculature and CNS. Felids become infected when they ingest these cysts after preying on an intermediate host. During digestion, the haploid bradyzoite forms of *T. gondii* are released from the cysts and invade enterocytes, progressing through

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successive phases of asexual reproduction, which produces successive generations of merozoites that reinvade the intestine before ultimately differentiating into microgametocytes (each able to differentiate into 16–30 motile, flagellated microgametes) or macrogametocytes (each becoming a single sedentary macrogamete). The microgametes fertilise the macrogametes, forming a diploid zygote. Mobilisation of sub-cellular structures – veil- and wall-forming bodies – within the macrogamete leads to the formation of a multi-layered wall around the zygote, which is now referred to as an oocyst. The oocyst wall is extremely resistant to environmental and chemical insult, enabling the parasite to pass safely out into the world in the felid host's faeces. The resilience of the oocyst wall also provides a haven for meiosis to occur and complete the sexual reproductive phase of the life cycle (Walker et al., 2013). This results in the formation of eight haploid, infectious sporozoites, contained within two sporocysts (i.e., four sporozoites per sporocyst), inside an oocyst, which can survive for months, even years, in soil or water (Freppel et al., 2019).

Herbivorous intermediate hosts, including humans, can become infected by drinking water, eating fruit or vegetables, or coming into contact with soil that is contaminated with oocysts of *T. gondii.* There is also evidence that the eating of raw or undercooked marine molluscs can result in infection because oocysts can accumulate in these animals after run-off of contaminated soil into oceans (Freppel et al., 2019; Shapiro et al., 2019). Sporozoites exit their sporocysts and oocysts during digestion, invade intestinal cells, transform into tachyzoites (which are also haploid) and disseminate throughout the body. Carnivorous or omnivorous intermediate hosts, again including humans, can also become infected by eating other infected intermediate hosts. In this case, it is bradyzoites that are released into the intestine and invade intestinal cells, differentiate into tachyzoites and disseminate throughout the body of the host. Tachyzoites reproduce asexually and rapidly, rupturing and reinvading cells as they do so, until the immune response of the host drives them into an evasion strategy that sees them transform back into bradyzoites, which become encased and protected by a cyst wall inside host cells. A single mature cyst may contain large numbers of bradyzoites (Ferguson, 2009), and a single bradyzoite can establish infection in either an intermediate or definitive host. Other means by which infection can occur is via transfer of tachyzoites congenitally (El Bissati et al., 2018) or via organ transplants (Derouin et al., 2008).

The life cycle of *T. gondii* underpins its successful dispersal to all corners of the globe. However, humans have been unwitting contributors to this success. There appear to have been two major events in the evolution of *T. gondii.* First, as hypothesised by Bertranpetit et al. (2017), based on compelling molecular phylogenetic data, the most recent common ancestor of present-day strains of *T. gondii* appeared ~1.5 million years ago in South America, coincident with an expansion of both potential murine intermediate hosts and potential feline definitive hosts in South America. This diversification of hosts favoured diversification of *T. gondii* too, leading to its spread into North America, via the Isthmus of Panama, which formed 2–3 million years ago. From North America, further spread could occur, across the Bering Strait into Asia. The second event was far more recent – between 11,000 and 4,000 years ago – that being the genesis of agriculture, in particular the cultivation of grains in Asia and the Middle East (Shwab et al., 2018), before further dissemination into Europe, Africa and Southeast Asia. The adoption of agrarian practices also marked the start of commensal relationships between humans and house mice (*Mus musculus musculus, Mus musculus domesticus* and *Mus musculus castaneus*), and between humans and *Felis catus,* the house cat, setting in train the rise of the domestic cat and mouse life cycle for *T. gondii.* This domestic cycle has since, arguably, dominated the epidemiology of the parasite in humans and our livestock, due to the concentrated high levels of oocyst contamination of farm land that accompanies our commensal relationship with cats and mice (Shwab et al., 2018). It has, seemingly, even resulted in the relatively recent introduction of the domestic life cycle of *T. gondii* into North America with a concomitant domination of *T. gondii* clonal strains in that part of the world (Shwab et al., 2018).

Today, around 2 billion people are chronically infected with *T. gondii* across the world, though seroprevalence rates are particularly high in parts of tropical South America, Southeast Asia and Africa (Pappas et al., 2009). In an immunocompetent host, *T. gondii* infection usually causes mild symptoms only and rarely requires intervention. This is because the disease-causing tachyzoites are suppressed quickly by an immune response in which interferon-gamma (IFN-γ) produced by $CD4^+$ T cells plays a central role, supported by a range of other immune cells, cytokines (including, in particular, ILs 12, 18, 1 and 2, and tumour necrosis factor), and innate immune recognition and effector molecules and mechanisms (Sher et al., 2017). However, the consequent differentiation of tachyzoites into bradyzoites leads to the formation of long-lived tissue cysts in the muscles and CNS (Montoya and Liesenfeld, 2004). These possibly persist for the life of the host (Rougier et al., 2017), also under the control of IFN-γ-dependent processes (Sturge and Yarovinsky, 2014). Tissue cysts are capable of reactivating into the acute tachyzoite stage and this is of particular concern for immunocompromised individuals, particularly AIDS patients, whose $CD4^+$ T cell populations are drastically reduced. Reactivation can lead to the formation of brain lesions and associated toxoplasmic encephalitis, which is fatal if left untreated (Luft and Remington, 1992). A first infection with *T. gondii* during pregnancy is also of grave concern. It can lead to congenital toxoplasmosis, which may cause miscarriage or stillbirth or have serious effects for the new-born, which can be life-long and may include impairment of mental development, hearing or sight (Weiss and Dubey, 2009). Clinical disease can also occur in otherwise healthy, immunocompetent adults, in particular manifesting as ocular toxoplasmosis, one of the most frequently identified causes of uveitis (Weiss and Dubey, 2009). Infection is also associated with inflammatory disease (Lidar et al., 2009; Severance et al., 2012; Shapira et al., 2012), schizophrenia (Torrey et al., 2012), cancer and, potentially, several other disorders of the brain (Thomas et al., 2012; Ngo et al., 2017), although it is often difficult to determine whether the associations between these diseases and *T. gondii* infection are causal or correlative. Globally, it is likely that only a small percentage of people infected with *T. gondii* ever develop any manifestations of toxoplasmosis; however, the simple fact that around 2 billion people are infected by this parasite means that large numbers of people inevitably suffer significantly. In tropical South America, severe forms of toxoplasmosis are more prevalent than in other parts of the world, possibly reflecting the greater diversity of virulent strains of *T. gondii* (Bertranpetit et al., 2017; Shwab et al., 2018) combined with the high occurrence of infection via ingestion of oocysts (which have undergone recent sexual recombination and are therefore more genetically diverse than clonal infections derived from bradyzoites) in that part of the world (Galal et al., 2019). Hence, toxoplasmosis is one of the most damaging zoonotic diseases in the world, causing the loss of 2–8 million Disability Adjusted Life Years (Torgerson and Macpherson, 2011).

Despite the combined complexities of the ubiquity of the *T. gondii* life cycle and the multiple manifestations of human toxoplasmosis, control of this disease can potentially be narrowed in focus to strategies that target and prevent: congenital disease; reactivation of cysts (especially in immunocompromised people); formation of tissue cysts in food animals; and, release of oocysts into the environment. In this review, we present educational, food

and water safety, and therapeutic and prophylactic options to control human toxoplasmosis, and comment on the prospects for developing new approaches.

2. Public education, food and water safety to control toxoplasmosis

The governments of many nations, through their health and/or agricultural departments, issue clear advice and maintain educational websites explaining how individuals can prevent toxoplasmosis. Exemplifying this is the information provided by the Centers for Disease Control and Prevention, U.S.A. (https://www.cdc.gov/parasites/toxoplasmosis/prevent.html), which details that, to avoid toxoplasmosis, people need to:

- feed pet cats canned or dried commercial food only, never raw or undercooked meat;
- train pet cats to use cat litter;
- change cat litter daily, wearing disposable gloves whilst doing so;
- keep pet cats indoors, especially at night, to minimise their hunting activities and outside defecation;
- wear gloves during gardening or any other contact with soil;
- cover sandpits when not in use (as cats prefer sand or loose soil for defecation);
- wash fruit and vegetables thoroughly before eating;
- not eat undercooked molluscs;
- freeze meat for at least 2 days (at −12 °C or lower) before cooking;
- cook meat to minimum temperatures (e.g., 74 °C for poultry, 71 °C for minced or ground meat, and 63 °C for other cuts of meat, followed by a minimum 3 min rest period) before eating;
- wash thoroughly any cutting boards, counters, cutlery, crockery and other kitchen utensils that have been in contact with any uncooked food of any type;
- wash hands thoroughly after (i) contact with soil or sand, (ii) food preparation or contact with uncooked food, (iii) contact with pets or pet faeces (including cat litter), and reinforce with children the importance of handwashing; and
- avoid drinking untreated water.

Education about toxoplasmosis and how to prevent it is reinforced by health professionals, particularly as part of pre- and neo-natal education and with immunocompromised people. However, its effectiveness depends on, first, the completeness and accuracy of the advice being given and, second, how closely this is adhered to by patients. There is some doubt about the effectiveness of educational efforts on toxoplasmosis, with reported variability in people's understanding of the existence of the disease and the risks it poses (Gollub et al., 2008; Pereboom et al., 2013; Andiappan et al., 2014; Millar et al., 2014; Elsafi et al., 2015; Chandrasena et al., 2016; Smereka et al., 2018; Velázquez-Hernández et al., 2019) and a lack of rigorous evaluation of programs (Di Mario et al., 2015). This is, arguably, particularly true in poorer nations but difficulties in alleviating the burden of, e.g., congenital toxoplasmosis have also been observed in the U.S.A. (Jones et al., 2003; Ogunmodede et al., 2005; Montoya and Remington, 2008; El Bissati et al., 2018). France has been suggested as an exemplar of success (El Bissati et al., 2018) due to the integration of education, mandatory regular screening and diagnosis throughout pregnancy, and consequential timely treatment options (described in depth by McLeod et al., 2014). At the same time, however, the cost-benefit of screening and diagnosis and the effectiveness of treatment have been questioned (Opsteegh et al., 2014).

Systematic responses to ensure that food is free from contamination with *T. gondii* have been proposed (Kijlstra and Jongert, 2008; Opsteegh et al., 2014; Alizaddeh et al., 2018). These measures can be classified as pre- or post-harvest. The former aims to ensure minimal contamination of farm land or fodder with oocysts of *T. gondii*. The latter is aimed at removing or inactivating cysts or oocysts in or on meat, fruit and vegetables, but is mainly focused on cysts in meat since oocysts are resistant to heat, cold and a variety of chemicals. Moreover, washing of fruit and vegetables by individual consumers is already a widespread habit and probably reasonably effective at removing oocysts, assuming clean water is available (Shapiro et al., 2019), though it should be noted that the increasing availability of pre-packaged, "ready to eat" salads is a potential source of infection (Caradonna et al., 2017). Pre-harvest efforts are, thus, focused on biosecurity to prevent or restrict the presence of cats and rodents, and ensure that water for livestock and for irrigation of crops is clean. This has significant capacity to be successful for animals raised indoors under controlled husbandry conditions, the reduction in prevalence of *T. gondii* cysts in pork with adoption of modern farming techniques being a prime example (Opsteegh et al., 2014). Such success is, however, less likely for animals raised on pasture or under free range organic practices. Programs to ensure neutering of pet cats, combined with effective programs to reduce the population of stray cats, would potentially help to reduce oocyst contamination of the environment but would be extremely difficult and costly to initiate and police (Opsteegh et al., 2014). Vaccination of cats to prevent oocyst excretion is conceivable, and a live, attenuated vaccine has been tested on pig farms (Mateus-Pinilla et al., 1999, 2002); the prospects for this approach will be discussed in more detail in Section 5. Vaccination of livestock to prevent infection with *T. gondii* (discussed in Section 5) or chemoprophylactic prevention of infection, a la the use of coccidiostats in the treatment of poultry coccidiosis (see Chapman et al., 2013), may be conceivable approaches but the necessarily long-term administration of effective drugs would be expensive and would also likely cause unwanted side-effects or even toxicity (Sanchez-Sanchez et al., 2018). Post-harvest measures involve the inactivation of *T. gondii* tissue cysts in meat. This can be achieved through a number of treatments including freezing, irradiation and high-pressure processing (Kijlstra and Jongert, 2008; Opsteegh et al., 2014; Alizaddeh et al., 2018), but lack of acceptance by consumers due to significant effects on meat tenderness, texture and perceived quality would likely prove a major impediment to widespread implementation. Opsteegh et al. (2014) have proposed that selective post-harvest treatment, i.e., targeting only meat destined to be consumed raw or undercooked, or from known infected animals or high-risk (i.e., non-biosecure) farms may be more achievable, whilst acknowledging that the logistics would not be simple and that screening for confirmed infection would be costly.

Major outbreaks of toxoplasmosis have been traced to water contaminated with oocysts for nearly 50 years (Benenson et al., 1982; Bowie et al., 1997; Bahia-Oliveira et al., 2003; de Moura et al., 2006; Ferreira et al., 2018; Shapiro et al., 2019). The same measures listed above to protect individuals and farms from exposure to oocysts of *T. gondii* also apply to reducing contamination of the environment and, therefore, waterways. Detection of *T. gondii* oocysts in water relies on a combination of microscopy, bioassays and molecular techniques but, in general, has lagged behind development of methods for detecting other waterborne parasites such as *Giardia* and *Cryptosporidium* (Shapiro et al., 2019). Oocysts are resistant to chemical disinfection procedures generally employed in ensuring sanitary water supplies (e.g., they are resistant to strong acids, detergents, chlorine, ozone, ultraviolet radiation) and, therefore, filtration systems are a necessity (Freppel et al., 2019).

3. Chemotherapy for the control of toxoplasmosis

Current chemotherapeutic options for toxoplasmosis are limited (Dunay et al., 2018), notwithstanding a concerted effort in the last decade to harness high throughput screening strategies to discover new chemicals, including natural products, with anti-*Toxoplasma* activity or to repurpose or improve existing, approved drugs (Deng et al., 2019). The frontline treatment for toxoplasmosis is a combination of pyrimethamine and sulfadiazine, drugs that act synergistically by targeting two steps in folic acid metabolism. Other therapies combine pyrimethamine with clindamycin, azithromycin or atovaquone, or trimethoprim with sulfamethoxazole. However, all treatments are commonly associated with adverse side effects and toxicity (Ben-Harari et al., 2017); for example, in one study, 60% of toxoplasmic encephalitis patients treated with pyrimethamine and sulfadiazine experienced adverse effects including hematologic toxicity, rash and fever, which led to 45% discontinuing therapy (Haverkos, 1987). In immunocompetent patients with persistent or severe symptoms, pyrimethamine/sulfadiazine treatment duration is usually 4–6 weeks. Immunocompromised patients require long-term maintenance therapy after the initial 6 week treatment, since tissue cysts are not eliminated by any current treatments. The long duration of treatment and inability to eliminate infection, combined with frequency of side effects, make current chemotherapeutic options less than ideal and highlight the need for new anti-*Toxoplasma* chemotherapeutics. The ideal anti-*Toxoplasma* agent would have the following properties:

- decreased toxicity compared with existing drugs;
- safe for use during pregnancy;
- enhanced efficacy compared with existing drugs;
- the ability to target tachyzoites to treat acute infections;
- the ability to target bradyzoites to eliminate tissue cysts; and
- the ability to reach sufficient concentrations in the CNS to eliminate parasites in the brain and eye.

