A Comprehensive Review of Toxoplasma Gondii Biology and Host-Cell Interaction: Challenges for a Plant-Based Vaccine

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Abstract Toxoplasmosis is a worldwide-distributed infection caused by *Toxoplasma gondii*, which causes a wide range of clinical syndromes in humans, mammals and birds. *T. gondii* is considered a parasite of veterinary and medical importance, because it may cause abortion or congenital disease in its intermediate hosts. Despite the economic losses associated with *T. gondii* infection in farm animals and the socio-economic impact caused by this zoonotic disease in the human population, there is no effective treatment available for humans or animals able to eliminate the parasite from the host once the chronic infection has been established. The only commercial vaccine is the S48 strain of attenuated tachyzoites for use in sheep. However, this vaccine causes side effects, has a short life time and induces a short-term immunity. So far, no acellular vaccine against toxoplasmosis has been commercialized. In fact, future challenges include the development of an effective vaccine to prevent toxoplasmosis. Most parasitologists and vaccinologists agree that future efforts should be concentrated on developing multi-antigen vaccines and more efficient delivery systems able to express heterologous proteins abundantly as well as on searching for immunization schedules and adequate adjuvants to enhance the protective responses. To achieve this, platforms for the production of acellular vaccines based on the use of plants can have an important role.

Keywords Plant vaccine · Toxoplasmosis · Immune response
SAG1 · GRA4

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1 Toxoplasma Gondii

1.1 General Concepts

Toxoplasma gondii is an intracellular protozoan parasite member of the phylum Apicomplexa. It was found for the first time by Nicolle and Manceaux (1908) in the blood, spleen and liver of the rodent *Ctenodactylus gondii* and named with its definitive species designation in 1909. *T. gondii* is the sole etiological agent of toxoplasmosis and has been characterized as the most successful infecting parasite, since it can infect any warm-blooded animal, including livestock and humans (Dubey 2008). Toxoplasmosis causes a wide range of clinical syndromes in humans, mammals and birds. Since this infection has a worldwide distribution, virtually the whole population can be exposed to it, and it has been estimated that one third of the world's human population is seropositive (Miller et al. 2009). However, the seroprevalence and clinical severity of this disease vary according to age and is unevenly distributed across geographical areas and different socioeconomic strata of a specific population (Rosso et al. 2008); with South America exhibiting the highest burden (Bertranpetit et al. 2017).

1.2 Life Cycle and Infectious Stages

The life cycle of *T. gondii* can be divided into sexual replication, which takes place only in felids, such as the domestic cat (definitive hosts), and asexual replication, which occurs in other mammals and birds (intermediate hosts) (Dubey 1996). In nature, the parasite circulates primarily in three infectious stages: (i) the tachyzoite, a rapidly multiplying form which morphologically resembles a half-moon, (ii) the bradyzoite, a quiescent form with the ability to form tissue cysts, and (iii) the sporozoite, which is formed inside oocysts, and is shed in feces by the definitive host.

The sexual cycle occurs when the definitive host ingests environmental (water or soil) oocysts or meat contaminated with tissue cysts. After sexual reproduction, unsporulated oocysts, containing non-infective forms (sporozoites), are shed in feces, and a few days will be needed to form two infectious sporozoites and become a "sporulated oocyst" (Frenkel et al. 1970). Sporulated oocysts are highly resistant and can survive in the soil or water for several months (Dubey 1996). Like in the definitive host, in the intermediate host, the infection also occurs by ingestion of cysts or oocysts. In the intermediate host, the bradyzoites or sporozoites released in the small intestine penetrate the epithelial cells and multiply asexually, converting into tachyzoites (Fig. 1) (Blader et al. 2015). Tachyzoites may disseminate to extra-intestinal tissues through the lymph and blood by invading the same leukocytes that are recruited to respond against the parasite (Denkers et al. 2012). Tachyzoites are able to enter and multiply in any nucleated host cell, ultimately

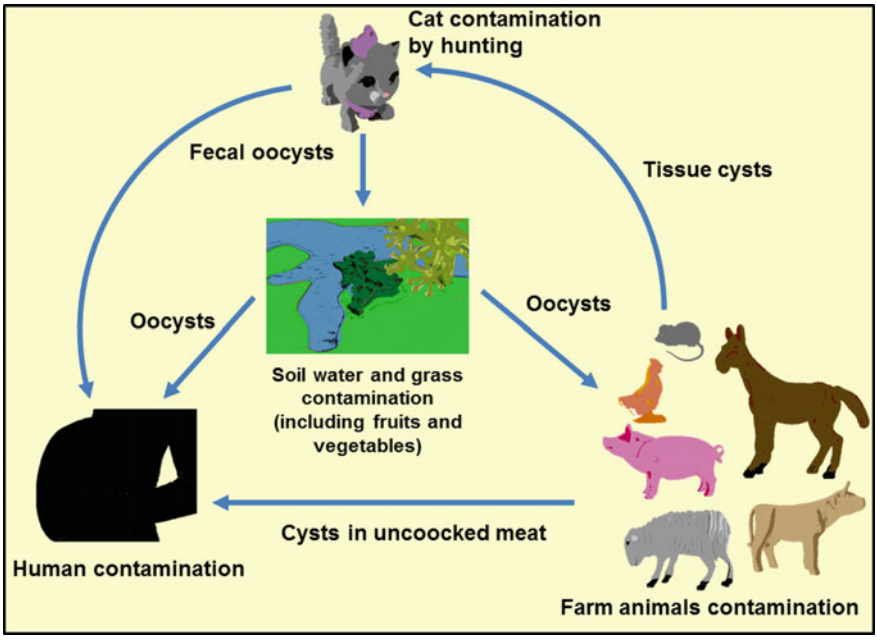

Fig. 1 Life cycle of *Toxoplasma gondii*. Felines are the definitive hosts in which the sexual replication phase of *T. gondii* occurs, resulting in the generation of thousands of highly resistant oocysts containing non-infective sporozoites that are spread by feces. Once in the environment, sporozoites become infective. Other mammals and birds are intermediate hosts. Both definitive and intermediate hosts can be infected by ingestion of oocysts present in the soil, unwashed vegetables or water. The asexual cycle of *T. gondii* occurs in the intermediate hosts, with two stages: highly replicative tachyzoites and latent bradyzoites, the latter forming tissue cysts. In addition to oocyst infection, animals and humans can be infected by ingestion of tissue cysts. Finally, vertical transmission can be observed in a variety of hosts, including humans

leading to cell death to continue dissemination to the spleen, liver, lungs, lymphoid tissue, central nervous system, retina and cardiac and skeletal muscles (Cañedo-Solares et al. 2013). This is called "the acute phase of infection", which is associated with a strong inflammatory response. Bradyzoites are the slow replicating form of the parasite, mainly in central nervous system tissues and skeletal muscles, leading to the latent or "chronic phase of infection" for the life of the host (Skariah et al. 2010).

1.3 Transmission

Transmission occurs when tissue cysts, present in raw or undercooked meat, or oocysts, present in unwashed vegetables, soil or water, are ingested. In humans, consumption of raw or undercooked meat is the main transmission route and is

responsible for an important annual cost of illness and quality-adjusted life year loss in the USA (Cook et al. 2000; Scallan et al. 2011). Jones et al. (2009) found that ingestion of raw clams and oysters may also cause infection due to contact with contaminated water. A recent study on risk factors for toxoplasmosis indicated that the prevalence of *T. gondii* is higher in lamb and pork meat than in beef and poultry, and that organic management systems result in a higher prevalence of the parasite than conventional management systems, probably because of the access of these animals to the outdoors (Guo et al. 2015). Although more accurate studies about risk factors are needed, it can be noted that raw or undercooked meat from cattle, pigs, sheep, horses and goats is a potential source of *T. gondii* and should not be consumed by at-risk groups in the population (Belluco et al. 2016).

Since untreated water has been reported to be the source of major outbreaks of acute toxoplasmosis in Canada (Bowie et al. 1997) and Brazil (Vaudaux et al. 2010), waterborne outbreaks of *T. gondii* have aroused attention on the importance of the oocysts shed in the feces of infected cats. Regarding unwashed vegetables, a recent study made in Brazil demonstrated that almost 4% of fresh-leaf vegetables destined for human consumption and obtained directly from production sites and stores were contaminated with *T. gondii* DNA (Marchioro et al. 2016). In rural communities, consumption of unwashed raw vegetables or fruits is also a risk factor of toxoplasmosis (Rostami et al. 2016). Regarding the presence of oocysts in the environment, a survey made in California (USA) estimated that the annual oocyst burden is 3–434 oocysts per square foot (Dabritz et al. 2007). Considering that a single oocyst is able to cause toxoplasmosis, this oocyst burden represents a major potential public health problem.

In humans, congenital transmission involves the transmission of *T. gondii* tachyzoites from a newly infected mother (primary infection) to the fetus through the placenta or to the baby during vaginal delivery (Gras et al. 2005; Thiébaut et al. 2007). However, it has been reported that congenital transmission to the fetus also occurs from mothers chronically infected and reinfected with virulent strains of *T. gondii* during pregnancy (Elbez-Rubinstein et al. 2009), from highly immunocompromised mothers (Azevedo et al. 2010; Lindsay and Dubey 2011), and from women who acquire the infection some months before pregnancy (Hennequin et al. 1997). Congenital toxoplasmosis also occurs in many other animal species, particularly cattle, sheep, goats and rodents such as mice (Dubey 2008). In most classes of livestock, congenital toxoplasmosis has been recognized as being responsible for major economic losses through abortions, stillbirths and neonatal mortality (Raeghi et al. 2011). The clinical aspects of congenital toxoplasmosis in humans and other animals will be widely discussed in the next section.

2 Toxoplasmosis: Disease Symptoms and Occurrence of Infection

Because *Toxoplasma gondii* may cause abortion or congenital disease in its intermediate hosts, it is considered a parasite of both veterinary and medical importance (Tenter et al. 2000).

2.1 T. gondii *Infections in Humans*

The seroprevalence of *T. gondii* varies according to the age, geographical location and socio-economic strata of a specific population (Rosso et al. 2008). While one third of the world's human population is seropositive to *T. gondii*, clinical disease is largely confined to risk groups.

In the majority of immunocompetent individuals, toxoplasmosis is asymptomatic (Feustel et al. 2012). When symptoms are manifested, almost all cases are due to the tachyzoite form. Occasionally, toxoplasmosis may present with lymphadenopathy, fever, muscle aches and headaches (Dubey 1996), whereas severe manifestations such as encephalitis, myocarditis, hepatitis and sepsis syndrome very rarely occur (Tenter et al. 2000). The severity of the infection may depend on the genotype of the strain, where atypical genotypes are associated with worse clinical outcomes (Robert-Gagneaux and Dardé 2012).

In humans, congenital toxoplasmosis is one of the main clinical problems of Toxoplasma infection. Congenital toxoplasmosis can cause spontaneous abortion, as well as blindness and mental retardation in congenitally infected children (Shaapan 2016). In 2016, the World Health Organization estimated a global incidence rate of 1.5 cases of congenital toxoplasmosis per 1000 live births (Torgerson and Mastroiacovo 2013). The highest burden is found in South America (3.4 per 1000 live births) and is driven by the more pathogenic genotypes that circulate in that part of the world (Torgerson and Mastroiacovo 2013). Other regions with high incidence of congenital toxoplasmosis include parts of the Middle East (2.5 per 1000 live births) and some low income countries in Africa (2.4 per 1000 live births) (Torgerson and Mastroiacovo 2013). The risk of transplacental transmission increases with gestational age, from less than 10% in the first trimester to 30% in the second and 70–90% in the third trimester (Dunn et al. 1999). In contrast, the severity of congenital toxoplasmosis is inversely correlated with the time of gestation when maternal infection was acquired, with infection in early pregnancy displaying more severe consequences, such as stillbirths, abortions, substantial brain necrosis and hydrocephalus (Moncada and Montoya 2012; Xiao and Yolken 2015). In addition to the gestational age, the parasite genotype may also play a role in congenital disease, with atypical strains causing severe damage even when acquired during the third trimester (Delhaes et al. 2010). Near 70–90% of infants born with congenital *Toxoplasma* infection are asymptomatic at birth and therefore

their infection is not recognized. However, during the first year of life or more, most of these children will develop sequelae such as ocular lesions and neurological symptoms (Wilson et al. 1980). Other factors involved in congenital toxoplasmosis transmission and clinical outcome are the genetics of the host, the size of the inocula, the infecting form of the parasite and the maternal treatment (Moncada and Montoya 2012).

Ocular toxoplasmosis, a particular form of the infection, can be postnatally acquired by immunocompetent individuals as well as by reactivation of congenital infection (Perkins 1973; Matias et al. 2014). Its incidence varies from 2–3% in the USA and Europe to nearly 18% in Southern Brazil (Glasner et al. 1992; Holland 2003). Ocular toxoplasmosis may result in inflammation in the retina, choroid, and uvea, and consequently lead to complications such as glaucoma, cataract, and posterior synechiae (Jasper et al. 2017). This type of toxoplasmosis is considered the most frequent cause of infectious posterior uveitis (Pleyer et al. 2014). Since ocular toxoplasmosis is a preventable cause of blindness, it is necessary to assess factors that have the potential to control this disease.

Different factors, including HIV infection, certain types of cancers including lymphomas, immunosuppressive therapies (Robert-Gagneaux and Dardé 2012), and chronic inflammatory diseases such as diabetes or obesity (Esch and Petersen 2013), can profoundly impair cellular immunity, leading to severe toxoplasmosis. Reactivation of a latent infection or de novo infection in immunocompromised individuals can cause the development of clinical illness with varied presentations, including encephalitis, pneumonitis, chorioretinitis, meningitis, and disseminated toxoplasmosis with multi-organ involvement (Khurana and Batra 2016). In this sense, toxoplasmosis represents one of the main opportunistic infections in HIV/AIDS, associated with high morbidity and mortality rates (Basavaraju 2016). In transplant patients, the severity of toxoplasmosis is clearly associated with the degree of induced immunosuppression (Robert-Gagneaux and Dardé 2012), as well as with the timing of appropriate anti-Toxoplasma therapy (Coster 2013). About 10–25% of transplant recipients with toxoplasmosis show central nervous system manifestations, mainly encephalitis. When toxoplasmosis is severe or disseminated, patients present myocarditis, encephalitis, pneumonitis, or multi-organ failure, and its fatality rate is >80%.

Since *T. gondii* has a strong predilection to infect the central nervous system, over the past decade, chronic *T. gondii* infection has been increasingly associated with psychiatric disorders (Torrey and Yolken 2003; Dickerson et al. 2009; Zhu 2009; Shibre et al. 2010), such as attention-deficit hyperactivity disorder, obsessive compulsive disorder, schizophrenia, major depression, and bipolar disorder (Halonen and Weiss 2013; Elsheikha et al. 2016; Del Grande et al. 2017). Three putative mechanisms for *T. gondii* infection of the central nervous system have been proposed: (i) the direct effect position, which is based on the fact that *T. gondii* bradyzoites are present mainly in neurons, affecting their function (Ferguson and Hutchison 1987); (ii) a neuroimmunological mechanism based on the fact that proinflammatory cytokines may modulate dopaminergic and glutamatergic

neurotransmission (Mastropaolo et al. 1989; Emery et al. 2007); (iii) a proposal that suggests that bradyzoites are able to synthesize and release the dopamine neurotransmitter (Ayaz et al. 2016).