In the following sections, we review current and emerging targets (see also Table 1 and Fig. 1) and challenges of anti-parasitic chemotherapy for treating human toxoplasmosis.

3.1. Folic acid metabolism inhibitors

Apicomplexans, like many protozoans, encode a fused dihydrofolate reductase (DHFR)-thymidylate synthase enzyme that is important for folate metabolism and nucleotide synthesis (Ivanetich and Santi, 1990; Trujillo et al., 1996). The frontline treatment for toxoplasmosis, pyrimethamine, targets DHFR by acting as a folic acid antagonist (Fig. 1). Pyrimethamine acts synergistically with sulfonamides, which act on dihydropteroate synthase, another enzyme in the folate synthesis pathway. However, treatment with pyrimethamine and sulfadiazine frequently causes toxic side effects in patients including hypersensitivity to sulfadiazine treatment, resulting in skin rashes and fevers (McLeod et al., 2006), and bone marrow suppression and adverse effects on developing foetuses caused by folate depletion associated with pyrimethamine treatment (Serranti et al., 2011). Pyrimethamine is, therefore, not always recommended for treating congenital infections during the first trimester of pregnancy.

DHFR-targeting compounds with greater potency against *T. gondii* compared with humans have been investigated to overcome issues associated with pyrimethamine toxicity. For instance, WR99210 is a *T. gondii* DHFR inhibitor that is active both in vitro and in a mouse infection model, and exhibits ~10-fold greater potency than pyrimethamine (Mui et al., 2005). Further studies identified the WR99210 analogue, JPC-2067-B, as a potent inhibitor of tachyzoite proliferation in vitro (50% inhibitory concentration (IC_{50}) 20 nM), with minimal toxicity against human cells (Mui et al., 2008). JPC-2067-B can be administered as an orally bioavailable prodrug called JPC-2056, which is active in vivo, and exhibits better pharmacological characteristics than WR99210.

Computational structure-based, rational drug design approaches have also been utilised to identify compounds with greater selectivity for *T. gondii* DHFR over human DHFR. Using

Table 1
Diverse classes of compounds have activity against *Toxoplasma gondii* in vitro and in vivo in mouse models of acute and chronic infection. Plus symbol (+) indicates active, minus symbol (–) indicates inactive and (ND) is not determined against *T. gondii* in the indicated experimental system (either against tachyzoites in vitro, acute tachyzoite infection in vivo or chronic bradyzoite brain cyst infection in vivo).

Compound	In Vitro (Tachyzoite)	In Vivo (Acute)	In Vivo Cyst (Chronic)
Bumped Kinase Inhibitors			
BKI 1294[a]	+	+	ND
Compound 32[b]	+	+	+
Compound 24[c]	+	+	+
Compound 3a[d]	+	+	+
Electron Transport Chain Inhibitors			
Atovaquone[e]	+	+	+
ELQ271[f]	+	+	+
ELQ334 (ELQ316)[g]	+	+	+
ELQ400[h]	+	+	ND
Fatty Acid Synthesis Inhibitors			
Triclosan[i]	+	+	+
Folic Acid Synthesis Inhibitors			
JPC-2056 (JPC-2067-B)[j]	+	+	ND
TRC-19[k]	+	ND	ND
Compound 3[l]	+	+	ND
Histone Modification Inhibitors			
FR235222[m]	+	ND	ND
W363 and W399[n]	+	ND	ND
Isoprenoid Pathway Inhibitors			
Risedronate[o]	+	+	ND
Compound 1[p]	+	+	ND
Atorvastatin[q]	ND	+	ND
Protein Synthesis Inhibitors			
Clindamycin[r]	+	+	–
Azithromycin[s]	+	+	–
Guanabenz[t]	+	+	+

[a] Doggett et al. (2014), Müller et al. (2017).
[b] Vidadala et al. (2016).
[c] Rutaganira et al. (2017).
[d] Janetka et al. (2020).
[e] Araujo et al. (1991a), Araujo et al. (1991b), Araujo et al. (1992), Romand et al. (1993), Ferguson et al. (1994), Dunay et al. (2004).
[f] Doggett et al. (2012).
[g] Doggett et al. (2012), Doggett et al. (2020), McConnell et al. (2018).
[h] Doggett et al. (2012), McConnell et al. (2018).
[i] McLeod et al. (2001), El-Zawawy et al. (2015a, b).
[j] Mui et al. (2008).
[k] Welsch et al. (2016).
[l] Hopper et al. (2019).
[m] Bougdour et al. (2009), Maubon et al. (2010).
[n] Maubon et al. (2010).
[o] Martin et al. (2001), Yardley et al. (2002).
[p] Ling et al. (2005).
[q] Li et al. (2013).
[r] Araujo and Remington (1974).
[s] Araujo et al. (1991a), Araujo et al. (1991b), Dumas et al. (1994).
[t] Benmerzouga et al. (2015), Martynowicz et al. (2019), Martynowicz et al. (2020).

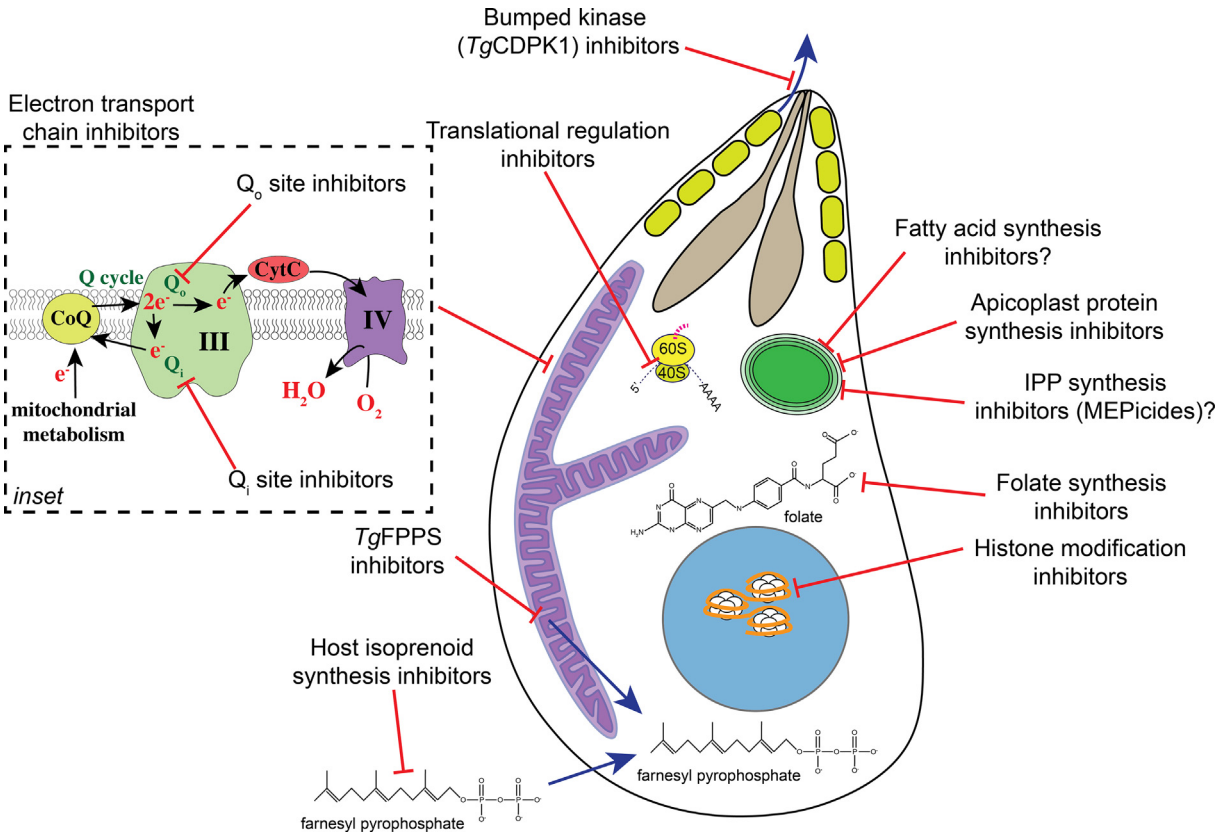


Fig. 1. Summary of some of the targets for chemotherapeutic interventions against *Toxoplasma gondii*. Targets include processes that occur in the apicoplast, mitochondrion, and nucleus, as well as the regulation of cytosolic translation and the synthesis of folates and isoprenoids. See Section 3 and Table 1 for further details. Inset depicts the Q cycle of the mitochondrial electron transport chain (ETC). Electrons (e^-) derived from mitochondrial metabolism are donated to coenzyme Q (CoQ), then to the Q_o site of Complex III. From here, electrons are transported via cytochrome *c* (CytC) and Complex IV to O_2, or back to CoQ at the Q_i site of Complex III. Most inhibitors of the electron transport chain in these parasites target either the Q_i or Q_o sites of Complex III. CDPK, calcium-dependent protein kinase; FPPS, farnesyl diphosphate/geranyl geranyl-diphosphate synthase; IPP, isopentenyl pyrophosphate; MEP, methylerythritol phosphate.

pyrimethamine as a lead structure, one study identified TRC-19, a compound with ~25-fold greater potency against the *Tg*DHFR enzyme than pyrimethamine (9 nM versus 230 nM IC_{50}) and a ~90-fold greater selectivity for *T. gondii* DHFR over the human enzyme (Welsch et al., 2016). A recent structure–activity relationship study further optimised TRC-19 to develop the 2-methoxypyrimidine, "Compound 3", with a resulting IC_{50} of 1.6 nM against *Tg*DHFR activity, an ~200-fold selectivity for *T. gondii* DHFR over the human enzyme, and an 50% effective concentration (EC_{50}) of 13 nM against *T. gondii* proliferation in an in vitro culture model (Hopper et al., 2013). In acute *T. gondii* infection of mice, treatment with Compound 3 improved survival in a dose-dependent manner, the highest dose yielding 100% survival over 30 days, as opposed to the vehicle-treated mice, which succumbed to infection after 6 or 7 days (Hopper et al., 2019). It remains to be seen whether Compound 3 is effective against chronic infection or bradyzoite cysts but preliminary pharmacokinetic studies in mice suggest Compound 3 is able to cross into the CNS (Hopper et al., 2019). These studies are a promising start in the bid to identify more selective and potent inhibitors of *T. gondii* DHFR with the physicochemical characteristics needed to avoid the toxicity associated with current pyrimethamine and sulfadiazine treatment.

3.2. Mitochondrial electron transport chain inhibitors

The mitochondrial electron transport chain consists of a series of protein complexes embedded in the inner mitochondrial membrane and plays a key role in pyrimidine biosynthesis and oxidative phosphorylation, a process by which *T. gondii* parasites can generate adenosine triphosphate (ATP) (Hayward and van Dooren, 2019). Several enzymatic reactions that take place in the mitochondrion (e.g., reactions in the tricarboxylic acid cycle) result in the transfer of electrons to coenzyme Q (CoQ), an electron carrier embedded in the inner mitochondrial membrane (Fig. 1, inset). Complex III (also known as the cytochrome bc_1 complex) facilitates the net transfer of electrons from CoQ to cytochrome *c*, a mobile electron carrier in the mitochondrial intermembrane space (Fig. 1, inset). Electrons from cytochrome *c* are transferred to Complex IV of the electron transport chain, where they ultimately reduce O_2 (Fig. 1, inset). Electron transport through Complexes III and IV is coupled to the translocation of protons across the inner mitochondrial membrane, generating a proton gradient that can be utilised by ATP synthase (Complex V) for the synthesis of ATP. The cytochrome *b* protein of Complex III interacts with CoQ in a process known as the "Q-cycle" (Fig. 1, inset). Reduced CoQ docks at the so-called Q_o site of cytochrome *b*, donating electrons to either the iron-sulfur cluster of the Rieske protein of Complex III (from where it is transported onward through the remainder of the electron transport chain), or to a second CoQ docking site on cytochrome *b* called the Q_i site (Fig. 1, inset). At the Q_i site, oxidised CoQ is reduced, removing protons from the mitochondrial matrix and thereby contributing to the proton gradient across the inner membrane.

The Q_o and Q_i sites of cytochrome *b* are targets of a broad range of anti-parasitic drugs, including compounds that target *T. gondii* (Fig. 1, inset). The best studied of these is atovaquone, which tar-

gets the Q_o site of cytochrome *b* (Fry and Pudney, 1992; Pfefferkorn et al., 1993; McFadden et al., 2000). Early studies showed that atovaquone has activity against tachyzoites and tissue cysts both in vitro and in vivo (Araujo et al., 1991a, 1991b, 1992; Romand et al., 1993; Ferguson et al., 1994; Dunay et al., 2004). However, clinical trials have had mixed results regarding the ability of atovaquone to prevent relapse of infection, with between 12 and 26% of patients experiencing relapse after 1 year, and up to 75% after 6 years of therapy (Katlama et al., 1996; Pearson et al., 1999). Instances of failure of atovaquone treatment have also been reported (Baatz et al., 2006; Megged et al., 2008). These studies suggest that atovaquone does not completely eliminate tissue cysts in humans. Given that many clinical trials were retrospective, non-randomized and non-comparative, more clinical data is required to evaluate the true efficacy of atovaquone, ideally comparing the rates of relapse in patients given atovaquone or pyrimethamine and sulfadiazine, or combinations of these treatments.

There has been much interest in developing Complex III inhibitors with better potency and bioavailability than atovaquone. The endochin-like-quinolone (ELQ) series of 4(1H)-quinolone-3-diarylethers can target the Q_i site and/or the Q_o site depending on their chemistry. The Q_i site inhibitors ELQ-271 and ELQ-316 are potent inhibitors of *T. gondii* proliferation in vitro (IC_{50} values of 0.1 and 0.007 nM, respectively) and in treating acute infection in mice, with a high selectivity in targeting the *T. gondii* complex over the human Complex III (>90,000-fold and $>10^6$-fold, respectively) (Doggett et al., 2012). Notably, ELQ-271 and ELQ-316 treatment reduced *T. gondii* brain cysts by up to 88% in mice (Doggett et al., 2012), suggesting these compounds are active against both tachyzoites and bradyzoites in vivo. ELQ-400 targets both the Q_o and Q_i sites of cytochrome *b*, and exhibits greater efficacy in treating acute *T. gondii* infections in mice than both atovaquone and ELQ-316 (McConnell et al., 2018; Song et al., 2018).

The aforementioned ELQs exhibit low aqueous solubility and high crystallinity, which may limit their oral bioavailability. A carbonate ester prodrug of ELQ-316, called ELQ-334, was created and shown to both improve bioavailability and prolong the survival of mice in an acute infection model (Doggett et al., 2020). Oral treatment with ELQ-334 also reduced the brain cyst burden by up to 83% in mice chronically infected with *T. gondii* parasites (Doggett et al., 2020). While smaller than non-treated cysts, the tissue cysts that survived ELQ-334 treatment were viable and capable of establishing an infection in naïve mice (Doggett et al., 2020), suggesting that ELQ-334 treatment does not entirely clear brain cysts.