2.2 *Toxoplasmosis in Other Animals*

As mentioned above, almost every warm-blooded animal species can be infected with *T. gondii*. Although most of them show no clinical signs of the disease, their latent infection is still of veterinarian/zoonotic relevance, since their meat could be the source of de novo infections by carnivorous animals, including humans.

2.2.1 Toxoplasmosis in Pets

The first reported case of fatal toxoplasmosis in a domestic animal was in a dog in 1910 (Dubey 2008). In dogs, the seroprevalence of *T. gondii* varies according to the geographical location, age and gender, ranging from 9.1% in dogs from Shangai (Jiang et al. 2015) to 24% in hunting dogs from Italy (Machacova et al. 2016), 42.2% in dogs from St Kitts in the Caribbean (Dubey et al. 2016), 48.75% in dogs from Fernando de Noronha Island in Brazil (Magalhães et al. 2017) and 51.9% in dogs from Zhanjiang city in southern China (Jiang et al. 2015). As in humans, the clinical presentation of the infection varies from total lack of symptoms to affections in different organ systems, including encephalitis, and even severe disease, involving the lungs and liver, which may kill dogs within a week (Dubey et al. 2009). Transplacental transmission in dogs can cause spontaneous abortion and fetal death (Bresciani et al. 1999, 2001), and even reinfection of pregnant females can lead to clinical alterations such as fever, lymphadenopathy, miscarriage, and fetal death (Bresciani et al. 2009).

The first report of toxoplasmosis in a cat was in 1942 (Dubey 2008) and nearly 30 years later, Frenkel et al. (1970) demonstrated that cats are the definitive host of *T. gondii*. The estimated seroprevalence for *T. gondii* in domestic cats worldwide is 30–40% (Muñoz et al. 2011), but antibodies to *T. gondii* may be detected in up to 74% of adult cat populations, mainly depending on the type of feeding and whether the cat remains indoors or outdoors (Tenter et al. 2000). As in other hosts, most of the postnatally acquired infections in cats are asymptomatic and vertical transmission is infrequent (Tenter et al. 2000). However, when congenital toxoplasmosis occurs, clinical illness is common and may result in stillbirth or kitten death before weaning. The clinical presentation is often characterized by inflammation of the liver, lungs and central nervous system. In postnatally acquired infections, anorexia, lethargy and dyspnea are common (Dubey et al. 2009). The zoonotic relevance of cats, especially related to the shed of oocysts in the environment, has been previously discussed (Sect. 1.2).

2.2.2 Toxoplasmosis in Animals Destined to Human Consumption

The study and control of toxoplasmosis in meat-producing animals is of major relevance mainly for two reasons: first, because, as previously mentioned, toxoplasmosis is one of the main foodborne diseases because tissue cysts of *T. gondii* contained in meat of livestock are considered the main source of infection in humans (Cook et al. 2000), and second, because the disease is responsible for major economic losses in most classes of livestock through abortions, stillbirths, early embryonic death, resorption, mummification, and neonatal mortality or birth of live but weak offspring (Buxton et al. 2007; Raeghi et al. 2011), especially in sheep (Hiszczyńska-Sawicka 2014), goats and lambs (Dubey 2008; Dubey et al. 2009). Congenital toxoplasmosis in sheep and goats is slightly different, since, in sheep, it occurs only in primary infections of pregnant sheep, whereas, in goats, the same goat can transplacentally infect its fetuses in successive pregnancies (Dubey 1982). In the UK, congenital toxoplasmosis is responsible for 1–2% of neonatal losses in sheep and goats per annum (approximately 300,000 sheep/goats lose their fetuses) (Menzies et al. 2008), while, in the European Union, pregnancy losses in these species are between 0.7 and 1.4 million annually (Katzer et al. 2011).

In pigs, symptomatic toxoplasmosis is rare, but clinical signs include anorexia, fever, dyspnea, limb weakness, encephalitis, pneumonitis, lymph node necrosis, hepatic necrosis and even death (Dubey 2009). In addition, abortions related to congenital toxoplasmosis in pigs have also been reported (Kim et al. 2009).

In cattle and horses, there are no confirmed cases of clinical toxoplasmosis (Dubey 2007). However, a recent experimental study has shown that dams inoculated with tachyzoites of the *T. gondii* RH strain aborted on days 6 and 11 post-inoculation, which demonstrates that vertical transmission of toxoplasmosis in cattle can occur (Wiengcharoen et al. 2011). The role of toxoplasmosis in economic losses due to bovine abortion warrants further investigation.

In poultry, there is a high prevalence of *T. gondii* infection but neither chickens nor turkeys develop any clinical sign of the disease (Guo et al. 2015), and toxoplasmosis does not cause any economic losses in these species (Dhama et al. 2013). However, chickens are considered one of the most important hosts in the epidemiology of *T. gondii* infection and the importance of chicken meat as a source of human toxoplasmosis must not be ignored.

Finally, in rabbits, toxoplasmosis is one of the most important diseases in commercial herds (Dubey et al. 2011). *T. gondii* transmission in rabbits occurs through food contaminated with parasite oocysts, transplacental transmission and interaction between rabbits and domestic cats infected with *T. gondii* (Dubey et al. 1992).

3 Mechanism of Infection and Immune Response

3.1 *Mechanism of Host-Cell Invasion and Egression*

Toxoplasma gondii is an apicomplexan parasite, and, as any other member of the group, it is characterized by the presence of an "apical complex" consisting of a closed, truncated cone called the conoid, which is composed of unique fibers of tubulin polymers. This apical complex acts as an organizing center, and a cluster of apical secretory organelles including rhoptries, micronemes and dense granules (Hu et al. 2006; Sibley 2011; Okamoto and Keeling 2014). In addition, the apical complex is involved in the processes of host attachment and invasion, being fundamental for *T. gondii* infection (Okamoto and Keeling 2014).

Apicomplexan parasites lack cilia and flagella and their mode of locomotion, called gliding, propels them across the substrate and supports active penetration of host cells. Gliding relies on a unique form of substrate-dependent motility based on the actin-myosin motor of the parasites (Barragan and Sibley 2002; Sibley 2011).

T. gondii invades host cells in several orchestrated steps that begin with the molecular interaction between the parasite and the host cell before internalization, first mediated by transient interactions followed by sequential secretion of specialized proteins (micronemes and rhoptry proteins) to establish the "parasitophorous vacuola" (Peng et al. 2011; Blader et al. 2015). The "parasitophorous vacuola" protects *T. gondii* from being eliminated by host cells, especially by endosomal acidification and lysosomal fusion. Once inside, tachyzoites induce the host cell to arrest at the G2/M phase of the cell cycle and to provide a nutrient pool to the parasite that enables *T. gondii* proliferation and replication by endodiogeny (Fig. 2a). After several rounds of replication, the parasite activates motility and exits the host cell by rupturing the vacuola and breaching the host cell plasma membrane (Fig. 2a) (Melzer et al. 2008; Peng et al. 2011).