Mutations in the Q_o and Q_i sites of cytochrome *b* are common causes of resistance against compounds that target these sites (McFadden et al., 2000; Alday et al., 2017). The potential of inhibitors that target both the Q_o and Q_i site, such as ELQ-400, or combination therapy with Q_o and Q_i site inhibitors as dual Complex III inhibitors, could slow the emergence of drug resistance and thereby provide more effective treatments against tissue cysts. While yet to be tested in *T. gondii* infection, a combination of ELQ-334 (Q_i site inhibitor) and atovaquone (Q_o site inhibitor) cleared mice of infection with *Babesia microti*, a related apicomplexan parasite, with no recrudescence after 122 days (Lawres et al., 2016).

3.3. Calcium-dependent protein kinase-1 inhibitors

Calcium-dependent protein kinases (CDPKs) are a family of serine/threonine protein kinases found in plants and numerous protozoans, including apicomplexans (Billker et al., 2009). The *T. gondii* genome encodes 14 CDPKs, and these are predicted to function in a range of calcium signaling-dependent processes across the life cycle of the parasite (Long et al., 2016). *Tg*CDPK1 is important for tachyzoite proliferation, where it functions in calcium-dependent microneme secretion, a process that is critical for parasite egress from host cells, gliding motility and invasion of parasites into new host cells (Lourido et al., 2010). *Tg*CDPK1 can be effectively inhibited by so-called bumped kinase inhibitors (BKIs; Fig. 1), which are considered to be promising potential therapies against apicomplexan parasites (Choi et al., 2020). BKIs target the ATP binding pocket of *Tg*CDPK1, and the selectivity of BKIs for *Tg*CDPK1 over host kinases (and other CDPKs encoded by *T. gondii*) lies in the presence of a small "gatekeeper residue" (a glycine) in the ATP binding site of *Tg*CDPK1, as opposed to bulkier residues such as methionine found in human kinases. Several BKI scaffolds have been identified, with pyrazolopyrimidines (PPs) and 5-aminopyrazole-4-carboxamides (ACs) the two main classes. The structure of TgCDPK1 has shown that BKIs act as competitive inhibitors in the ATP binding pocket (Ojo et al., 2010), which has facilitated several structure-based drug development programs (Cardew et al., 2018) that have led to improved pharmacology, efficacy and safety of BKIs (Choi et al., 2020).

The PP compound, BKI 1294, had an in vitro IC_{50} of 140 nM and showed efficacy against acute infection in mice when administered orally (Doggett et al., 2014). Oral administration of BKI 1294 also increased the survival of pups from 31% to 100% in a congenital infection of mice, with only 7% of pups positive for brain infection (Müller et al., 2017). However, a potential for cardiotoxicity via inhibition of the ion channel human Ether-a-go-go-Related Gene (hERG) was discovered and prevented progression of this compound into clinical development (Ojo et al., 2014). To overcome this, a line of optimized PP-based BKIs were developed to avoid hERG inhibition (Vidadala et al., 2016). "Compound 32" from this study had an in vitro IC_{50} of 60 nM and, importantly, was able to cross into the CNS and reduce the number of brain cysts by 88% in a chronic infection of mice (Vidadala et al., 2016). Another structure–activity relationship study into PP molecules identified a compound ("Compound 24") that reduced dissemination of parasites from the peritoneal cavity to the brain in acute infection of mice (Rutaganira et al., 2017). Compound 24 also reduced brain cyst burden and cured 40% of mice or delayed death in 60% of mice in a reactivation model of parasite infection in immunocompromised mice (Rutaganira et al., 2017).

Structure-activity relationship studies have also progressed the development of BKIs with an AC scaffold (Zhang et al., 2014). In one study, structural optimisation of compounds with an AC scaffold led to the production of "Compound 35", a molecule that is able to cross into the CNS with a lower in vitro IC_{50} (89 nM) and improved pharmacokinetic properties compared with the starting compound (Huang et al., 2015). The development of improved BKIs via the incorporation of AC and PP scaffolds highlights the promise of developing *Tg*CDPK1-targeting compounds with activity against both acute and chronic stages of parasite infection.

3.4. Fatty acid synthesis (FAS) inhibitors

Fatty acids are major components of biological membranes, and also serve as important signaling molecules. Early work indicated that *T. gondii* parasites are able to scavenge lipids, including fatty acids, from host cells (Charron and Sibley, 2002). Sequencing of the parasite genome found that *T. gondii* encodes enzymes of the so-called FAS II pathway of fatty acid biosynthesis (Seeber and Soldati-Favre, 2010). Enzymes of the FASII pathway localise to the apicoplast organelle (Waller et al., 1998; Seeber and Soldati-Favre, 2010), a non-photosynthetic plastid that is widespread throughout apicomplexans. Notably, the apicoplast FAS II pathway differs from the FAS pathway found in the mitochondria of mammals, and also from the cytosolic single multifunctional fatty acid synthase I (FAS I) enzyme for FAS.

Conditional depletion of acyl carrier protein, a central enzyme in the FAS II pathway, led to reduced parasite proliferation in vitro and reduced virulence in an acute infection of mice (Mazumdar et al., 2006). Coupled with its absence from the mammalian host, this suggested that the FASII pathway may represent a promising drug target (Fig. 1). Indeed, drugs such as thiolactomycin and triclosan, which target the FASII pathway in bacteria, were shown to inhibit parasite proliferation (McLeod et al., 2001; Mazumdar et al., 2006). A more recent study, however, demonstrated that parasites are able to proliferate in vitro, and cause disease in an acute infection model, upon the knockout of genes encoding key FASII enzymes (Liang et al., 2020a, 2020b). In vitro proliferation in these FASII mutants was slower than in wild type parasites, and this proliferation defect could be rescued by supplementation with excess fatty acids in the growth medium. This mirrors another recent study that revealed that the FASII pathway is upregulated when parasites are cultured in a lipid-poor growth medium (Amiar et al., 2020). Together, these findings point to a considerable degree of metabolic flexibility in lipid acquisition in *T. gondii*, suggesting that the FASII pathway may be particularly important in intracellular niches or stages of infection where fatty acids in the environment are limiting. Nevertheless, doubts remain about whether FASII represents a feasible drug target in these parasites.

3.5. Histone modification inhibitors

Histones are DNA binding proteins that function in packaging DNA in the nucleus of eukaryotic cells. Post-translational histone modifications play many and various roles in regulating gene expression, as well as facilitating DNA replication and repair. In *T. gondii*, histone deacetylase (HDAC) and histone acetylase enzymes are important for controlling gene expression, including in the interconversion between tachyzoites and bradyzoites (Saksouk et al., 2005). A HDAC inhibitor, FR235222, targets the *Tg*HDAC3 protein in *T. gondii* (Fig. 1), and is a potent inhibitor of tachyzoite proliferation (Bougdour et al., 2009). Treatment with FR235222 induces the upregulation of bradyzoite-specific genes and results in the differentiation of tachyzoites into bradyzoites. Interestingly, pretreatment of ex vivo bradyzoite-containing cysts rendered them incapable of converting to tachyzoites when inoculated into mice (Maubon et al., 2010). This suggests that HDAC3 inhibitors hold particular promise in preventing reactivation of brain cysts. A potential limitation is that FR235222 has only 10-fold selectivity for *T. gondii* over host cells. The related HDAC-targeting compounds W363 and W399, however, exhibit similar potency to FR235222 in inhibiting parasite proliferation and are less toxic to human cells (Maubon et al., 2010).

3.6. Isoprenoid biosynthesis inhibitors

Isoprenoids are lipids that contribute to various aspects of cellular biology. In apicomplexan parasites such as *T. gondii*, they function as precursors for molecules such as the mitochondrial electron carrier, CoQ, and a class of lipids termed dolichols, as well as in the prenylation of proteins that function in the endomembrane system (Imlay and Odom, 2014). The basic building blocks of isoprenoids are two five-carbon molecules termed isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The synthesis of IPP and DMAPP occurs in the apicoplast of apicomplexans via a seven-enzyme pathway termed the methylerythritol phosphate (MEP) pathway (Imlay and Odom, 2014). The MEP pathway, sometimes referred to as the non-mevalonate pathway, is fundamentally different from the so-called mevalonate pathway that synthesises IPP and DMAPP in mammalian cells and, hence, it has been proposed as a promising therapeutic target in apicomplexans (Fig. 1). Indeed, the antibiotic fosmidomycin, which inhibits 1-deoxy-D-xylulose-5-phosphate (DOXP) reductoisomerase, the second enzyme in the MEP pathway, is a potent inhibitor of proliferation of blood-stage *Plasmodium* parasites (Jomaa et al., 1999). Curiously, however, fosmidomycin has no effect on the growth of *T. gondii*, despite DOXP reductoisomerase being critical for *T. gondii* proliferation and activity of recombinant DOXP reductoisomerase being sensitive to fosmidomycin (Ling et al., 2005; Baumeister et al., 2011; Nair et al., 2011). This observation can be explained by the inaccessibility of fosmidomycin to its site of action. Overexpression in *T. gondii* of a bacterial protein capable of fosmidomycin transport rendered these parasites sensitive to fosmidomycin both in vitro and in vivo (Nair et al., 2011). Similar observations of limited fosmidomycin accessibility have been made in liver-stage *Plasmodium* parasites (Baumeister et al., 2011; Nair et al., 2011). To overcome these drug accessibility issues, a series of so called "MEPicides", which are lipophilic ester prodrugs structurally related to fosmidomycin and designed to improve entry into target cells, have been synthesised recently; they were shown to be potent inhibitors of *Plasmodium* proliferation in vitro and in vivo (Wang et al., 2018a, 2018b) as well as being able to inhibit other intracellular pathogens, such as *Staphylococcus* bacteria, in a transporter-independent manner (Edwards et al., 2020). It will now be of particular interest to test MEPicides for anti-*Toxoplasma* activity.

Following its synthesis in the apicoplast, IPP is exported into the cytosol where it can be metabolised further to generate a range of isoprenoids. A dual-function farnesyl diphosphate/geranyl geranyldiphosphate synthase (*Tg*FPPS) enzyme in *T. gondii* generates 15- and 20-carbon isoprenoids from IPP, and is important for the biosynthesis of isoprenoids downstream of the DOXP pathway (Ling et al., 2007). The bisphosphonate compound, risedronate, inhibits this enzyme (Fig. 1) and shows activity against *T. gondii* proliferation in vitro (Martin et al., 2001). Risedronate also improved the survival of mice infected with tissue cysts by 55% over 30 days of infection at the highest dose used (Yardley et al., 2002). Other bisphosphonates with a greater therapeutic index and potency than risedronate have been identified, with one compound improving the survival of infected mice by up to 80% over a 30 day period (Ling et al., 2005; Shubar et al., 2008).

Genetic disruption of the gene encoding the *Tg*FPPS protein did not affect proliferation of parasites in fibroblasts in vitro but did affect proliferation under stress conditions such as growth in macrophages and when parasites were extracellular (Li et al., 2013). The dispensability of *Tg*FPPS was attributed to the ability of *T. gondii* to scavenge isoprenoids from the host cell. In support of this, concomitant inhibition of the host mevalonate isoprenoid biosynthesis pathway using the drug atorvastatin (Fig. 1) increased survival of mice infected with a *Tg*FPPS knockout strain from 20% to 90% (Li et al., 2013). Inhibitors of the host mevalonate pathway, including statins such as atorvastatin, are in widespread use for the treatment of humans at risk of heart disease. A dual inhibition strategy using drugs to target both the parasite and host isoprenoid biosynthesis pathways could yield synergistic effects in vivo, and future studies examining the therapeutic feasibility of such an approach for the treatment of *T. gondii* infections are of particular interest.

3.7. Protein synthesis inhibitors

The endosymbiotic origin of the apicoplast of *T. gondii* means that this compartment houses a bacterially-derived translational machinery (e.g., 70S ribosomes). Numerous inhibitors of bacterial translation have been shown to inhibit the proliferation of *T. gondii* (Fig. 1) and other apicomplexans (Goodman et al., 2016); for example, clindamycin, an inhibitor of the bacterial 50S (large) ribosomal

subunit, inhibits *T. gondii* proliferation (Pfefferkorn et al., 1992) and has been used in clinical treatment of toxoplasmic encephalitis patients in combination with pyrimethamine (Dannemann et al., 1992). Clindamycin-resistant *T. gondii* strains have mutations in the large rRNA that is encoded on the apicoplast genome (Camps et al., 2002), suggesting that clindamycin targets protein translation in the apicoplast of these parasites.

Parasites treated with clindamycin and other compounds targeting apicoplast translation exhibit a peculiar phenotype known as delayed death (Fichera et al., 1995; Camps et al., 2002), where parasite proliferation remains unaffected by drug treatment until they have egressed from existing host cells and reinvaded new host cells. This phenomenon is tied to apicoplast loss that occurs upon treatment with these inhibitors – it appears that the parasites are able to tolerate apicoplast loss until they reinvade new host cells, leading to the proposal that a product of apicoplast metabolism is required to establish a new infection (He et al., 2001). Interestingly, co-treatment with clindamycin and the host mevalonate pathway inhibitor, atorvastatin, results in a more immediate death phenotype compared with treatment with clindamycin alone (Amberg-Johnson and Yeh, 2019). This leads to the proposal that scavenging of host isoprenoids compensates for the loss of the apicoplast that occurs in the first lytic cycle after clindamycin treatment but that host isoprenoid scavenging is not sufficient to facilitate longer-term parasite proliferation following apicoplast loss (Amberg-Johnson and Yeh, 2019).

The macrolide, azithromycin, is another inhibitor of apicoplast protein translation, and it can be used alone or in combination with pyrimethamine to treat acute episodes of ocular toxoplasmosis (Rothova et al., 1998; Bosch-Driessen et al., 2002; Balaskas et al., 2012). Azithromycin has many favourable properties including good oral bioavailability, long half-life and fewer side-effects than pyrimethamine and sulfadiazine (Alday and Doggett, 2017). While patients experienced fewer side effects with azithromycin treatment than with pyrimethamine and sulfadiazine, one study noted that 27% of patients experienced recurrence within a year of azithromycin monotherapy (Rothova et al., 1998). This suggests that azithromycin does not fully eliminate *T. gondii* cysts in patients. In agreement with this, while azithromycin treatment prolonged survival of mice in both acute and chronic mouse infection models (Araujo et al., 1991a, 1991b; Dumas et al., 1994), azithromycin treatment of mice chronically infected with *T. gondii* parasites did not lessen brain cyst burden compared with controls (Dumas et al., 1994).

Cytosolic protein synthesis is also a potential target of therapeutic intervention in *T. gondii* (Fig. 1). Studies have revealed that the conversion of tachyzoites to bradyzoites is concomitant with the phosphorylation of the α subunit of the *T. gondii* eukaryotic initiation factor 2 (*Tg*eIF2; Sullivan et al., 2004; Narasimham et al., 2008). *Tg*eIF2 has a key role in initiating translation at cytosolic ribosomes and, as in other organisms, its phosphorylation impairs cytosolic translation, leading to a global reduction in parasite protein synthesis (Sullivan et al., 2004; Narasimhan et al., 2008). Treatment of parasites with the Food and Drug Administration (U.S.A.)-approved anti-hypertension drug, guanabenz, inhibits *Tg*eIF2 dephosphorylation, which in turn promotes the formation of bradyzoite-containing cysts and prevents reactivation into tachyzoites in vitro (Konrad et al., 2013). Guanabenz treatment prolonged the survival of mice in an acute infection model by several days (Konrad et al., 2013; Benmerzouga et al., 2015). Interestingly, the effects of guanabenz on brain cyst burden seems to vary between mouse strains, with guanabenz treatment leading to a decrease in brain cysts in BALB/c mice but leading to an increase in brain cysts in C57BL/6J mice (Martynowicz et al., 2019). The authors speculated that these differences could arise from differences in the reactivation of tissue cysts, differences in susceptibility of mice to *T. gondii* infection, and/or differences in the immune response elicited by these two mouse strains. Regardless of the reasons, this study highlights the importance of investigating the contributions of host biology to disease outcomes following drug treatments.