3.2 T. gondii *Dissemination Through the Host*

T. gondii primary infection generally occurs by the ingestion of oocysts or tissue cysts, which release sporozoites or bradyzoites to the lumen of the gut from where the parasite spreads throughout the body of the individual (Fig. 2b). The dissemination of *T. gondii* to a large variety of other organs in the body occurs via intracellular and extracellular mechanisms (Harker et al. 2015). *T. gondii* can flow freely in host fluids, migrate on cell layers (endothelium, epithelium), cross them (paracellular route) or use motile host cells (intracellular route) to achieve distant or hardly accessible organs (Długonska 2014). As a consequence of oral infection, innate immune cells such as neutrophils, monocytes and dendritic cells (DCs) are recruited to the small intestine (Cohen and Denkers 2014). During the acute phase of the infection, *T. gondii* shows a certain preference for cells of the immune

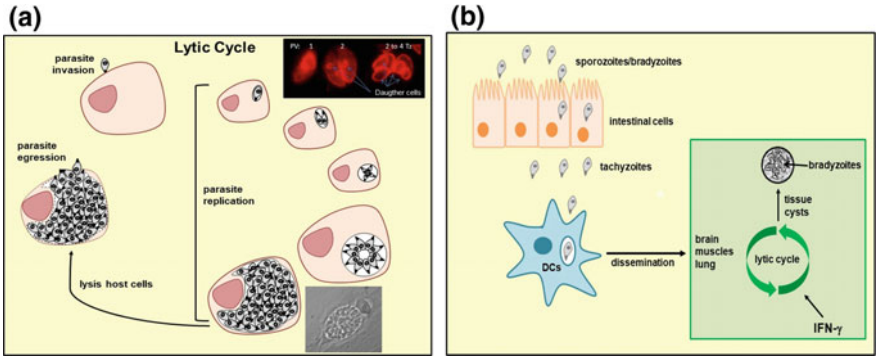

Fig. 2 Toxoplasma lytic cycle and infection. **a** *T. gondii* invades the host cells by an active process that results in the formation of an intracellular parasitophorous vacuole (PV). Once inside the cell, tachyzoites begin to replicate by a process called endodiogeny, in which the gestation of two daughter cells within the mother cell can be observed (see upper panel). After several replication rounds, it is possible to observe the presence of 2, 4, 8 and so on tachyzoites per PV until the generation of a large PV (see lower panel) near the egression step to resume the lytic cycle. Upper panel: Replicating intracellular tachyzoites were labeled with anti-tubulin antibodies and detected by epifluorescence microscopy. Lower panel: intracellular PV observed by phase contrast. **b** The parasite enters the lumen of the intestine by ingestion of cysts or oocysts releasing bradyzoites or sporozoites, respectively. The bradyzoites/sporozoites penetrate the intestinal epithelium and convert to the highly replicative tachyzoites, which disseminate within the new host. Tachyzoites invade every nucleated cell, including dendritic cells, which give them mobility, allowing the spread of *T. gondii* throughout the body of the host. *T. gondii* also has the ability to modulate the immune response in such a way as to allow its dissemination. Finally, the IFN-γ-dependent cell-mediated immune response is able to kill the intracellular parasite but also to trigger the conversion of tachyzoites to the latent form of bradyzoites, generating tissue cysts, mainly at the central nervous system and muscle. The immune response is also important to maintain the latent infection. The lack of a good control of infection may lead to serious clinical consequences, including death of the infected individual, as observed in immunocompromised patients

system, mainly DCs (Sanecka and Frickel 2012). DCs play a key role in the immune function, being the link between innate and adaptive responses, as well as in actively secreting IL-12 and a number of other cytokines that attract and activate other cells of the immune system such as pathogen-specific T cells (Sanecka and Frickel 2012; Długonska 2014). On the other hand, DCs have motile properties, resulting in a very effective vehicle to cross many biological barriers that have exaggerated immune responses (the blood brain barrier, blood-eye barrier and the placenta) to enter immune privileged organs like the brain and the eye or even to infect the fetus (Bierly et al. 2008).

3.3 *Immune Control of* T. gondii

The immune response against *T. gondii* strongly depends on the strain of the infecting parasite (Xiao and Yolken 2015), as well as on the genetic background and immune status of the host (Suzuki and Remington 1993). However, the salient features of this complex immune reaction have been elucidated mainly based on the study of murine experimental models of toxoplasmosis (Dupont et al. 2012).

3.3.1 Immune Response During the Acute Phase of Infection

When *T. gondii* reaches the gut of the host, monocytes, granulocytes (mainly neutrophils), DCs and lymphocytes residing in the small intestinal epithelium are rapidly recruited to the site of infection (Muñoz et al. 2011; Dupont et al. 2012). Among them, neutrophils are very important during the initial response (Bliss et al. 2001) and their recruitment depends on the chemokine receptors CXCR2 (Del Rio et al. 2001) and CCR1 (Khan et al. 2001). In *T. gondii* infection, the main pattern-recognition receptors involved are the Toll-like receptors 2, 4 (Debierre-Grockiego et al. 2007; Bereswill et al. 2014) and 11 (Yarovinsky et al. 2008). After recognition, mainly DCs but also neutrophils and activated macrophages secrete IL-12 (Muñoz et al. 2011; Dupont et al. 2012), which in turns stimulates natural killer cells (Sturge and Yarovinsky 2014). Natural killer cells are the main source of IFN-γ during the initial phase of infection and are also able to kill the parasite by apoptosis (Muñoz et al. 2011). DCs and macrophages increase the expression of major histocompatibility complex type II molecules, stimulated by IFN-γ (Denkers et al. 1996), becoming mature antigen-presenting cells. Activated macrophages and neutrophils are also able to secrete reactive oxygen species and reactive nitrogen intermediaries that help to control the parasite, limiting infection (Gazzinelli et al. 1993; Alves et al. 2013). Therefore, the innate immune response limits tachyzoite replication and drives the generation of a specific T-cell response.

As previously discussed, DCs then migrate to the lymph nodes, both helping the parasite to disseminate to other tissues and acting as antigen-presenting cells, leading to the proliferation of *T. gondii*-specific T lymphocytes (TLs). In the presence of co-stimulatory molecules and in an IL-12 context, CD4+ and CD8+ *T. gondii*-specific TLs proliferate and differentiate into populations that produce pro-inflammatory cytokines, particularly IFN-γ, TNF-α, IL-6 and IL-1 (Sibley et al. 1991; Langermans et al. 1992). In addition, CD4+ TLs secrete IL-2, which in turn helps CD8+ TLs to proliferate and become cytotoxic lymphocytes, which act as effectors lysing the parasites, promoting apoptosis by CD40-CD40L and secreting IFN-γ (Dupont et al. 2012).

The role of humoral immunity in toxoplasmosis is not so clear. Specific immunoglobulins (Igs) against *T. gondii* antigens, including IgM, IgA, IgE and IgGs, have been found in infected individuals (Robert-Gagneaux and Dardé 2012).

In this sense, in vitro assays have demonstrated that *T. gondii*-specific antibodies are able to opsonize the parasite for phagocytosis, block invasion and activate the complement (Dupont et al. 2012).

3.3.2 Limitation of the Immune Response Against *T. gondii* and Immune Evasion and Anti-apoptotic Reactions of the Parasite in the Host Cell

It is important to note that the immune response against *T. gondii* is a double-edged sword, since a potent pro-inflammatory reaction may kill the parasite, but it also can be detrimental and even fatal for the host, as in the case of *T. gondii* intestinal ileitis (Mennechet et al. 2004). The invasion of host cells by *T. gondii* triggers a potent immune response that eliminates most parasites during the acute phase of infection. However, some tachyzoites can evade this response, convert to bradyzoites (the low replicative form of the parasite), and encyst in non-replicative cells for the lifespan of the host, in the chronic phase of the infection. To achieve this delicate balance between induction and suppression of the host immune responses, *T. gondii* induces modifications in the expression and secretion of immunomodulatory cytokines and in the viability of immune cells, as well as mechanisms to abolish antimicrobial effector mechanisms (Muñoz et al. 2011). In this sense, *T. gondii* is able to significantly decrease the responsiveness of host infected cells to IFN-γ signaling by blocking STAT1 transcriptional activity (Kim et al. 2007). A recent report has also demonstrated that the secretion of ROP16 by the tachyzoite is associated with prolonged phosphorylation of the transcription factors STAT3 and STAT6, enhancing IL-4 and IL-6 production while down-regulating IL-12 production, thus avoiding a strong inflammatory response and leading to an anti-inflammatory one, which is less harmful for the parasite (Saeij et al. 2007). Moreover, *T. gondii* enhances production of anti-inflammatory cytokines, including IL-10 and TGF-β, which inhibit IFN-γ production, impair macrophage activation and inhibit the activity of natural killer cells (Muñoz et al. 2011).

Another cell signaling pathway modulated by *T. gondii* is the transcription factor NF-kB signaling pathway, critical in immune response and inflammation (Du et al. 2014). The role of *T. gondii* in regulating this transcription factor is controversial. Some studies have demonstrated that invasion of cells by *T. gondii* fail to activate NF-κB signaling in macrophages and fibroblasts (Butcher et al. 2001; Shapira et al. 2005), enabling the parasite to invade cells without triggering pro-inflammatory cytokine induction. On the other hand, other studies have demonstrated that *T. gondii* activates NF-κB in mouse embryonic fibroblasts (Molestina et al. 2003) and mouse spleen cells (Kim et al. 2006), finally leading to the up-regulation of the expression of anti-apoptotic genes resulting in the enhanced survival of the parasite. Although the mechanism of *T. gondii* in regulating the NF-κB signaling pathway needs further elucidation, parasite-specific molecules likely induce this pathway as a means of disrupting host cell immune responses (Sibley 2011).

3.3.3 Immune Response During the Chronic Phase of Infection

Tachyzoites that avoid the immune reaction during the acute phase of infection reach immune privileged sites and convert into encysted bradyzoites, initiating the chronic phase of the infection. The persistence of *T. gondii* in this phase depends on immune control, since it has been shown that, in immunocompromised individuals, reactivation of the infection may occur and result in life-threatening toxoplasmic encephalitis (Israelski and Remington 1993). However, the mechanisms by which the immune system maintains a latent *T. gondii* infection are not completely understood. Resistance during chronic toxoplasmosis involves at least secretion of IFN-γ (Suzuki et al. 1989) and TNF-α (Gazzinelli et al. 1993), and the interaction between CD40 and CD40L (Reichmann et al. 2000).

4 Comparison of Plant-Made Vaccine Candidates with Current and Alternative Treatments for Toxoplasmosis

4.1 Current Treatments for Toxoplasmosis

Despite the economic losses associated with *T. gondii* infection in farm animals and socio-economic impact caused by this zoonotic disease in the human population, treatment is not able to eliminate the parasite from the host once the chronic infection has been established (Innes 2010). An ideal drug for prophylaxis and/or treatment of toxoplasmosis should show effective penetration and concentration in the placenta, transplacental passage, parasiticidal properties against the different parasitic stages, penetration into cysts, and distribution in the main sites of infection. Unfortunately, so far, no available drug fulfills these criteria (Derouin and Santillana-Hayat 2000; Montoya and Liesenfeld 2004). Even if a drug with these characteristics were available, it could hardly be used in animals since there is a risk related to the increment of drug resistance and to drug residues entering in the food chain (Hiszczyńska-Sawicka et al. 2014). In addition, a drug-based treatment would not be as well-suited to control toxoplasmosis in farm animals, since the main goal of toxoplasmosis prevention in livestock is related to the control of abortions and to prevent the transmission of the parasite to humans through the consumption of meat. In this sense, it is the development of a prophylactic treatment based on vaccination seems to be the most effective method to avoid the spread of the disease in livestock.

The current anti-*T. gondii* chemotherapy for humans is based on the prophylactic administration of pyrimethamine and sulfadiazine (Montazeri et al. 2017). However, it is a deficient treatment since it is not well-tolerated by immunocompromised patients (Rodriguez and Szajnman 2012). In addition, the use of these drugs has several side effects, including renal calculi (McGettigan et al. 2012),

neutropenia, severe drop of platelet count, thrombocytopenia, leukopenia, increase in serum creatinine and serum liver enzymes, hematological abnormalities, and hypersensitivity reactions (Montazeri et al. 2017). In congenital toxoplasmosis, spiramycine, a macrolide that has not been shown to be teratogenic, is used to treat infection. However, prenatal spiromycine treatment is still controversial (Kieffer and Wallon 2013), since multicenter studies conducted in Europe have shown conflicting results. Some studies have shown that prenatal administration of spiromycine decreases the severe neurological sequelae of congenital toxoplasmosis, whereas other studies have shown that prenatal treatment is not able to prevent fetal transmission (Gilbert and Gras 2003; Gras et al. 2005; Fricker-Hidalgo et al. 2013).

Many of the current treatment regimens are based on case series and case studies and there are no large-scale trials on drugs used to treat toxoplasmosis. Thus, there is a need to evaluate the available evidence with regard to the efficacy and safety of the drugs used to treat symptomatic toxoplasmosis in immunocompetent and immunocompromised hosts, pregnant women, and patients with ocular toxoplasmosis (Rajapakse et al. 2007).

In recent years, a highly valuable goal for global toxoplasmosis control is the development of well-tolerated and safe specific immunoprophylaxis against the disease (Lim and Othman 2014). Immunotherapeutic strategies to improve toxoplasmosis control could be a vaccine able to induce either strong protective immunity or passive immunization in cases of disease recrudescence. So far, the only commercial vaccine is the S48 strain of attenuated tachyzoites (Toxovax®, MSD Animal Health, Summit, NJ, USA) for use in sheep (Buxton et al. 1991). Toxovax vaccine is used in the UK, New Zealand, France, and Ireland (Garcia et al. 2014). Vaccination of sheep with this vaccine allows a reduction of abortions and neonatal mortality, and improves the birth weight of lambs (Buxton et al. 1991). However, this vaccine causes side effects, has a short life time, induces a short-term immunity, and has a high cost of production because it depends on the growth of the parasite in mammalian cell cultures. Moreover, there are some concerns about its safety because this type of attenuated live vaccines carries the risk of reverting to a pathogenic strain and can infect humans (Garcia et al. 2014).

4.2 *Plant-Made* T. gondii *Vaccine Candidates: Rationale, Efficacy and Yield*

Despite the potential of vaccine antigens expressed in plant systems, the possibility of using this strategy for the expression of parasite antigens has aroused greater interest in recent years (Matsumoto et al. 2009; Clemente and Corigliano 2012; Sathish et al. 2012; Jacob et al. 2013; Hernandez et al. 2014; Ganapathy et al. 2014; Monreal-Escalante et al. 2016; Kesik-Brodacka et al. 2017; Wilbers et al. 2017). Antigens of *Plasmodium* sp. (Clemente and Corigliano 2012), *Entamoeba histolytica* (Chebolu and Daniel 2007) and *T. gondii* (Clemente 2014) were the

pioneer examples of parasitic antigens expressed in plants. Although the results obtained are highly promising, *T. gondii* antigen expression in plants is just beginning and only a few *T. gondii* antigens have been successfully expressed.

4.2.1 Dense Granule Protein 4 (GRA4) of *T. gondii*

The dense granule protein 4 (GRA4) of *T. gondii* belongs to the family of proteins secreted into the lumen of the parasitophorous vacuole by the tachyzoite (Labruyère et al. 1999). GRA4 is involved in the parasite-host interaction, which is associated with a intravacuolar network that participates in the transport of nutrients and proteins into the parasitophorous vacuole (Labruyère et al. 1999).

GRA4 is considered a highly feasible candidate for vaccine development against toxoplasmosis, since recombinant GRA4 protein produced in bacteria or GRA4 DNA vaccines have been shown to induce both a humoral and a cellular response during *T. gondii* oral infection (Desolme et al. 2000; Martin et al. 2004; Mévélec et al. 2005; Chen et al. 2009; Sánchez et al. 2011). In fact, results of mouse immunizations with the recombinant protein or its gene have shown that both are able to confer protection against Toxoplasma infection (Mévélec et al. 1998; Desolme et al. 2000; Martin et al. 2004, Mévélec et al. 2005). In addition, Mévélec et al. found that oral immunization of mice with truncated soluble forms of recombinant GRA4 in association with cholera toxin induces a significant Th2-like mucosal response and partial resistance to oral infection with *T. gondii* (Mévélec et al. 1998) and that the recombinant GRA4 protein is able to induce a specific Th1 humoral and cellular immune response in a murine model (Mévélec et al. 2005).