3.8. Challenges and prospects for better chemotherapeutic treatment options

While much progress has been made towards identifying new anti-*Toxoplasma* chemotherapeutics, there are still many challenges to translate these findings into better clinical outcomes for patients. The ideal chemotherapeutic against *T. gondii* would cure patients of infection, which would require the compound to have activity against both the disease-causing tachyzoite form and the slower-growing bradyzoite form. One of the key challenges is to obtain a broader understanding of the biology of bradyzoite-containing tissue cysts, and why drugs fail to eliminate tissue cysts in patients. Recent advances in facilitating the in vitro differentiation of tachyzoites into bradyzoites en masse may aid in these efforts (Waldman et al., 2020), although it will be important to verify that in vitro models recapitulate the biology of bradyzoites and tissue cysts in vivo. Notably, the replicative capacity and physiology of bradyzoites in vivo seems to be more heterogeneous and dynamic than appreciated previously (Watts et al., 2015; Sinai et al., 2016), implying that some cysts (potentially those that are latent/dormant) may be more resistant to drugs than others. Understanding the causes of this heterogeneity may uncover new avenues for therapeutic intervention, in particular determining what factors drive bradyzoites to latency/dormancy.

Another roadblock is the potential for strain-specific effects of drugs in both *T. gondii* and mice. A striking example of this is the mouse strain-dependent disease outcomes observed upon guanabenz treatment (see Section 3.7) (Konrad et al., 2013; Benmerzouga et al., 2015; Martynowicz et al., 2019). These studies highlight the benefit and, perhaps, necessity of testing compound efficacy in multiple mouse and *T. gondii* strains to better inform the possible outcomes when compounds are progressed to clinical trials. This also questions the extent to which the performance of a compound in mouse models resembles the outcome in human disease.

In the bid to identify new drug targets and novel chemotherapies, high throughput screening methods are increasingly utilised to assess large compound libraries for activity against *T. gondii* proliferation in vitro (Boyom et al., 2014; Murata et al., 2017; Adeyemi et al., 2018a). One challenge that limits the progression of hit compounds is differences in their in vitro versus in vivo effectiveness, as many compounds that show promise in preliminary screens exhibit unfavorable pharmacokinetic properties or, ultimately, target processes that are less important for parasite survival in vivo than in vitro. Structure-activity relationship studies and the development of prodrugs with more desirable in vivo properties hold particular promise in the development of promising "hits" that emerge from high throughput screens.

A final challenge is understanding the extent to which anti-*Toxoplasma* treatments can "piggy-back" on treatment efforts in other apicomplexan parasites, most notably *Plasmodium* parasites, for which extensive drug screening efforts exist. Several current anti-*Toxoplasma* agents, including pyrimethamine and atovaquone, are also potent inhibitors of *Plasmodium* parasites. Nevertheless, there are major differences in the host cell types and physiological environments that these parasites inhabit, and also major differences in the biology of the different life cycle stages of these parasites. This means that drugs that are highly efficacious in one parasite will not necessarily be potent inhibitors in another parasite. Emphasising this point, a screen of antimalarial drugs in late

preclinical development revealed that most anti-malarials tested exhibited only a limited efficacy against *T. gondii* proliferation (Radke et al., 2018). As a more specific example, cipargamin, a potent inhibitor of *P. falciparum* parasites that targets the *Pf*ATP4 sodium pump (Spillman et al., 2013), shows limited efficacy against *T. gondii* (Radke et al., 2018; Lehane et al., 2019). This can be explained by the differences in sodium concentrations experienced by the disease-causing stages of these parasites within their host cells, with the *T. gondii* homologue of *Pf*ATP4 being dispensable for intracellular proliferation (Lehane et al., 2019). Therefore, while the idea of developing pan-apicomplexan therapeutics that act on conserved pathways is appealing, it is likely that focusing early drug development to optimise efficacy against *T. gondii* will lead to better therapeutics for treatment of toxoplasmosis.

4. Immunotherapies for the control of toxoplasmosis

The activation or suppression of distinct elements of the immune system via targeted host-directed immunotherapy has gained significant attention in the field of infectious diseases, including toxoplasmosis (Kaufmann et al., 2018a, 2018b; Singh et al., 2019). Immunotherapies are less prone to the emergence of resistance and often have fewer side effects compared with antimicrobial drugs. Whilst there are currently no immunotherapies in use for human toxoplasmosis, murine models have provided insight that may prove crucial for future developments (Fig. 2). In the following section, we review recent research advances in immunotherapies against toxoplasmosis, including the role of passive immunisation, monoclonal antibody therapy, chimeric antigen receptor (CAR) T cells, cytokines and chemokines, immunomodulatory drugs and trained immunity, any of which could conceivably become the basis for future treatments of immunosuppressed people.

4.1. Passive immunisation, monoclonal antibodies and CAR T cells

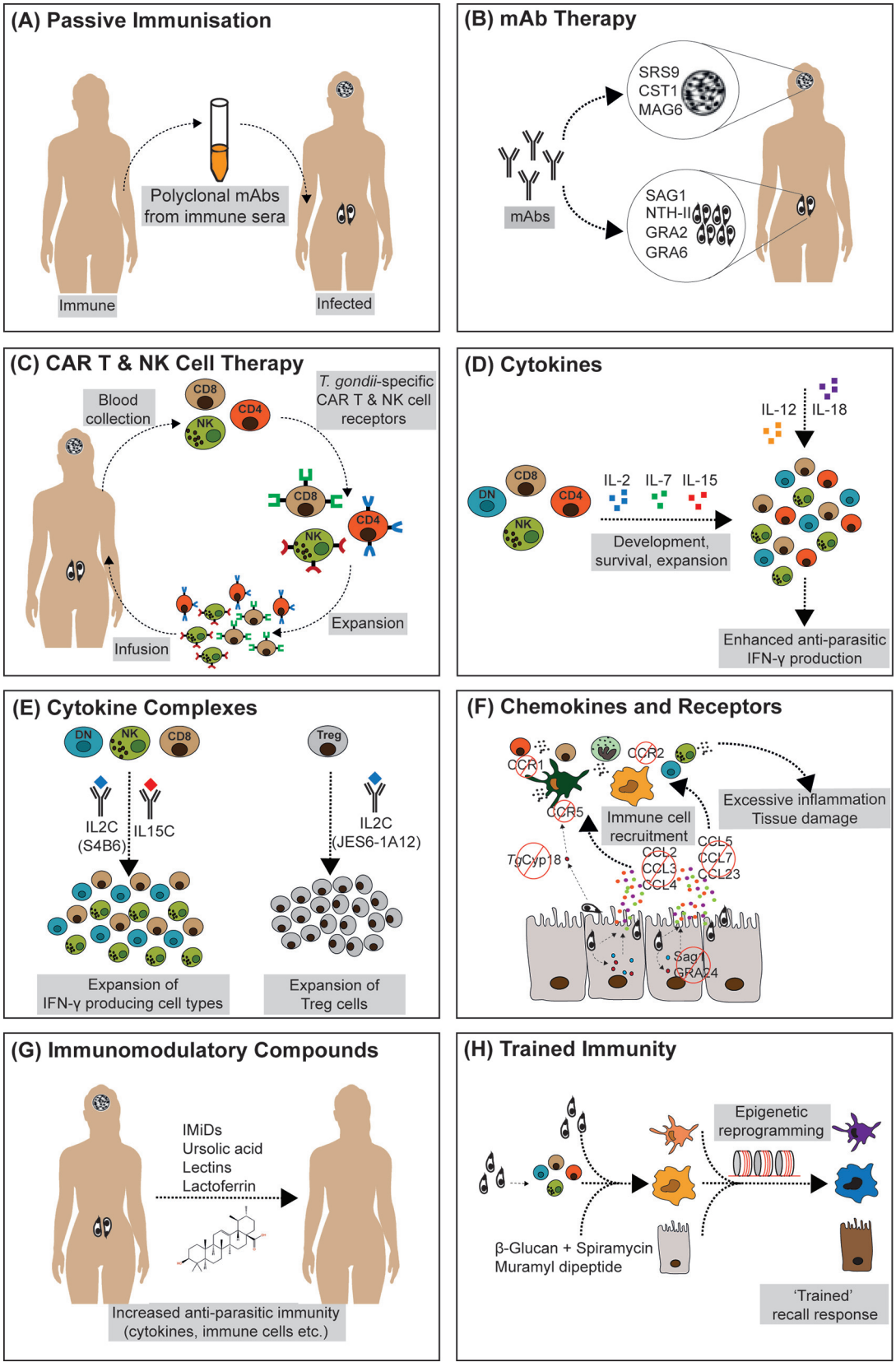
Passive immunisation, the transfer of polyclonal antibodies from immune sera (Fig. 2A), has been used to treat and prevent infectious diseases such as tetanus, rabies and hepatitis B (Sparrow et al., 2017). Early adoptive transfer studies in a hamster model of *T. gondii* infection suggested that antibodies contribute only marginally to protection after transfer (Frenkel, 1967). These findings laid the foundation for the concept that immunity to *T. gondii* is dominated by IFN-γ producing $CD8^+$ T cells (Suzuki and Remington, 1990; Kang et al., 2000; Sayles et al., 2000; Casciotti et al., 2002). However, it was later shown that, in murine toxoplasmosis, antibodies contribute to controlling long-term persistence and vaccination-induced resistance (Kang et al., 2000; Sayles et al., 2000). With the more recent and widespread use of monoclonal antibody (mAb) therapy in the treatment of cancer (Byun et al., 2017), passive immunisation with mAbs in toxoplasmosis has been revitalised. It was shown that the transfer of human mAb Fab fragments specific to the *T. gondii* surface antigen 1 (SAG1) protein into mice significantly improved survival rates (Fu et al., 2011). Similarly, the transfer of mAbs against dense granule proteins GRA2, GRA6 or nucleoside triphosphate hydrolase-II into mice promoted prolonged survival (Cha et al., 2001; Tan et al., 2010). Even though none of these transfers provided sterile immunity, it is possible that the identification of additional bradyzoite- and cyst wall-specific antigens, such as SRS9, CST1 and MAG2 (Kim et al., 2007; Tomita et al., 2013; Tu et al., 2020), may pave the way for future combination mAb therapies targeting several stage-specific molecules for attachment, invasion, proliferation and persistence simultaneously (Fig. 2B). Similarly, mAbs that target the inhibition of receptors, such as CTLA-4 and PD-1, on CD8+ T cells and natural killer (NK) cells have been shown to enhance immunity during chronic *T. gondii* infection (Hunter et al., 1997; Bhadra et al., 2011, 2012; Xiao et al., 2018), though these findings require further validation (Splitt et al., 2018).

Although cellular immunotherapies, such as the transfer of immune $CD8^+$ T cells and NK cells, can protect animals from lethal *T. gondii* challenge (Casciotti et al., 2002; Ivanova et al., 2019), these strategies are not suitable for large scale human application. However, the advent of commercial-scale CAR T cell and NK cell therapy may provide a new avenue for cell-mediated immunotherapy in toxoplasmosis (Maldini et al., 2018; Liu et al., 2020). CAR T cell therapy involves the transfer into patients of genetically modified T cells that not only recognise specific protein targets but also co-express T cell activation functions in a single engineered receptor. CAR T cell therapy has revolutionised cancer treatment over the last decade and, despite non-cancer applications still being in their infancy, one could envisage that the re-engineering of patient-derived $CD8^+$ T cell or NK cell receptors specific for stage-specific *T. gondii* antigens could significantly improve feasibility and success of cellular immunotherapy for (latent) toxoplasmosis (Fig. 2C). Nevertheless, a vigilant evaluation of these immunotherapies in the context of infection will be required, as neurological complications and disseminated toxoplasmosis have been reported in CAR T cell-treated patients (Kersten et al., 2019; Kator et al., 2020).

4.2. Cytokines

Immunity to toxoplasmosis is critically dependent on ILs and IFN-γ, in particular the IL-12/IFN-γ axis (Yarovinsky, 2014); many studies have demonstrated beneficial outcomes after the delivery of recombinant IL-12 (Hunter et al., 1995; Araujo et al., 1997) or IFN-γ (Suzuki et al., 1988; Suzuki et al., 1990; Benedetto et al., 1991; Delemarre et al., 1993; Delemarre et al., 1994) in mouse models of toxoplasmosis and in ex vivo infection of human cells. Similarly, IL-18, formerly known as IFN-γ enhancing factor, also plays a critical role in immunity to T. gondii, by synergising with IL-12 (Cai et al., 2000). The development, expansion and survival of IFN-γ-producing cell types depends on the γ chain cytokines, IL-2, IL-7 and IL-15 and, thus, immunotherapies based on said cytokines have also shown improved outcomes of toxoplasmosis in animal models (Fig. 2D). While the exact role of IL-15 in immunity to T. gondii remains somewhat unresolved (Khan et al., 2002; Lieberman et al., 2004), IL-2 deficient mice are highly susceptible to *T. gondii* infection (Villegas et al., 2002), and administration of recombinant IL-2 enhances survival of Toxoplasma-infected mice (Sharma et al., 1985; Shirahata et al., 1993). Delivery of recombinant IL-15 also protects against lethal infection by enhancing IFN-γ production (Khan and Casciotti, 1999; Khan and Kasper, 1996) but IL-15 deficient mice are able to develop protective immunity (Lieberman et al., 2004). IL-7 and IL-15 appear to synergise during acute infection (Kasper et al., 1995; Bhadra et al., 2010), although the recall response of memory $CD8^+$ T cells to *T. gondii* is almost exclusively dependent on IL-15 (Bhadra and Khan, 2012).