Considering that the portal of entry of *T. gondii* is the mucosa, the stimulation of an efficient local response of mucosal membranes as well as that of a systemic response constitute a priority which could be achieved by the administration of an oral/nasal vaccine. Therefore, GRA4 is an interesting protein to express in plants for the production of eukaryotic immunoprophylactic antigens and for the assessment of its effectiveness in oral vaccines against intracellular pathogens.

The region of GRA4 that contains the B-cell and T-cell epitopes ($Gra4_{163\text{-}345}$) (Mévélec et al. 1998) displays antigenic properties (Nigro et al. 2003; Altcheh et al. 2006) and confers immune protection against toxoplasmosis in mice (Martin et al. 2004). Thus, this region has been chosen for expression in tobacco plants. Ferraro et al. (2008) transiently expressed $GRA4_{163\text{-}345}$ in tobacco leaves using both a Potato Virus X (PVX)-based amplicon and an apoplast-targeting system, and found that the targeting to the secretory pathway increased the production of GRA4 (0.01% vs. 0.001% of total soluble proteins using PVX amplicon system). Although yields were not very high, $GRA4_{163\text{-}345}$ successfully accumulated into the apoplastic space. They also found that extracellular targeting allowed the recovery of recombinant products from apoplastic washing fluids (in the case of leaf infiltration systems) or from the nutrient medium (in the case of hydroponically cultivated transgenic plants), thus simplifying the extraction of fractions enriched in the recombinant protein. In addition, the authors showed that plant-derived GRA4 was

immune reactive with seropositive human sera (Ferraro et al. 2008). Later, Del L Yácono et al. (2012) analyzed the expression of $GRA4_{163\text{-}345}$ by chloroplast transformation (chlGRA4) in tobacco plants, and found that this transformation allowed a significant 30-fold increase in GRA4 protein accumulation in the plant. In the work by Ferraro et al., the yields of GRA4 in tobacco infiltrated leaves were around 0.2 μg/g of fresh weight (Ferraro et al. 2008), whereas in the work by Del L Yácono et al., the chlGRA4 expression levels in transplastomic plants were up to 6 μg/g of fresh weight (or 0.2% of total proteins) (Del L Yácono et al. 2012). These results support the idea that the chloroplast would be the best compartment to express this protein. In addition, oral immunization with the chloroplast-derived GRA4 resulted in a decrease of 59% in the brain cyst load of mice and elicited both a mucosal and systemic immune response characterized by the production of specific IgA and IgG, and secretion of IFN-γ, IL-4 and IL-10. The authors suggested that the antigen expressed in the chloroplasts would have been recognized by the antigen-presenting cells and presented in the gut-associated lymphoid tissues, resulting in the activation of T helper cells that would trigger a partial protection against Toxoplasma infection (Del L Yácono et al. 2012). This protection correlates with a mucosal and systemic balanced Th1/Th2 response.

4.2.2 Main Surface Antigen 1 (SAG1) of *T. gondii*

The proteins present on the surface of the tachyzoite, called surface antigens (SAGs), are the ligands through which the tachyzoite recognizes and adheres to the host cells (Pollard et al. 2008). Since cellular invasion is essential for the survival and spread of the parasite, the proteins involved in this process have been the principal target of the study for vaccine development against *T. gondii* (Jongert et al. 2009).

SAG1 is well conserved at the immunological and amino acid sequence levels, making it an attractive antigen for immunoprophylaxis of toxoplasmosis (Wang and Yin 2014). SAG1 is able to stimulate IFN-γ production by T cells in seropositive individuals through the action of CD8 + T cells that have cytotoxic activity (Khan et al. 1988). In addition, several studies have identified B- and T-epitopes in the SAG1 coding sequence, which would be recognized by the immune system after the infection. These epitopes would be able to induce a humoral and/or cellular immune response and, specifically, stimulate cytotoxic T lymphocytes cells (Siachoque et al. 2006; Cardona et al. 2009; Wang et al. 2013). In this context, numerous studies have established the potential of SAG1 as a candidate to produce an anti-*T. gondii* vaccine (Wang and Yin 2014). Previous attempts to express SAG1 using various classical heterologous expression systems have been impaired by the low expression levels or poor antigenicity due to misfolding or an excessive glycosylation of the recombinant SAG1 protein (Burg et al. 1988; Makioka and Kobayashi 1991; Harning et al. 1996; Nigro et al. 2003). Thus, SAG1 was a good candidate to optimize an antigen production strategy using an alternative protein

expression system such as plant-based protein production. SAG1 was the first antigen of *T. gondii* expressed in plants (Clemente et al. 2005).

The transient transformation of SAG1 was evaluated using a vacuum infiltration system via recombinant Agrobacterium in tobacco plants (Clemente et al. 2005). In that study, this expression system was used to test the performance of three different constructs carrying the *SAG1* gene (Clemente et al. 2005). Two of the constructs were based on a PVX amplicon, whereas in the third construct, the *SAG1* gene was fused to an apoplastic peptide signal under the CaMV 35S promoter. The expression levels of SAG1 in infiltrated leaves ranged from 0.06 to 0.1% of TSP (~6–10 μg/g of fresh weight). Contrary to the results for GRA4, the infiltrated leaves with the version of SAG1 targeting to the apoplast space showed the lowest expression levels (Clemente et al. 2005). Thus, the better replication capacity of the amplicons could explain the higher SAG1 levels compared with the construct that targeted SAG1 to the apoplast.

Later, Laguía-Becher et al. (2010) designed and synthesized a plant-codon-optimized version of SAG1, and transiently expressed it in tobacco leaves. Both plant-optimized SAG1 and native SAG1 genes were fused to the apoplast or endoplasmic reticulum peptide signals for stable protein accumulation (Laguía-Becher et al. 2010). Surprisingly, the authors observed that leaves agroinfiltrated with an unmodified SAG1 gene accumulated 5- to 10-fold more than leaves agroinfiltrated with a codon-optimized SAG1 gene, although transcript accumulation was similar. The expression level of the unmodified SAG1 was 1.3 μg/g of fresh weight, while that of the codon-optimized SAG1 was 0.4 μg/g of fresh weight. In addition, the endoplasmic reticulum localization allowed the accumulation of higher levels of unmodified SAG1 compared to localization in the apoplast (1.3 μg vs. 0.7 μg/g of fresh weight, respectively). The authors suggested that the endoplasmic reticulum provided a relatively protective environment, which resulted in an increase in protein stability and an enhanced level of protein accumulation (Laguía-Becher et al. 2010).

Finally, Albarracín et al. (2015) expressed SAG1 in transplastomic tobacco plants. In addition, to improve expression in transplastomic plants, these authors expressed the 90-kDa heat shock protein (HSP) of *Leishmania infantum* (LiHsp83) as a carrier for the SAG1 antigen. Fusion of SAG1 to LiHsp83 significantly increased the level of SAG1 accumulation in tobacco chloroplasts (by up to 500-fold). The authors showed that LiHsp83-SAG1 protein accumulation did not decrease significantly as the plant aged, yielding up to approximately 100 μg/g of fresh weight. These authors proposed that LiHsp83 is a promising candidate to function as a carrier protein for the expression of vaccine antigens in plants.

Studies on the immunogenicity of the plant-made SAG1 have shown that, when this antigen is expressed in plants, it is able to elicit an immune response by subcutaneous (s.c.) or oral vaccination in a murine model (Clemente et al. 2005; Laguía-Becher et al. 2010; Albarracín et al. 2015). These findings provide a rationale for the development of a plant-made oral vaccine against toxoplasmosis. Our group was the first to demonstrate the immunogenic properties of SAG1 (Clemente et al. 2005). We found that s.c. immunization with SAG1-infiltrated leaf extracts emulsified with Freund's incomplete adjuvant induced a significant

increase in the systemic-specific antibodies and a reduction of number of cysts (55%) against *T. gondii* (Clemente et al. 2005). Then, Laguía-Becher et al. (2010) demonstrated that plant-made SAG1 is able to induce a reduction in the brain cyst burden (30%) when delivered s.c. or orally without any adjuvant. In addition, Laguía-Becher et al. (2010) showed that this protection is associated with the secretion of significant levels of IFN-γ and that the protection can be increased with a significant reduction in the parasite load (54%) when mice were intradermally boosted with rSAG1 (SAG1 + boost). Similarly, in an oral immunization assay, these authors demonstrated that the SAG1 + boost group showed a significantly lower brain cyst burden (50%) than the rest of the groups. More recently, Albarracín et al. (2015) found that oral immunization of mice using SAG1 fused to LiHsp83 elicited an effective immunity against Toxoplasma infection, suggesting that the LiHsp83-SAG1 fusion protein retains the structural integrity to elicit immunological responses in mice.

4.3 Plant-Derived Vaccines as an Alternative Treatment

Several factors, including the economic losses associated with *T. gondii* infection in farm animals, the risk of transmission of the parasite to animals and humans, the unsatisfactory chemotherapy associated with increasing drug resistance, and the drug residues entering the food chain, justify the current attempts to develop an effective prophylactic *T. gondii* vaccine for both humans and animals. For all these reasons and since a vaccine against *T. gondii* is considered the most efficient method to prevent this infection, in the last few years, much progress has been made in research on DNA vaccination, protein vaccination, live attenuated vaccination and heterologous vaccination. New vaccine candidates, including SAGs and secretory antigens (ROP, MIC, and GRA organelles), have been tested either individually or as multi-antigen vaccines and novel adjuvants. However, researchers have not been able to find a proper vaccine for prevention of toxoplasmosis in animals and/or humans (Zhang et al. 2013, 2015; Montazeri et al. 2017). One possible explanation for this is that most studies have used single or only a few antigen candidate vaccines eliciting only partial protective immunity against *T. gondii* and never allowed a complete protection against tissue cysts (Zhang et al. 2013). This could be due to the low number of T lymphocytes epitope generated after administrating vaccines with a single antigen. In this way, experimental vaccines should include many antigens (Jongert et al. 2009; Hiszczyńska-Sawicka et al. 2014).

Our laboratory is mainly focused on studying the potential of plant-produced *T. gondii* antigens as a mucosal vaccine. SAG1 and GRA4 are considered the antigens with the greatest potential to be incorporated in a multicomponent vaccine against *T. gondii* (Lim and Othman 2014). Therefore, the achievement of efficiently expressing these antigens in plants is a success per se. In this sense, GRA4 and SAG1 antigens have been correctly expressed in tobacco using various strategies

(Ferraro et al. 2008; Del L Yácono et al. 2012; Clemente et al. 2005; Laguía-Becher et al. 2010; Albarracín et al. 2015). When transiently expressed in tobacco leaved via agroinfiltration, yields of GRA4 were around 0.2 μg/g of fresh weight (Ferraro et al. 2008), while GRA4 expression levels in transplastomic plants were up to 6 μg/g of fresh weight (Del L Yácono et al. 2012). Therefore, the best yields were obtained by plastid transformation (Del L Yácono et al. 2012). Similarly, yields of SAG1 from transient expression via agroinfiltration were approximately 1.3 μg/g of fresh weight (Laguía-Becher et al. 2010), while SAG1 accumulated in transplastomic plants at around 0.2 μg/g of fresh weight (Albarracín et al. 2015). However, the best attained yields for SAG1 protein were achieved when it was fused to the carrier/adjuvant LiHsp83 chaperone and this fusion protein was expressed in chloroplasts (100 μg/g of fresh weight) (Albarracín et al. 2015). This result suggests that the fusion of the protein of interest to chaperones like Hsp90 could be implemented as a new strategy. Since plants are considered a new platform to produce drugs and vaccines, the ability of HSPs to chaperone peptides could provide stability to the recombinant protein, increasing the production yields and providing added value to plant-based platforms.

However, the protection achieved can be also improved expressing various antigens in the same plant tissue. This can be accomplished through transient expression by vacuum agroinfiltration, which has been demonstrated to be a versatile system that allows the simultaneous expression of several recombinant proteins. The main advantage of the transient expression plant platform is that antigen-encoding genes can be cloned in different constructs and then later introduced in leaves by a single event of agroinfiltration. However, the expression levels of different antigens are very dissimilar and this generates variability in the antigens ratios. An alternative is to produce each antigen in a separate plant and then combine this material to reach the required dose. In this case, different expression systems such as nuclear and/or chloroplast transformation could be used. These strategies allow several antigens of *T. gondii* to be simultaneously accumulated within the same delivery system and to obtain a multicomponent vaccine that addresses one of the main challenges for the production of a vaccine against toxoplasmosis. In the future, new strategies of immunization should be implemented to improve the degree of protection. The combination of GRA4-SAG1 mixed plant material, as well as the incorporation of new antigens and/or the use of heterologous vaccination protocols like intradermal or intranasal boost, could contribute to achieving a higher level of protection against Toxoplasma infection.

A remarkable feature of *T. gondii* is that the main route of host infection is oral. The main form of infection in herbivores is with oocysts and the route of infection in pigs and humans is through tissue cysts. So, local immunity in the gut via lymphocytes (mainly intraepithelial lymphocytes with CD8+ activity) and IgA is of fundamental importance in host resistance to the parasite (Bourguin et al. 1993). A potential strategy to resolve this problem would be the implementation of plant tissue as a vehicle for vaccine antigens (Fig. 3). The production of plant-derived vaccines has been widely assessed in the last 20 years, with several antigens from human and animal pathogens being correctly expressed and shown to produce a

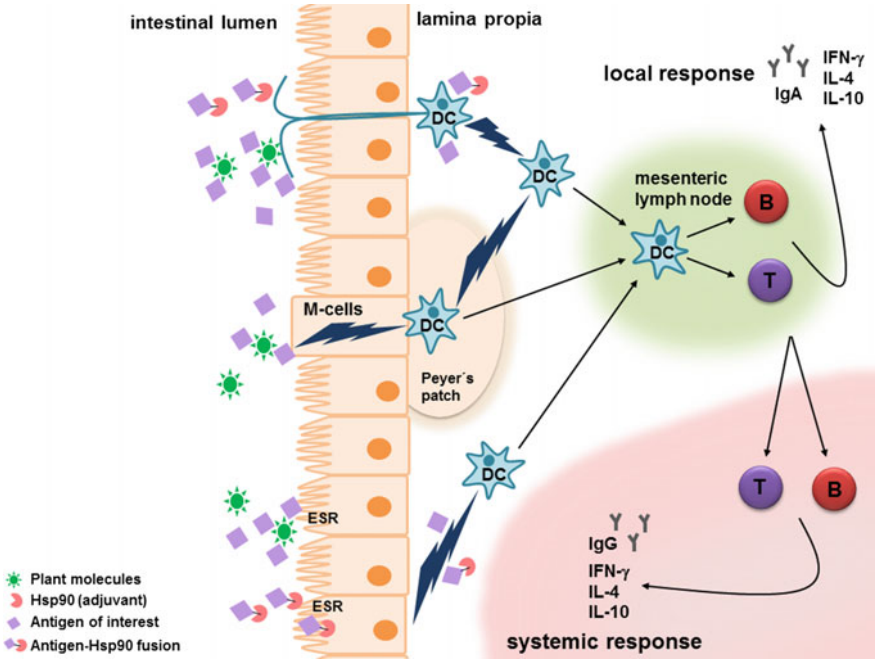

Fig. 3 Hypothetical mechanism to explain the immune response triggered by antigens of *T. gondii* expressed in plants in oral administration. Plant molecules with adjuvant properties or Hsp90 (90-kDa Heat shock protein) as a carrier enhance the antigenicity of the co-administered soluble antigen. Plant molecules and/or Hsp90 proteins as carriers/adjuvants facilitate the delivery of encapsulated antigens and can promote interactions with immune-responsive cells at mucosal surfaces through epithelial surface receptors (ESR), M cells with a subsequent processing by dendritic cells (DCs) and/or a direct uptake by DCs through dendrites that cross the epithelium. Plant-based vaccines carrying *T. gondii* antigens stimulate local immune responses, inducing the production of IgAs, IL-4, IL-10 and IFN-γ and the systemic immune responses by the stimulation of production of IgGs and IL-4, IL-10 and IFN-γ

specific humoral response, and in some cases, a protective response against infection in murine models (Kong et al. 2001; Clemente et al. 2005; Gómez et al. 2008; Santi et al. 2008; Kostrzak et al. 2009; Zhang et al. 2010; Laguía-Becher et al. 2010; Gonzalez-Rabade et al. 2011). One of the main advantages of plants is that their tissues provide a natural environment for antigen encapsulation, which protects the antigen from degradation (Limaye et al. 2006; Hayden et al. 2012). In this way, when the plant tissue is digested, a sufficient quantity of antigen can be captured from the mucous membranes and stimulate an immune response (Berinstein et al. 2005; Kapusta et al. 2010). For this reason, plants are an ideal vehicle for oral vaccine administration.