IL-2 and IL-15 are presented to the cytokine receptor complex beta and the common γ chain in the context of cell-bound high-affinity alpha chains of the cytokine receptor (Stoklasek et al., 2006; Stonier and Schluns, 2010). This process has been termed trans-presentation. Combining IL-2 with anti-IL-2 mAbs to form an IL-2 complex (IL2C) or combining IL-15 with a chimeric receptor (IL-15RαFc) to form an IL-15 complex (IL15C) significantly enhances the biological activity of these cytokines in vivo (Boyman et al., 2006; Votavova et al., 2014). The binding site of the anti-IL2 mAb used in the IL2C determines whether a preferential expansion of regulatory T cells (T_{Reg}) or $CD8^+$ T cell and NK cells occurs; anti-IL-2 mAb JES6-1A12 causes expansion of T_{Reg} cells;


(A) Passive Immunisation
Polyclonal mAbs from immune sera
Immune
Infected
(B) mAb Therapy
SRS9
CST1
MAG6
mAbs
SAG1
NTH-II
GRA2
GRA6
(C) CAR T & NK Cell Therapy
Blood collection
T. gondii-specific CAR T & NK cell receptors
CD8
CD4
NK
Infusion
Expansion
(D) Cytokines
IL-12
IL-18
DN
IL-2
IL-7
IL-15
Development, survival, expansion
Enhanced anti-parasitic IFN-γ production
(E) Cytokine Complexes
DN
NK
CD8
IL2C (S4B6)
IL15C
Treg
IL2C (JES6-1A12)
Expansion of IFN-γ producing cell types
Expansion of Treg cells
(F) Chemokines and Receptors
CCR1
CCR2
CCR5
Immune cell recruitment
Excessive inflammation Tissue damage
TgCyp18
CCL2
CCL3
CCL4
CCL5
CCL7
CCL23
Sag1
GRA24
(G) Immunomodulatory Compounds
IMiDs
Ursolic acid
Lectins
Lactoferrin
Increased anti-parasitic immunity (cytokines, immune cells etc.)
(H) Trained Immunity
Epigenetic reprogramming
β-Glucan + Spiramycin
Muramyl dipeptide
'Trained' recall response

whereas anti-IL-2 mAb S4B6 causes expansion of $CD8^+$ T cells and NK cells (Boyman et al., 2006; Shevach, 2012). In animal studies, JES6-1A12-containing IL2C improved control of RH strain *T. gondii* infection (Akbar et al., 2015) and reduced immunopathology and morbidity during acute type II *T. gondii* ME49 infection (Oldenhove et al., 2009), likely by preventing the competition for bioavailable IL-2 between regulatory and effector T cells. We have also shown recently that S4B6-containing IL2C-mediated expansion of non-CD4 immune cells that produce IFN-γ can be harnessed to rescue mice from acute lethal toxoplasmosis (Kupz et al., 2020). Intriguingly, although this IL-12- and IL-18-dependent effect of treatment with SB46-IL2C leads to reduced acute pathology, it does so without affecting T_{Reg} cells or parasite burden. Together, these IL2C studies suggest that cytokine complex immunotherapy may be a viable future adjunct treatment in the context of chronic toxoplasmosis, in particular for immunosuppressed people with impaired $CD4^+$ T cells, e.g., as caused by HIV co-infection in AIDS (Fig. 2E). However, caution is needed because data on the clinical use of IL2C and IL15C treatments in humans are lacking and it needs to be acknowledged that treatment with cytokine complexes could lead to hyperinflammatory responses. Such an undesirable potential outcome should be taken into careful consideration before embarking on translational studies in humans.

4.3. Chemokines

Many parasitic infections, including toxoplasmosis, are characterised by excessive or imbalanced inflammation and pathology (Menzies et al., 2016). These events are tightly linked to the recruitment, influx and persistence of leukocytes to the site of infection, events that are regulated by chemokines and their receptors (Menzies et al., 2016). Similar to most intracellular pathogens, *T. gondii* benefits from leukocyte recruitment, as it can use infected cells to spread through the host (Da Gama et al., 2004; Lambert et al., 2009). Hence, interfering with the chemokine-chemokine receptor axis may provide novel targets for host-directed therapies (Fig. 2F). To illustrate, mouse studies have shown that the absence of chemokine receptor type 5 (CCR5) leads to a reduction in tissue damage due to reduced NK cell recruitment and IFN-γ production, but also to an increased parasite burden (Khan et al., 2006). CCR5 signalling is also involved in the *T. gondii*-induced interruption of pregnancy in mice (Nishimura et al., 2017), and human polymorphisms in CCR5 have been linked to a greater risk of developing ocular toxoplasmosis (de Faria Junior et al., 2018). The *T. gondii* secreted effector molecule, cyclophilin 18 (*Tg*Cyp18), binds to CCR5 and mimics CCR5 ligand binding (Aliberti et al., 2003), further supporting the idea that CCR5 activation favours *T. gondii* survival. However, no therapeutic or prophylactic treatments with antibodies or inhibitors targeting CCR5 or its ligands CCL3, CCL4 and CCL5 in the context of toxoplasmosis have been reported to date. Given that CCR5 receptor antagonism inhibits hepatitis C virus replication (Blackard et al., 2019) and limits the progression of gastric cancer (Aldinucci and Casagrande, 2018), further studies into its effect on toxoplasmosis are warranted.

CCL2, CCL3, CCL4 and CCL5 are produced by intestinal cells after they become exposed to tachyzoites (Gopal et al., 2011). In particular, the parasite-derived molecules GRA24 and SAG1 have been implicated in this process (Rachinel et al., 2004; Brenier-Pinchart et al., 2006; Braun et al., 2013), indicating that *T. gondii* actively manipulates chemokine secretion pathways to its advantage. The double-edged sword of leukocyte recruitment for the initiation of immunity against *T. gondii* and the parasite's requirement to induce inflammation, is also demonstrated by studies using CCR1 and CCR2 knockout mice, both of which are highly susceptible to lethal toxoplasmosis (Khan et al., 2001; Dunay et al., 2008). Chemokine and anti-chemokine therapies are actively being pursued in the field of inflammatory diseases (Castellani et al., 2007; Mohit and Rafati, 2012; Ambade et al., 2019) and cancer (Ruffini et al., 2007; Arakaki et al., 2016; Lim et al., 2016). However, similar to CCL3, CCL4 and CCL5, no targeted therapies with CCL2, CCL7, CCL23, or synthetic CCR1 or CCR2 agonists and antagonists have been reported in the context of toxoplasmosis. This area of research would benefit from further investigations.

4.4. Immunomodulatory drugs

Immunomodulatory drugs comprise different classes of compounds with direct or indirect impact on the immune system (Gao et al., 2020). The imide-containing class of immunomodulatory drugs (IMiDs), such as thalidomide and several analogues, are widely used in the treatment of autoimmune diseases and cancer (Abe and Ishida, 2019; Fuchs, 2019). To date, none of these IMiDs have been reported to have an impact on toxoplasmosis. However, there is growing interest in deciphering the role of various immunomodulatory compounds derived from chemical libraries as well as from natural products on infectious diseases, including toxoplasmosis (Sepulveda-Arias et al., 2014; Sharif et al., 2016) (Fig. 2G); for example, in one study, it was shown that treatment of mice with ursolic acid, a compound commonly found in various fruits, increased a number of critical anti-toxoplasmosis cytokines in a mouse model of toxoplasmosis (Choi and Lee, 2019). Similarly, beneficial effects on murine toxoplasmosis have been reported after treatment with lectins (Ramos et al., 2016), *Echinacea purpurea* extracts (Gasparotto Junior et al., 2016) and lactoferrin (Anand et al., 2015).

4.5. Trained immunity

In recent years, the concept of "trained immunity" has gained significant attention (Netea et al., 2016, 2020). It has been shown

Fig. 2. Summary of potential immunotherapies for the control of toxoplasmosis. (A) Isolation and transfer of polyclonal antibodies from sera of *Toxoplasma gondii* immune individuals could be used for passive immunisation. (B) Monoclonal antibody (mAb) therapy with mAbs could be used to target different antigens expressed by tachyzoites, bradyzoites and the cyst wall. (C) Isolation of T and natural killer (NK) cells from *T. gondii*-infected individuals followed by re-programming of these cells with chimeric antigen receptors (CAR) specific for *T. gondii* epitopes. Expanded chimeric antigen receptors T and natural killer cells are re-infused into infected individuals to target quiescent and actively replicating parasites. (D) The synergistic effects of IL-2, IL-7, IL-12, IL-15 and IL-18 in mediating development, differentiation, expansion, survival and interferon-γ production by T cell subsets and natural killer cells could be used to boost immunity to *T. gondii*. (E) Cytokine complexes of IL-2/anti-IL-2 and IL-15/anti-IL-15 could be used to expand interferon-γ (IFN-γ)-producing immune cells to boost immunity against acute toxoplasmosis. Expansion of regulatory T cells could be used to reduce immunopathology and morbidity. (F) Interference with the chemokine-chemokine receptor axis may be used to target excessive and imbalanced inflammation and pathology during acute toxoplasmosis. The *T. gondii* secreted effector molecules, *Tg*Cyp18, SAG1 and GRA24, either bind to CCR5 and mimic CCR5 ligand binding or induce chemokine secretion by epithelial cells, respectively. Blocking these effector molecules in addition to CCL2, 3, 4, 5, 7 and 23, as well as their receptors, CCR1, 2 and 5, may prevent excessive recruitment, influx and persistence of leukocytes to the site of infection. (G) Immunomodulatory compounds such as the imide-containing class of immunomodulatory drugs (IMiDs) and natural products such as lectins, lactoferrin and ursolic acid, which stimulate cytokines and immune cells, could be used as adjunct treatments of toxoplasmosis. (H) A further understanding of trained immunity in the context of toxoplasmosis may reveal potential therapies to specifically target epigenetic imprinting in immune cells and epithelial cells. Muramyl dipeptide and β-glucan in combination with spiramycin have shown therapeutic potential in murine models of toxoplasmosis. See Section 4 for further details.

that some live attenuated vaccines, adjuvants, microbial ligands and β-glucan have a strong effect on "training" hematopoietic stem cells and innate immune cells via epigenetic imprinting (Kaufmann et al., 2018a, 2018b). This trained immunity appears to be the reason for adaptive immunity-independent protection against a variety of viral, bacterial and parasitic pathogens (Arts et al., 2018; Dos Santos et al., 2019; Tarancon et al., 2020). To date, no study has addressed the specific role of trained immunity in treating, preventing or ameliorating *T. gondii* infection and disease, whether acute or latent. However, given the explosion of research activity in this field, it appears to be only a matter of time before the impact of trained immunity on toxoplasmosis will be investigated (Fig. 2H). In that context, it is important to note that Krahenbuhl et al. (1981) demonstrated that pre-treatment of mice with the synthetic adjuvant muramyl dipeptide afforded resistance to subsequent *T. gondii* challenge. The authors of that study concluded that the protection was independent of enhanced antibody production or macrophage activation, and must have been due to a yet to be identified mechanism. Interestingly, independently of assessing the impact on immunity, a synergistic effect of β-glucan with spiramycin in the treatment of toxoplasmosis has also been reported previously (Buyukbaba Boral et al., 2012). It has also been proposed that human *T. gondii* infection itself may have long-term effects on the phenotype and responsiveness of monocytes, implying a training effect that may also impact immunity to unrelated pathogens (Ehmen and Lüder, 2019).

5. Vaccination for the control of toxoplasmosis

Vaccination has been used extensively to control infectious diseases worldwide (Doherty et al., 2016). Due to the complexity of the life cycle of *T. gondii* and the parasite's ability to infect any warm-blood animal, the challenge to develop a vaccine to control toxoplasmosis involves consideration of: (i) which stage of the parasite life cycle and, hence, which host(s) to target; (ii) which parasite antigens to target; and (iii) the mode of delivery.

A vaccine for direct use in humans should, ideally, protect against both the acute and chronic phases of infection, needing to prevent the severe consequences of ocular toxoplasmosis, congenital toxoplasmosis caused by a primary infection in pregnant women, and the mortality caused by the reactivation of latent parasites in immunosuppressed individuals (Tenter et al., 2000; Machala et al., 2015; El Bissati et al., 2018). It is debatable whether a single vaccine could achieve all these requirements.

A vaccine targeting *T. gondii* in livestock would not only need to prevent bradyzoite cyst formation in order to minimise transmission of the parasite to humans through consumption of undercooked meat (Tenter et al., 2000) but would also need to reduce the economic loss caused by animal abortion, especially in sheep and goats (Stelzer et al., 2019), to encourage widespread use. Again, these different requirements might necessitate the identification of different targets in *T. gondii* and require different modes of delivery.

A vaccine targeting felids, the only definitive host of *T. gondii*, would be able to stop the shedding of highly infective oocysts into the environment and, consequently, reduce the incidence of infection via ingestion in both livestock and humans (Frenkel and Smith, 1982; Innes et al., 2009; Torrey and Yolken, 2013). Mathematical modelling highlights the attractiveness of the cat as a target for vaccination in order to prevent toxoplasmosis in livestock and humans (Mateus-Pinilla et al., 2002; Arenas et al., 2010; Jiang et al., 2012; Turner et al., 2013; Sykes and Rychtář, 2015; Bonačić Marinović et al., 2020) but the form and means of delivery to achieve sufficient coverage of such a vaccine arguably presents a potential obstacle to success (Bonačić Marinović et al., 2020).

5.1. Anti-Toxoplasma vaccines for intermediate hosts

5.1.1. Anti-Toxoplasma vaccines targeting humans

Virtually all individuals are at risk of acquiring toxoplasma infection at some point in their lifetime, therefore a vaccine to prevent toxoplasmosis in humans would benefit all (Tenter et al., 2000). Ideally, a *T. gondii* vaccine should target young girls before they reach fertile age and induce a protective immune response able to prevent the vertical transmission of the parasite from the mother to the unborn child. Such a vaccine would reduce the global incidence of congenital toxoplasmosis, which is estimated to be 1.5 cases per 1,000 live births (~190,000 new cases every year) (Jones et al., 2001; Torgerson and Mastroiacovo, 2013). A vaccine to prevent acute and chronic toxoplasmosis is desirable. Such a vaccine would prevent the severe illness caused by an acute infection in immunocompromised individuals, as well as protect against the life-threatening reactivation of latent bradyzoites present in tissue cysts of individuals who acquired toxoplasmosis when healthy but become immunocompromised afterwards (Pott and Castelo, 2013; Stajner et al., 2013; Wang et al., 2017).

There are no reports of *T. gondii* vaccine studies in humans. There is, however, a long list of experimental vaccines that have been tested recently in murine models, examples of which are summarised in Table 2 and Fig. 3. As is the case for any intermediate host, mice can be infected by oral administration of oocysts or cysts containing bradyzoites, mimicking natural routes of infection, or by the artificial injection of tachyzoites. Experimental models established in mice include acute and lethal *T. gondii* infection, the formation of bradyzoite tissue cysts in muscles and brain, and congenital toxoplasmosis (Subauste, 2012). Several vaccine candidates have been investigated including SAGs, rhoptry proteins (ROP), microneme proteins (MIC), GRA and other proteins secreted by tachyzoites (Fig. 3). Similarly, different formulations, adjuvants, immunisation routes and schedules have been tested, spanning killed or live attenuated parasites, subunit vaccines, recombinant proteins, DNA vaccines and live vectors to deliver *T. gondii* antigens (Fig. 3). Despite showing some promise – most candidates reporting some ability to reduce cyst formation and prolong survival of the host – there is no report of progression of a vaccine tested in mice to human clinical trials.

To date, the only commercial vaccine against *T. gondii* is based on an attenuated strain designed to prevent abortion in sheep which, due to potential safety and regulatory issues associated with a possible reversion to fully virulent parasites, is not considered suitable for administration to humans (Innes et al., 2019). However, provision of the genomes of the main *T. gondii* strains (Lorenzi et al., 2015) and transcriptomic data sets of different stages of the parasite life cycle (Behnke et al., 2014; Hehl et al., 2015; Ramakrishnan et al., 2019), combined with advances in the use of CRISPR/Cas9 genome editing strategies (Shen et al., 2017; Sidik et al., 2018; Markus et al., 2019; Young et al., 2019) that allow targeted and selectable marker-free deletion of genes, may lead to the development of optimised attenuated strains with reduced risks of reversion. Indeed, the prospects for development of a live attenuated vaccine for humans and other intermediate hosts have been enhanced by recent discoveries concerning how stage differentiation is regulated at a molecular level (Farhat et al., 2020; Waldman et al., 2020). In particular, the identification of a single transcription factor (the Myb-like transcription factor, BFD1) as a master-regulator for differentiation from tachyzoite to bradyzoite (Waldman et al., 2020) theoretically paves the way for development of a live vaccine incapable of forming cysts (Kochanowsky and Koshy, 2020). In addition, an advantage of CRISPR/Cas9 in the context of producing attenuated vaccine strains is that genes can be, effectively, knocked out without introducing foreign DNA into the parasite. This means that these strains would not neces-

Table 2
Examples of recent experimental murine vaccines against *Toxoplasma gondii*.

Live Attenuated Vaccines					
Gene deleted	*Toxoplasma* Immunising Strain	*Toxoplasma* Challenge Strain	Murine Host Strain	Immune Response	Vaccine Trial Results Summary
CDPK2[a]	PruΔcdpk2	RH, PRU	Kunming	IgG, Th1/Th2 cytokines	Protection of host against lethal challenge; partial reduction of brain cyst numbers.
LDH1 and LDH2[b,c]	PruΔldh[b]	RH[b]	BALB/c[b]		Protection of host against lethal challenge[b]
	ME49Δldh [c]	RH, ME49, VEG and a field isolate of Chinese 1[c]	ICR mice[c]	IgG, IFN-γ, IL-12	Protection of host against lethal challenge; partial reduction of brain cyst numbers[c]
Gra17 and NPT1[d]	RHΔgra17Δnpt1	RH, PRU	Kunming	IgG, IFN-γ, IL-2, IL-10 and IL-12	Protection of host against lethal challenge; partial reduction of brain cyst numbers; partial protection against congenital toxoplasmosis
Tyrosine kinase-like 1[e]	RHΔtkl1	RH, PRU	Kunming	IgG1, IgG2a, IFN-γ, IL-2	Protection of host against lethal challenge; partial reduction of brain cyst numbers; partial protection against congenital toxoplasmosis
Adenylosuccinate lyase[f]	ME49Δ ADSL	RH, ME49, VEG	ICR	IgG and IgA	Protection of host against lethal challenge
Subunit/Recombinant Vaccines					
Antigen	**Formulation/Adjuvant**	***Toxoplasma* Challenge Strain**	**Murine Host Strain**	**Immune Response**	**Vaccine Trial Results Summary**
Recombinant GRA2[g]	Monophosphorryl lipid A (MPL)	Tehran	C57BL/6	IgG, IFN-γ	Partial reduction of brain cyst numbers
Recombinant serine protease inhibitor-1 (*Tg*PI-1), ROP2 and/or GRA4[h]	Aluminium hydroxide or CpG-ODN	ME49	C3H/ HeN	IgG, Th1/Th2 cytokines	Partial reduction of brain cyst numbers
Recombinant calcium-dependent protein kinases family, *Tg*CDPK1[i]	BALB/c	RH	BALB/c	IgG, IFN-γ, IL-12, IL-10	Prolonged survival of host
Epitopes of GRA10[j]	Multiple antigenic peptide (MAP) in chitosan microspheres	RH, PRU	BALB/c and C57BL/6	IgG1, IgG2a, IFN-γ, IL-2	Protection of host against lethal challenge; partial reduction of brain cyst numbers
Epitopes of SAG1, AMA1, ROP2, and GRA4[k]	Poly lactic-co-glycolic acid (PLGA) nanoparticle + Alum	RH	BALB/c	IgG1, IgG2a, IFN-γ, IL-2	Prolonged survival of host
Apical membrane antigen 1 (AMA1)[l]	VLP containing influenza M1 protein	ME49	BALB/c	IgG, IgA	Prolonged survival of host; partial reduction of brain cyst numbers
DNA Vaccines					
Gene	**Formulation/Adjuvant**	***Toxoplasma* Challenge Strain**	**Murine Host Strain**	**Immune Response**	**Vaccine Trial Results Summary**
GRA7 and ROP2[m]		RH	BALB/c	IgG2a, IFN-y	Prolonged survival of host
T. gondii profilin (*Tg*PF)[n]	IL-15	PRU	Kunming	IgG, IgG2a, IFN-γ, IL-2, IL-4, IL-10	Partial reduction of brain cyst numbers
Perforin-like protein (PLP1) and ROP18[o]	IL-18	PRU	Kunming	IgG2a, IL-2, IL-12, IFN-γ	Prolonged survival of host; partial reduction of brain cyst numbers
ROP22[p]		RH	BALB/c	IgG1, IgG2a, IL-2, IFN-γ	Prolonged survival of host; partial reduction of brain cyst numbers
ROP21[q]		RH, PRU	BALB/c	IgG1, IgG2a, IFN-γ	Prolonged survival of host; partial reduction of brain cyst numbers
ROP5 and ROP18[r]	IL-33	RH	Kunming	IgG2, IFN-γ, IL-2, IL-12	Prolonged survival 0f host; partial reduction of brain cyst numbers
ROP 8 epitopes[s]		RH	BALB/c	IgG1, IgG2a, IFN-γ	Prolonged survival of host
Heterologous Vector Expressing *Toxoplasma* proteins					
Gene	**Vector**	***Toxoplasma* Challenge Strain**	**Murine Host Strain**	**Immune Response**	**Vaccine Trial Results Summary**
Apical membrane antigen 1 (AMA1)[t]	Adenovirus vaccine	RH	BALB/c	IFN-γ, IL-4	Increased survival of host

[a] Wang et al. (2018a).
[b] Abdelbaset et al. (2017).
[c] Xia et al. (2018).
[d] Liang et al. (2020a), Liang et al. (2020b).
[e] Wang et al. (2020a).
[f] Wang et al. (2020b).
[g] Babaie et al. (2018).
[h] Picchio et al. (2018).
[i] Huang et al. (2019).
[j] Guo et al. (2018).

[k] Roozbehani et al. (2018).
[l] Kim et al. (2020).
[m] Vazini et al. (2018).
[n] Gao et al. (2018).
[o] Chen et al. (2018).
[p] Zhang et al. (2019).
[q] Zhang et al. (2018).
[r] Zhu et al. (2020).
[s] Foroutan et al. (2020).
[t] Jia et al. (2018).

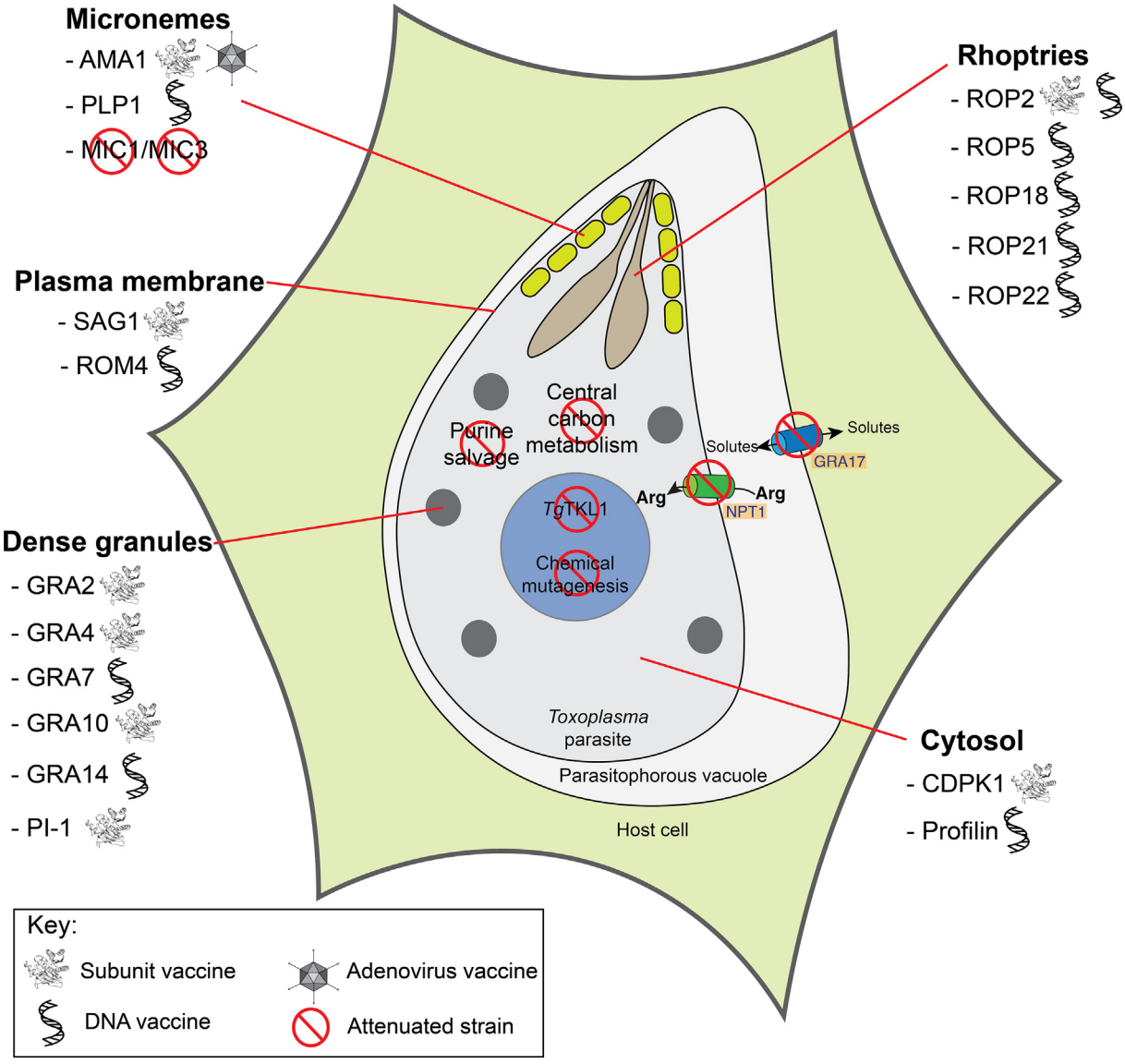


Fig. 3. Summary of some recent experimental murine vaccines against *Toxoplasma gondii* infection. Targets include proteins that function in the micronemes, rhoptries, dense granules, cytosol, nucleus, plasma membrane, or parasitophorous vacuole membrane. Vaccine strategies include the production of protein subunit vaccines, DNA vaccines, adenovirus vaccines, and attenuated parasite strains (refer to key for symbols). See Section 5 and Table 2 for further details.

sarily be classified as Genetically Modified Organisms (GMOs) in many countries (Zhang et al., 2020).

Another approach to vaccine development has been to combine bioinformatic tools with immunological approaches. Such studies have focussed on identifying peptide epitopes from proteins secreted during infection with *T. gondii* that can access the major histocompatibility complex class I pathway. These peptides can elicit the production of IFN-γ from $CD8^+$ T cells in peripheral blood mononuclear leukocytes of humans who are *T. gondii* seropositive (Cong et al., 2010, 2011). Immunisation of human leukocyte anti-

gen (HLA) transgenic mice with multi-epitope DNA or protein vaccines in combination with a universal $CD4^+$ epitope peptide and adjuvant induced high levels of IFN-γ and protected mice against challenge with type II parasites (Cong et al., 2010, 2011; El Bissati et al., 2016). Although the high polymorphism of HLA molecules in the human population may be an obstacle for epitope-based vaccines, studies have demonstrated that the specificity of peptide-binding shared by the HLA supertypes A02, A03, and B07 corresponds to ~90% of the world's population (Barber et al., 1995; Sidney et al., 1995).

5.1.2. Anti-Toxoplasma vaccines targeting livestock

The only commercial *T. gondii* vaccine (Toxovax®) was developed over three decades ago in New Zealand for control of abortion in sheep (O'Connell et al., 1988; Buxton and Innes, 1995). Toxovax® consists of attenuated tachyzoites of the S48 strain of *T. gondii*, which after over 3000 passages in mice lost its ability to develop bradyzoite tissue cysts (Buxton, 1993). This vaccine is recommended for immunisation of ewes as a single intramuscular injection at least 4 weeks before mating. Following immunisation, the attenuated parasites undergo limited replication cycles, mimicking a natural infection, and this is able to induce long-lasting protection, likely involving $CD4^+$ and $CD8^+$ cell responses along with IFN-γ production (Buxton et al., 1994). Toxovax® is commercialised through MSD Animal Health and is available in the UK, Ireland, France and New Zealand.

Vaccination with the S48 strain of *T. gondii* impairs the formation of bradyzoite tissue cysts in animals subsequently treated with wild type parasites; immunisation of lambs with the S48 strain reduced the presence of parasites detected by PCR in the heart (by 75%) and skeletal muscles (by 82%) of lambs challenged with wild type oocysts (Katzer et al., 2014). Similar results were observed in pigs immunised with the S48 strain before challenge with wild type oocysts (Burrells et al., 2015). A bioassay showed increased survival (48.5%) of mice that received tissue samples from vaccinated pigs compared with mice that received porcine tissue samples from the control group (Burrells et al., 2015). Although S48 vaccination did not completely prevent tissue cyst formation following challenge in lambs and pigs, these studies indicate that a vaccine able to reduce the cyst burden in livestock may reduce the risk of infection of humans through consumption of undercooked meat (Katzer et al., 2014; Burrells et al., 2015).

Since the approval of Toxovax®, other live attenuated and inactivated parasites have been investigated in order to develop an improved vaccine to prevent sheep abortion and protect against tissue cyst development. Immunisation of ewes with tachyzoites of a strain of *T. gondii,* wherein MIC1 and MIC3 were knocked out (MIC1-3KO) 2 months before mating, induced humoral responses and reduced the abortion of lambs in ~72% of ewes after a challenge with wild type oocysts mid-gestation (Mevelec et al., 2010). However, MIC1-3KO tachyzoites were reported to persist as tissue cysts in immunised animals (Mevelec et al., 2010). A temperature-sensitive mutant (TS-4) of the RH isolate of *T. gondii* (Pfefferkorn and Pfefferkorn, 1976), which does not persist as tissue cysts in the tissues of mice (Waldeland et al., 1983), was evaluated for its ability to prevent tissue cysts in pigs (Lindsay et al., 1993). A bioassay showed that immunisation of pigs with TS-4 tachyzoites by subcutaneous or intravenous routes did not protect against bradyzoite cyst formation in the tissues of pigs challenged with wild type oocysts (Lindsay et al., 1993; Dubey et al., 1994). Similarly, oral administration of oocysts inactivated by ^{137}Cs gamma irradiation (0.3 or 0.4 kGy) did not prevent bradyzoite tissue cyst formation in pigs challenged with live oocysts (Dubey et al., 1998).