Oral immunization with chlGRA4 showed that this antigen can elicit an immune protective response, like chlLiHsp83-SAG1, when administrated without any exogenous adjuvant supplement. Oral immunization with *T. gondii* GRA4 antigen

expressed in transplastomic tobacco elicits both mucosal and systemic immune responses (Del L Yácono et al. 2012). Several publications have suggested that plant tissues provide protection and prevent degradation of the antigen when it passes through the gut (Yusibov and Rabindran 2008; Paul and Ma 2010; Hayden et al. 2012). These data imply that orally delivered plant-made antigens can be processed to elicit systemic humoral and cellular responses. Evidently, additional adjuvants present in the plant material could contribute to the modulation of the immune response (Fig. 3). In fact, plant secondary metabolites including lectins, saponins, alkaloids, phenolic compounds, and flavonoids are beginning to be exploited as a source for adjuvant capacity (Granell et al. 2010; Vajdy 2011; Rosales-Mendoza and Salazar-González 2014). It has been demonstrated that some phytochemicals and proteins present in plants could synergistically affect the immunogenicity of plant-expressed antigens, acting as endogenous adjuvants (Licciardi and Underwood 2011; Buriani et al. 2011; Corigliano et al. 2011). In this context, plants are an ideal vehicle for oral vaccine administration and should continue to be explored for the development of an anti-*T. gondii* vaccine.

Another important challenge for the development of a successful vaccine against *T. gondii* infection is to find appropriate adjuvants that would facilitate the transport of the antigen from the gut lumen to gut-associated lymphoid tissues. In this context, the use of adjuvants for improving or enhancing the immune response induced by the antigen cocktail expressed in plants should be also explored. In plants, adjuvants and antigens could be expressed in the same plant as recombinant fusion proteins (Fig. 3). Thus, adjuvants could be either co-delivered with the antigen or incorporated to the delivery system with the antigen. In the *T. gondii* model, the most promising results were attained using LiHsp83-SAG1 in oral immunization since LiHSP83-SAG1-immunized mice showed a significant decrease of parasite load (60%) compared to the control group (Albarracín et al. 2015). These results suggest that oral immunization with leaf extract-expressed antigen could be improved by using efficient adjuvants (such as Hsp90) to enhance immunogenicity. Interestingly, Corigliano et al. (2013) demonstrated that the covalent linkage of plant Hsp90 (pHsp90) fused to maltose binding protein (MBP) as a reporter antigen is essential to induce anti-MBP antibodies, causing predominance of specific IgG2a isotype and IFN-γ secretion. In this context, these pHsp90 s were fused to antigenic proteins or peptides to assess their immunomodulatory properties in adjuvant-free immunizations. In addition, Buriani et al. (2012) showed that plant Hsp70 purified from plant tissue transiently expressing the influenza virus nucleoprotein is able to induce both the activation of major histocompatibility complex class I restricted polyclonal T-cell responses and antibody production in different mouse strains without the need of exogenous adjuvants addition. All these results suggest that HSPs could be used as novel carriers for vaccine antigen candidates to improve the immunogenicity property of the plants as delivery vehicles of such antigens (Fig. 3).

The choice of adjuvants for co-expression with antigens in plants depends upon the proved efficacy of the specific adjuvant with a selected antigen. In this way, the expression of adjuvant–antigen as a fusion protein would help to improve the

formulations based on plant-made vaccines. The benefit of using a plant system for antigen and adjuvant production is that plants are able to express, process and assemble complex proteins, favoring the formation of recombinant immunogenic complexes inside the plant, which could be subsequently purified or partially purified to then be orally or nasally administered. In addition, adjuvants and antigens can be co-produced directly in edible plants. The main advantage of edible plant-based vaccines is that they are highly safe and cost-effective. Several researchers have demonstrated the potential of edible vegetables and fruits as vaccines against cholera, measles, hepatitis-B, Norwalk virus and rabies virus (Ahmad et al. 2012; Mason and Herbst-Kralovetz 2012). In particular, some plant-based vaccines developed in edible crops have been tested in Phase I clinical trials against diarrheal disease, hepatitis B, and against rabies (Yusibov et al. 2011). Therefore, recombinant immunogenic complexes or adjuvant-protein fusion proteins expressed in edible tissues or seeds could be directly administered by oral delivery as lyophilized tissue or as seed bioencapsulations, thus reducing delivery costs. While expression of *T. gondii* antigens in edible plants has not yet been explored, oral immunization would be the perfect way to obtain the appropriate immunity against this pathogen and an edible vaccine should therefore be pursued in the future.

5 Pathway to Commercialization

Vaccination against bacterial and viral diseases is widespread, routine, and successful, but only a few vaccines for veterinary protozoan diseases have been developed successfully (Garcia et al. 2014). Development of an effective acellular vaccine against toxoplasmosis is a great challenge for medical and veterinary science. One point in favor of using vaccination as a method of toxoplasmosis control is that after the primary infection with *T. gondii*, the host develops an effective protective immunity against the disease (Innes and Vermeulen 2006). Much progress has been made in the understanding of how to induce and regulate protective immune responses, encouraging a real optimism in developing cost-effective new vaccines that may be suitable for large-scale production.

The target antigens chosen for the development of an effective vaccine formulation should: (1) limit acute infection and protect against congenital toxoplasmosis; (2) reduce tissue cysts; and (3) reduce oocyst shedding in cats to avoid environmental contamination (Innes and Vermeulen 2006; Hiszczyńska-Sawicka et al. 2014). As a main goal of anti-*T. gondii* vaccination in animals is to reduce infection in humans, a successful vaccine is needed for both consumption animals and cats. A vaccine for farm animals should prevent parasite transmission to other animals and humans, while a vaccine for cats should prevent environmental contamination and infection risk for intermediate hosts. Generally, vaccine studies have shown that multi-antigenic formulations confer better protection than single-subunit vaccines. Several antigens have been shown to efficiently stimulate an effective

response, which could be included in a multi-component vaccine. SAG1, GRA2, GRA4, GRA7, ROP2, ROP5, MIC2, MIC3, MIC4, M2AP and AMA1 are strong candidates to develop an effective acellular vaccine that prevents oocyst shedding by cats and tissue cyst formation in food animals, which combined would have great impact on environmental contamination and consequently on public health (Zhang et al. 2013). However, so far no acellular vaccine against toxoplasmosis has been commercialized, and an effective vaccine to prevent toxoplasmosis remains to be developed. Most parasitologists and vaccinologists agree that future efforts should be concentrated on developing multi-antigen vaccines, developing more efficient delivery systems able to express heterologous proteins abundantly, and determining appropriate immunization schedules and adjuvants to enhance the protective responses. To this end, the platforms for the production of acellular vaccines based on the use of plants can have an important role.

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