Extracts or purified fractions of *T. gondii* parasites in combination with adjuvants have been investigated as subunit vaccines. Microspheres containing proteins from a crude extract of RH strain tachyzoites encapsulated into poly(D,L-lactide-co-glycolide) were investigated as an intranasal vaccine for sheep with and without cholera holotoxin (Stanley et al., 2004). Although both systemic and mucosal humoral responses, together with cell-mediated immunity, were observed, immunisation with microspheres containing *T. gondii* proteins failed to protect sheep against clinical signs of infection upon challenge with oocysts of the M3 strain of *T. gondii*. Western blot analysis demonstrated that the main antibody elicited after immunisation was reactive against a protein of approximately 30 kDa, likely to be the major surface antigen of the parasite, SAG1 (Stanley et al., 2004). This suggests that SAG1 antibodies are not sufficient to protect against infection. In pigs, subcutaneous immunisation with two doses of crude rhoptry proteins formulated as immunostimulating complexes (ISCOMs), induced a systemic IgG response. Following challenge with oocysts, the animals immunised with rhoptry proteins showed the same clinical signs of infection as control pigs (including fever, secretions from the eye, coughing and loss of appetite) and only 20% of vaccinated pigs did not develop tissue cysts as indicated by a bioassay in mice (Garcia et al., 2005). Similarly, a further investigation showed that crude ROPs administered by the intranasal route in combination with the adjuvant, Quil A, did not induce strong humoral or cellular immune responses prior to challenge infection, and only one of four pigs tested negative for *T. gondii* by bioassays post-challenge (da Cunha et al., 2012). In contrast, another study showed that immunisation of pigs with a mixture of tachyzoite excretory/secretory antigens induced humoral and cellular immune responses, including IFN-γ and IL-4, and prevented the development of tissue cysts in four out of five pigs following challenge with tachyzoites injected intraperitoneally (Wang et al., 2013). However, the small number of animals tested and the unnatural route of challenge infection combined with the use FCA, a powerful inducer of cell-mediated immune responses that is not used commercially due to safety issues, restricts evaluation of this study's practical implications.

DNA vaccines encoding several *T. gondii* antigens have been investigated for their ability to induce an immunological response in sheep and pigs. Immunisation of sheep with a DNA vaccine encoding the bradyzoite-specific antigen, MAG1, alone or co-expressed with the cytokine, IL-6, induced a specific humoral response (Hiszczynska-Sawicka et al., 2010). Comparison of the immune response in sheep immunised with a DNA vaccine encoding SAG1 or ROP1 combined with unmethylated cytosine-phosphate-guanosine oligodeoxynucleotide motifs showed that ROP1-encoding DNA, but not SAG1-encoding DNA, was able to induce humoral and IFN-γ responses (Li et al., 2010). Similarly, plasmids encoding for ROP1 in fusion with CD154, a co-stimulatory molecule expressed by activated T-cells, induced a mixed Th1/Th2 response in sheep, with higher IFN-γ levels than was induced by plasmids encoding ROP1 protein only (Hiszczynska-Sawicka et al., 2011a). DNA vaccines encoding GRA1, GRA4, GRA6 or GRA7 showed that GRA7 can stimulate the ovine immune system and was able to induce IFN-γ and IgG2 responses (Hiszczynska-Sawicka et al., 2011b). In pigs, immunisation with a DNA vaccine encoding for GRA1 and GRA7 induced antibodies and cellular immune responses to both proteins (Jongert et al., 2008). Unfortunately, none of the DNA vaccines described above have ever been tested for efficacy against *T. gondii* challenge.

5.2. Anti-Toxoplasma vaccines for the definitive host

Felids are the only definitive host of *T. gondii*. After a primary infection with *T. gondii*, cats may shed millions of oocysts that can persist in soil and water as sources of infection for many

months/years (Ferguson, 2009). Upon reinfection, few or, more usually, no oocysts are observed in the faeces of challenged cats indicating that, even after a single infection, cats develop an immune response that is able to prevent repeated oocyst shedding (Dubey, 1995). Moreover, there is evidence that this immunity cross-protects against heterologous strains (Freyre et al., 2007). Thus, although the nature of this immune response is not known, there are grounds to believe that a vaccine that is able to block oocyst shedding by cats would reduce the number of oocysts in the environment and, consequently, reduce the prevalence of toxoplasmosis, providing that issues related to vaccine coverage of pets and stray or feral cats could be resolved.

Attempts to develop recombinant and subunit vaccines to prevent the shedding of oocysts by cats have been few and have shown only limited promise. A recombinant feline herpesvirus type 1 (FHV1) expressing the *T. gondii* ROP2 protein reduced the bradyzoite cyst burden in the brains of cats but failed to prevent oocyst shedding after challenge with bradyzoite-containing cysts of the Beverley strain (Mishima et al., 2002). Crude rhoptry proteins purified from tachyzoites were investigated as a vaccine candidate in combination with the adjuvant Quil A; administration via the intranasal or rectal routes followed by oral challenge with bradyzoite cysts of ME49 strain showed some ability to prevent oocyst shedding by cats compared with a control group receiving BSA and Quil A (Zulpo et al., 2012). Similarly, immunisation of cats with recombinant ROP2 plus Quil A via the nasal route reduced the total number of oocysts in only 23.9% of cats compared with the control group receiving BSA and the adjuvant (Zulpo et al., 2017).

In contrast, immunisation of cats by infection with live attenuated strains of *T. gondii* has proven highly effective at preventing oocyst shedding. The T-263 strain of *T. gondii*, which is a chemically-induced mutant that develops through to micro- and macrogametes but lacks the ability to produce oocysts (Dubey, 2017), was the first reported live attenuated vaccine investigated. Oral administration of two doses of T-263 tissue cysts or bradyzoites released by pepsin digestion induced antibody responses and prevented oocyst shedding in 84% (Frenkel et al., 1991) or 100% (Freyre et al., 1993) of kittens after challenge with an oocyst producing strain. The efficacy of the live attenuated T-263 strain as a transmission-blocking vaccine was also investigated in a long-term field trial in swine farms in the U.S.A. (Mateus-Pinilla et al., 1999). Cats present on these swine farms were caught and immunised by infection with bradyzoites of the T-263 strain. Immunisation of cats significantly reduced *T. gondii* infection in finishing pigs on these farms across time (Mateus-Pinilla et al., 1999). Despite the promising results achieved with the T-263 strain of *T. gondii*, difficulties associated with large-scale production of bradyzoites, cold chain transportation, and perceived risks to people handling and administering the vaccine inhibited its commercialisation (Choromanski et al., 1995).

Given the manner of its derivation, the T-263 strain of *T. gondii* is likely to possess multiple mutations. However, apart from some documentation ~40 years ago of enzymatic perturbations (Pfefferkorn, 1978; Pfefferkorn and Kasper, 1983), these mutations have not been defined. The advent of next-generation sequencing approaches may facilitate a better understanding of this strain in future. Other studies on live vaccines indicate that stage-specific antigens and administration routes may play an important role in the protective immune response against the enteric stages of *T. gondii*. For example, immunisation by oral or duodenal administration of T-263 tachyzoites failed to prevent oocyst shedding upon subsequent challenge of cats (Freyre et al., 1993). Similarly, immunisation of cats by the intramuscular injection of ^{60}Co-irradiated tachyzoites of the Beverley strain prevented oocyst shedding in only 43% of the immunised cats following challenge with homologous bradyzoites, and immunisation with ^{60}Co-irradiated tachyzoites of the RH strain failed to prevent oocyst shedding upon challenge with bradyzoites of the heterologous Beverley strain (Omata et al., 1996). Recently, the aforementioned MIC1-3KO strain was investigated as a live attenuated vaccine for cats. Although promising results were reported in mice and sheep, immunisation of cats with tachyzoites of the MIC1-3KO strain by either the subcutaneous or oral route did not protect against oocyst shedding (Le Roux et al., 2020).

There is reason to hope that targeted attenuated vaccines are a realistic proposition. RNA-sequencing data and analyses of cat enteric stages of *T. gondii* (Behnke et al., 2014; Hehl et al., 2015; Ramakrishnan et al., 2019) have provided valuable insights into genes expressed specifically in merozoites, microgametes and macrogametes with presumptive roles in sexual reproduction and oocyst formation. These studies bring with them the opportunity to design vaccines that specifically target the feline enteric stages of *T. gondii*. Indeed, to establish proof-of-principle, a CRISPR/Cas9 gene-editing strategy was used to engineer a *T. gondii* strain lacking the expression of a putative microgamete-specific fertilisation factor, HAP2 (Ramakrishnan et al., 2019). Infection of rats with HAP2-deficient tachyzoites led to the establishment of chronic infection and tissue cyst development. Primary infection of cats with bradyzoite-containing cysts from these rats demonstrated that HAP2-deficient *T. gondii* parasites were unable to complete fertilisation and, as a result, very few oocysts were produced and those that were had an aberrant morphology and could not undergo meiosis to form infectious sporozoites within oocysts. Moreover, all cats previously immunised by infection with the HAP2-deficient *T. gondii* parasites shed no oocysts upon challenge with bradyzoite-containing cysts from the wild-type parental strain (Ramakrishnan et al., 2019). An impediment to application of a targeted attenuated vaccine to block transmission of *T. gondii* oocysts is, as it was for the T-263 mutant strain, the provision of bradyzoites for immunising infection of kittens. However, the recent identification of a master switch for the conversion of tachyzoites to bradyzoites (Waldman et al., 2020) significantly improves the prospects for development of an in vitro "cell factory" system for the mass production of bradyzoites.

6. Nanotechnology for the control of toxoplasmosis

The use of nanomaterials in medicine has been termed nanomedicine (Soares et al., 2018) and, increasingly, nanoparticles are being assessed for inclusion in a range of diagnostic, therapeutic and preventative applications. Nanomaterials can be defined as organic or inorganic, crystalline or amorphous particles in the range of tens to hundreds of nanometres in size (Assolini et al., 2017; Soares et al., 2018). They can be organised as single particles, aggregates, powders, or dispersed in a matrix to form suspensions, emulsions or nanolayer films (Soares et al., 2018). A consequence of their size is a very large surface area to volume ratio, making them much more reactive than larger particles. This gives them a tendency to adsorb biomolecules when in contact with biological fluids so that a layer known as the corona forms on the surface of colloidal nanoparticles. Their size also allows them to penetrate cells and interact with intracellular molecules. Their biological interactions are also influenced by shape, chemical composition, surface charge and roughness, allowing them to be tailor-made for specific targets (Soares et al., 2018). Their diversity makes them very versatile and nanoparticles have been used to improve diagnostic assays, as a new way of delivering chemotherapy or vaccine formulations, or as therapeutic agents and adjuvants themselves.

6.1. Nanotechnology in the diagnosis of T. gondii infection

Diagnosis of acute infections with *T. gondii* is usually through the detection of anti-*T. gondii* IgM, IgA, IgE or IgG antibodies in the serum of patients by ELISA, indirect haemagglutination tests or immunosorbent agglutination tests (Ybañez et al., 2020). An increase in IgM, IgA and IgE is considered to be characteristic of an acute infection, whereas detection of only IgG is characteristic of a chronic or previous infection (Ybañez et al., 2020). A major drawback to these methods is low sensitivity, which results in false negatives. Accurate diagnosis can also be complicated by the presence of immunosuppressed patients who produce very low levels of IgM or patients who can produce IgM for up to a year after initial infection (Ybañez et al., 2020). A number of studies have aimed to increase the sensitivity of the assays by the inclusion of nanoparticles due to increased surface area available for adsorption of reagents (Medawar-Aguilar et al., 2019). Assays that have been developed include a microfluidic assay using zinc oxide nanoparticles coated with chitosan (Medawar-Aguilar et al., 2019) and an electrochemical immunosensor consisting of graphene sheets, goldmag (Au-Fe_3O_4) nanoparticles and thionine (Jiang et al., 2013). Both of these assays used *T. gondii* lysate conjugated to the nanoparticles to capture anti-*T. gondii* antibodies in serum samples and demonstrated increased sensitivity compared with conventional sandwich ELISAs. Li et al. (2015) used goldmag nanoparticles modified with polymethacrylic acid conjugated with anti-human IgM antibodies in a lateral flow immunochromatographic assay (LFIA) to test for the TORCH pathogens (*Toxoplasma*, rubella virus, cytomegalovirus and herpes simplex virus) in pregnant women (Li et al., 2015). This assay also displayed increased sensitivity and specificity compared with commercially available LFIA assays. TORCH pathogens were also targeted in the development of a microarray using quantum dots (photoluminescent nanomaterials) coated with antigens to detect infections in serological samples (Yang et al., 2009). This assay had comparable sensitivity and specificity to conventional ELISA but had the advantage of a shorter run time and a more stable signal (Yang et al., 2009).

Toxoplasmosis can also be diagnosed through direct detection of *T. gondii*. This is particularly useful in immunocompromised patients where a serological response is reduced and there is the risk of false negative results. These tests can be developed to detect either DNA or antigens of the parasite. As with assays to detect antibodies, nanoparticles were used to increase the sensitivity of diagnostic assays that detect parasite antigen. Aly et al. (2018) used silica particles coated with polyclonal antibodies to *T. gondii* lysate to detect circulating parasite antigen in serological and urine samples. Sensitivity was increased from 85.7% to 90% for serum samples and 78.6% to 82.6% in urine samples (Aly et al., 2018). Hegazy et al. (2015) used immunomagnetic beads coated with antibodies to SAG1 to detect parasite antigens in serological samples, increasing sensitivity from 92% to 98%. Xu et al. (2013) developed a probe using nickel-magnetic nanoparticles and fluorescence resonance energy transfer to detect *T. gondii* DNA, although it should be noted that the probe was not validated using patient samples. It is unlikely that sensitivity of this probe would exceed that of the real-time PCR assay developed by Lin et al. (2000), limiting its usefulness.

In addition to increased sensitivity and specificity of diagnostic tests incorporating nanoparticles, the speed, cost and convenience were also often improved compared with existing tests. These improvements have led to a more accurate diagnosis and faster implementation of appropriate treatments for patients identified as suffering from toxoplasmosis.

6.2. Nanotechnology in the treatment of T. gondii infection

As discussed above, the most commonly used drugs for human toxoplasmosis are sulfadiazine and pyrimethamine, both of which can cause serious side effects such as bone marrow suppression, allergy and renal and hepatic complications (Gaafar et al., 2014). Other drugs, including a range of antibiotics and anti-malarials, have also been used but they can also have serious side effects (Abou-El-Naga et al., 2017). The unique physicochemical properties of nanoparticles can be harnessed to improve delivery of drugs by altering their pharmacokinetics. This can lead to a slower drug delivery, better target specificity, increased efficacy and a reduction in side effects (Anand et al., 2015). Drugs that are toxic, or have poor solubility, or are easily degraded in the gastrointestinal tract, can be delivered into the body for more effective treatment at lower doses using nanotechnology-based approaches. Nanoparticles have been evaluated both as a way of delivering current anti-toxoplasmosis treatments more effectively and as antimicrobial agents themselves (Pissuwan et al., 2009).

Chitosan is a natural polysaccharide that has been shown to have anti-bacterial and anti-malarial effects, and has been investigated for anti-*Toxoplasma* effects (Teimouri et al., 2018). Low molecular weight (86 kDa), medium molecular weight (234 kDa) and high molecular weight (353 kDa) nanoparticles were synthesised and tested against RH strain *T. gondii* in vitro and in vivo. Anti-*T. gondii* activity was demonstrated in vitro with all nanoparticle sizes but killing of exposed tachyzoites was fastest with low molecular weight nanoparticles. Smaller nanoparticles also performed best in an in vivo model, resulting in a significant drop in parasite burden compared with infected, untreated mice; however, they were not as effective as treatment with sulfadiazine (Teimouri et al., 2018). Addition of anti-*T. gondii* drugs to chitosan nanoparticles did, however, increase effectiveness of parasite killing compared with chitosan or the drug alone. Spiramycin is an antibiotic that is used to treat toxoplasmosis in pregnant women but is limited in its effectiveness by low bioavailability and an inability to cross the blood–brain barrier. Loading of spiramycin into chitosan nanoparticles increased its absorption and permeation, increasing survival time and reducing parasite burden in mice compared with spiramycin or chitosan nanoparticles alone. The inflammatory response to infection was lessened in the treated animals and the parasites that were isolated showed morphological deformities, indicating a direct effect of the spiramycin-chitosan nanoparticles on the parasites themselves (Etewa et al., 2018; Hagras et al., 2019). Similarly, Abou-El-Naga et al. (2017) found that administration of the anti-retroviral lopinvar/ritonavir combination encapsulated in polylactic-*co*-glycolic acid (PLGA) nanoparticles to mice infected with *T. gondii* reduced parasite burden significantly compared with administration as free drug. Infectivity of tachyzoites exposed to lopinvar/ritonavir in PLGA nanoparticles was also reduced significantly compared with tachyzoites from mice treated with free drug, although mortality rates in the two groups were not significantly different (Abou-El-Naga et al., 2017).

Mixed results have been seen with other potential anti-*Toxoplasma* drugs loaded into nanoparticles, with effects seemingly dependent on the pharmacokinetics of individual drugs. Triclosan, an inhibitor of fatty acid synthesis, is a drug with promising activity against protozoan parasites in vitro, but its poor solubility has limited its use in vivo. Loading triclosan into nanoparticles comprised of lipids (liposomes) significantly reduced parasite burden in mice infected with a virulent strain of *T. gondii* compared with triclosan alone (El-Zawawy et al., 2015a). Surprisingly, similar experiments using an avirulent strain of *T. gondii* found no

difference in parasite burden after treatment with triclosan in liposomes compared with triclosan alone, although both groups had significant reductions in parasite burden compared with infected, untreated mice (El-Zawawy et al., 2015b). This may indicate a *T. gondii* strain effect on the mode of action of the drug, although different doses of triclosan and triclosan/liposomes were used in the studies, complicating comparisons. Bottari et al., (2015) compared brain cyst burdens in mice infected with *T. gondii* then treated with sulfamethoxazole/trimethoprim and reseveratrol packaged into nanoparticles or as free drug. They found a significant reduction in cyst burden in mice treated with sulfamethoxazole/trimethoprim and resveratrol compared with mice treated with sulfamethoxazole/trimethoprim only, but there was no significant difference between the two delivery methods of reseveratrol (Bottari et al., 2015).

Chitosan nanoparticles have also been combined with silver nanoparticles to increase their efficacy (Gaafar et al., 2014). Mice infected with *T. gondii* were treated with pyrimethamine, chitosan nanoparticles, silver nanoparticles, or a combination of chitosan and silver nanoparticles. Parasite burden was significantly reduced after treatment with silver nanoparticles or the combination of silver and chitosan nanoparticles, pyrimethamine or chitosan particles. However, there was no significant difference between silver nanoparticles and the combination of silver and chitosan nanoparticles, suggesting it is the silver nanoparticles that had the anti-*T. gondii* effect (Gaafar et al., 2014). This finding is supported by in vitro studies with gold, silver and platinum nanoparticles by Adeyemi et al. (2017) who found tachyzoite viability was reduced by 90% in the presence of the metallic nanoparticles. The selectivity index was $\geq$20, indicating marked toxicity towards the parasite without affecting the host cells. Silver and gold nanoparticles were more effective than platinum at killing the parasites (Adeyemi et al., 2017). In a subsequent study, *T. gondii* parasites exposed to metallic nanoparticles were impaired in converting from tachyzoite to bradyzoite in vitro (Adeyemi et al., 2019), suggesting that metallic nanoparticles target both tachyzoites and bradyzoites. Killing of parasites was linked to the production of reactive oxygen species stimulated by the metal nanoparticles (Adeyemi et al., 2017, 2019). A further study using combinations of the metals confirmed their *anti-T. gondii* activity but noted some potential safety concerns since the selectivity index of effect on parasite versus host cells was $\leq$ 2-fold (Adeyemi et al., 2018b).

Recently, plant extracts have been used as stabilising or reducing agents in the preparation of so-called "biogenic" nanoparticles. This manufacturing method has the advantages of being more cost effective, producing less toxic waste, and generating more reactive nanoparticles. Coating the particles with plant molecules also increases their biocompatibility (Sharma et al., 2019). Alajmi et al. (2019) used extracts of date seeds and nabka leaves to produce biogenic silver nanoparticles and examined their effect on survival rate and hepatic damage in mice infected with *T. gondii*. Mice treated with the biogenic nanoparticles or sulfadiazine survived for longer than infected, untreated mice. Mice given the biogenic silver particles showed less signs of hepatic damage due to the infection but the mortality rate was higher than in mice given sulfadiazine (Alajmi et al., 2019). This treatment may have been more effective if a less virulent strain of *T. gondii* had been used for the experiments. Machado et al. (2020) created biogenic silver nanoparticles with the fungus, *Fusarium oxysporum*, and assessed their effect on *T. gondii* in vitro. Treatment with the nanoparticles significantly reduced the ability of parasites to invade and proliferate inside HeLa cells (Machado et al., 2020). These results were consistent with those obtained by Gaafar et al. (2014) and Adeyemi et al. (2017), although how the silver nanoparticles are damaging the parasite remains to be determined. Biogenic selenium nanoparticles created with *Bacillus* were also found to be effective against *T. gondii* in chronically infected mice (Keyhani et al., 2020). A significant reduction in brain cysts was seen in chronically infected mice treated for 14 days with the selenium nanoparticles compared with untreated mice, although complete elimination was not achieved. No toxicity was observed in the mice although mRNA levels of inflammatory cytokines were raised, and the authors speculated that the reason for the decrease in brain cysts was due to induction of an inflammatory response by selenium (Keyhani et al., 2020). Another study by the same group showed that biogenic selenium nanoparticles administered prior to infection could significantly reduce parasite burden in a model of acute toxoplasmosis (Shakibaie et al., 2020).

Toxicity can be an issue with metal nanoparticles as they are not biodegradable like other types, such as chitosan or liposomes, and can accumulate in organs. However, the nanoparticles tested to date display toxicity towards the parasite and, apart from one study (Adeyemi et al., 2018b), show little toxicity towards host cells in vitro or in vivo. The alterations that packaging drugs in nanoparticles makes to the pharmacokinetics of those drugs may lead to lower doses being effective. This, in turn, gives hope that this approach will lead to more effective treatments with fewer side effects for patients. However, it does need to be acknowledged that nanoparticles can compromise pregnancy (Park et al., 2013; Elsharawy et al., 2020) and this will potentially limit their utility in control of congenital toxoplasmosis.

6.3. Nanotechnology in vaccines against T. gondii infection

Immunogenicity is an issue with subunit and DNA vaccines. The plasmids and antigens that make up these vaccines are prone to enzymatic degradation and can be poorly taken up by the relevant cells so that only a weak immune response is stimulated (Maeta et al., 2020). Nanoparticles can act as adjuvants and have been shown to increase immunogenicity of a number of *T. gondii* antigens that have potential as vaccine candidates including ROP18 (Nabi et al., 2017) and SAG1 (Naeem et al., 2018). Peptides corresponding to epitopes of key antigens (SAG1, AMA1, ROP2 and GRA4) have been encapsulated in nanoparticles and tested for immunogenicity (Roozbehani et al., 2018). Unlike commonly used adjuvants, such as alum, nanoparticles appear able to induce cellular and humoral responses that are more likely to produce a protective response. Another benefit of encapsulating subunit vaccines in nanoparticles is that encapsulation can guard against degradation. In addition to this, nanoparticles can be targeted for delivery to certain cells by the adsorption of antibodies on their surfaces, and lower amounts of DNA and protein can be used in the formulation, reducing the risk of toxicity and side effects (Wang et al., 2019).

The properties of nanoparticles allow efficient delivery of vaccine candidates via routes other than subcutaneous or intramuscular. Dimier-Poisson et al. (2015) used nasal delivery to immunise mice with a total extract *T. gondii* antigens loaded onto nanoparticles made of maltodextrin and challenged mice with lethal and chronic doses of *T. gondii*. All mice immunised with the antigen/-nanoparticle combination survived a lethal challenge infection and had reduced cyst burdens in the chronic model compared with mice immunised with antigen alone. Ducournau et al. (2017) found the same formulation could also reduce vertical transmission in immunised mice infected during pregnancy.

DNA vaccines can stimulate a cellular response that is most effective for intracellular pathogens (Maeta et al., 2020) and nanoparticles have been shown to increase their effectiveness. Issues affecting the success of DNA vaccines include degradation of the DNA by extracellular nucleases, poor uptake by cells, and inability to escape the endosome once inside the cell (Maeta et al., 2020). Nanoparticles protect DNA from degradation during

delivery and are more easily taken up by macrophages. Addition of calcium phosphate nanoparticles to DNA vaccine formulations based on GRA14 and ROM4 have been shown to increase their immunogenicity in mice and prolong survival against a challenge with *T. gondii* RH (Ahmadpour et al., 2017; Rahimi et al., 2017; Pagheh et al., 2019). Maeta et al. (2020) were also able to demonstrate significant protection against *T. gondii* in mice immunised with DNA encoding *T. gondii* profilin encapsulated in liposomal nanocarriers.

A risk with DNA vaccines is their potential integration into the host genome. To overcome this possibility, a modified dendrimer nanoparticle that encapsulated RNA encoding for six *T. gondii* antigens (GRA6, ROP2A, ROP18, SAG1, SAG2A and AMA1) was developed. This vaccine was able to stimulate a cellular adaptive immune response and protect mice against a lethal challenge with *T. gondii* (Chahal et al., 2016). Luo et al. (2017) also encased RNA coding for *T. gondii* NTPase II in synthetic lipid nanoparticles and found a significantly increased cellular and humoral response in mice immunised with the RNA in lipid nanoparticles compared with the RNA alone. Mice immunised with the nanoparticles had prolonged survival rates when challenged with *T. gondii* RH although there was no significant advantage conferred by the nanoparticles when mice were challenged with *T. gondii* Pru. Cyst burden was reduced compared with untreated, infected mice but was not significantly different from mice immunised with only the RNA (Luo et al., 2017).

As was noted for diagnostic tests and administration of therapeutic drugs, nanoparticles have the potential to increase the effectiveness of vaccine formulations and, therefore, should be considered for inclusion in any development of vaccines to target toxoplasmosis.

7. Concluding remarks

Toxoplasma gondii has been described, with justification, as "the world's most successful parasite" and "the model apicomplexan" (Weiss and Kim, 2020). It is a parasite that can seemingly infect any nucleated cell in any warm-blooded animal, has spread across the Earth from origins in South America, adapting to human agricultural development in the process, and causes only minor, if any, symptoms in the majority of its hosts. However, the sheer enormity of the numbers of people and animals infected with this parasite make it one of the most globally significant zoonotic diseases. On health, economic and ecological grounds, the control of toxoplasmosis is therefore a necessity, not a luxury.

The success of *T. gondii* is underpinned by an exquisitely complex life cycle, which means that the manifestations of toxoplasmosis are also complex. Public education is, therefore, critically important, notwithstanding doubts about its effectiveness. It is particularly important for pregnant women and immunocompromised people but it must be clever, informative, detailed and practical enough to enable clear communication between medical professionals and their patients, and to encourage adherence to measures and habits that will reduce exposure to oocysts and cysts of *T. gondii* for those most at risk. Additionally, freely available accurate and practical information about toxoplasmosis is useful for both medical professionals and the general public. There is some evidence that this can have an effect since education is considered at least partly responsible for an apparent reduction, over recent decades, in prevalence of infection with *T. gondii* in, e.g., the U.S.A. (Jones et al., 2007, 2014), the Netherlands (Hofhuis et al., 2011), France (Nogareda et al., 2014) and Germany (Wilking et al., 2016), particularly through improved understanding of food and water hygiene.

Protection of food and water from contamination with *T. gondii* is a requisite for control of human toxoplasmosis. Biosecurity measures to minimise the presence of cats and rodents on farms is important, particularly since post-harvest detection and removal of the parasite in or on food is not straightforward. Availability of water that is oocyst-free is absolutely essential, not just for human consumption but also for raising livestock and producing crops. By 2020, access to clean water should be a right, not a privilege but, sadly, this is not the case and it is no coincidence that the significant incidence of toxoplasmosis traceable to oocyst-derived infection is often linked to socio-economic status (Bahia-Oliveira et al., 2003; Carellos et al., 2014; Singh et al., 2014; Mareze et al., 2019; Shapiro et al., 2019). Thus, a genuine global effort to address poverty and ensure provision of clean water for all people would go a long way to reducing the incidence of toxoplasmosis.

Timely treatment following diagnosis of infection with *T. gondii* is important, particularly for pregnant women and immunosuppressed people – it saves lives even if current drugs do not possess all the characteristics and efficacy of the ideal therapy. The prospects for new, better targeted drugs are real, fueled by insights into the parasite's genome, transcriptome and biochemistry. Additionally, nanotechnological advances have the potential to improve delivery of existing and future chemotherapeutic agents. Similarly, new insights into the immunobiology of toxoplasmosis are paving the way for the development of immunotherapeutics, particularly to prevent toxoplasmic encephalitis resulting from immunosuppression.

Live attenuated vaccines have proven to be effective in both intermediate and definitive hosts of *T. gondii* but subunit vaccines have not. This probably tells us something about the importance of cell-mediated immunity in both intermediate and definitive hosts (Innes et al., 2019), and perhaps also exposes our current lack of understanding and options for antigen presentation in the delivery of subunit vaccines; nanotechnology may help address this. In the meantime, recent research has utilised the availability of robust data sets for the genome and transcriptomes of *T. gondii*, combined with the gene editing power of CRISPR/Cas9 protocols to demonstrate that: (i) single transcription factors largely control the switch to sexual stage development (Farhat et al., 2020) and bradyzoite development (Waldman et al., 2020) in *T. gondii*; and (ii) kittens can be rendered totally incapable of transmitting viable oocysts following immunisation with a parasite strain gene-edited to prevent expression of a parasite fertilisation factor (Ramakrishnan et al., 2019). The discovery of a master-regulator of conversion of tachyzoites to bradyzoites opens the door to development of an attenuated vaccine for intermediate hosts that will have an inbuilt safeguard of not being able to progress into a long-lived tissue cyst. This is particularly attractive since cultivation of tachyzoites in vitro is not difficult and may well be amenable to commercial scale-up. This same discovery also paves the way for potential scale-up of in vitro production of bradyzoites, which would enable two things: first, the development of high-throughput approaches for the discovery and testing of drugs to treat chronic infection with *T. gondii,* treatments for which we lack currently; and second, an in vitro mass production system for bradyzoites, which could overcome one of the main obstacles to the implementation of a transmission-blocking vaccine program for cats using attenuated parasites. A vaccine to stop transmission of oocysts of *T. gondii* is attractive because it targets the core of the life cycle of *T. gondii* – without sexual reproduction and oocysts, all routes of infection with this parasite ultimately cease to exist. Completely preventing transmission of oocysts worldwide is, seemingly, impossible as vaccine coverage of all pet, community, stray and wild cats is a challenge that would necessitate delivery technologies and regimens we have not yet imagined. But, similar

to the provision of clean water for all, it is surely an ambition worth our effort.

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