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***Toxoplasma* Effectors that Affect Pregnancy Outcome**

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Abstract

As an immune-privileged organ, the placenta can tolerate the introduction of antigens without inducing a strong inflammatory response that would lead to abortion. However, for the control of intracellular pathogens, a strong Th1 response characterized by the production of interferon- γ is needed. Thus, invasion of the placenta by intracellular parasites puts the maternal immune system in a quandary: The proinflammatory response needed to eliminate the pathogen can also lead to abortion. *Toxoplasma* is a highly successful parasite that causes lifelong chronic infections and is a major cause of abortions in humans and livestock. Here, we discuss how *Toxoplasma* strain type and parasite effectors influence host cell signaling pathways, and we speculate about how this might affect the outcome of gestation.

***Toxoplasma*, Placenta, and Immune Response**

Toxoplasma gondii is an obligate intracellular parasite that infects most warm-blooded animals and is the causative agent of toxoplasmosis (Box 1). To be transmitted, *Toxoplasma* needs to form orally infectious tissue cysts in intermediate hosts. Hence, it is in the parasite's best interest to ensure that its host survives infection, which is achieved by the induction of an immune response able to control parasite multiplication. However, to ensure that not all parasites are eliminated by this immune response, *Toxoplasma* uses a dedicated arsenal of effectors that prevents its killing. This balance between activating the immune response to drive the formation of the chronic tissue cyst stages and the inhibition of the immune response to ensure its survival determines *Toxoplasma*'s success in a particular animal species. It is unlikely that every *Toxoplasma* strain can optimally balance activation of the immune response with inhibition of its killing in every animal species. Different *Toxoplasma* strains (Box 2) likely evolved to perfect this balancing act in particular animal species. Thus, *Toxoplasma* virulence leading to the death of its host could be seen as a mismatch between host species and *Toxoplasma* strain. Similarly, in some host genetic backgrounds infected with particular strains, the strong immune response induced by the parasite could lead to immunerelated pathology. Together with other protozoan parasites, such as *Neospora caninum* or *Trypanosoma cruzi*, *Toxoplasma* can invade the placenta, infect the fetus, and cause abortions in animals and humans. Specific *Toxoplasma* strain–host combinations may lead to detrimental outcomes during pregnancy. A strain that is very efficient in evading the immune response could easily multiply in the placenta and be

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transmitted to the fetus, causing its death and the subsequent abortion. By contrast, a strain that induces a strong immune response could elicit exacerbated inflammation in the placenta that disrupts the normal course of gestation, resulting in a failure to maintain the fetus. In this opinion article, we cover the different factors that could affect the outcome of gestation during *Toxoplasma* infection, focusing on parasite effectors and host immune genes.

Toxoplasma Dissemination to the Placenta

Toxoplasma resides within a nonfusogenic vacuole called the ‘parasitophorous vacuole’ (PV). The key to *Toxoplasma*’s successful co-option of the host resides in its three secretory organelles: micronemes, rhoptries, and dense granules. Microneme proteins (MICs) and rhoptry neck proteins mediate the mechanics of host cell invasion, while rhoptry bulb proteins (ROPs) and dense granule proteins (GRAs) are involved in modulating host cell signaling pathways and evasion of the immune response [1]. Once *Toxoplasma* forms its replicative niche inside the host cell, it multiplies by endodyogeny (i.e., a form of cell division in which two daughter cells are produced within the mother cell) until it egresses, lysing the host cell in the process, and spreads to invade neighboring cells [2].

After crossing the intestinal epithelial barrier, *Toxoplasma* disseminates throughout its host and reaches immune-privileged sites such as the brain, eyes, or placenta. The *Toxoplasma* WAVE regulatory complex (WRC)-interacting protein (*Tg*WIP) plays a pivotal role in parasite dissemination [3]. This effector is an ROP secreted upon invasion that interacts with the host WRC and SHP2 phosphatase, both of which regulate actin dynamics [4,5]. It affects the morphology of dendritic cells (DCs) and mediates the dissolution of **podosomes** (see Glossary), which are used by these cells to adhere to extracellular matrices. Hence, it enhances the motility of DCs, likely providing the parasite with a **Trojan horse** mechanism to disseminate throughout the host [6]. The *Toxoplasma* orthologue of the 14-3-3 protein family (*Tg*14-3-3) can also mediate hypermotility in infected DCs in a γ -amino butyric acid-dependent fashion [7]. Another parasite effector that plays an important role in *Toxoplasma* dissemination is the secreted kinase ROP17, which promotes the migration of infected monocytes [8]. Although the specific monocyte subsets affected and exact mechanisms involved are not fully understood, the process seems to occur through host Rho-associated protein kinase signaling. Regardless, upon parasite dissemination throughout the organism, *Toxoplasma* can use a variety of infected cells, including monocytes and natural killer (NK) cells, as carriers [9,10] or use paracellular/transcellular mechanisms allowing extracellular parasites to transmigrate across biological barriers such as ocular, placental, or blood–brain barriers [6,11]. Leukocytes have been described to use both **integrin**-dependent and -independent mechanisms during interstitial migration, both of which are well suited to promote *Toxoplasma* dissemination [11]. Although integrins were once thought to be critical for leukocyte migration, recent studies have shown that DCs, neutrophils, and T cells can still migrate in the interstitial tissue in the absence of integrins [12,13]. In this sense, *Tg*WIP would favor this latter mechanism because it promotes the dissolution of podosomes in infected DCs, which are needed for the integrin-dependent transmigration [3]. Likewise, ROP17 supports an integrin-independent interstitial migration by inducing changes in monocyte motility to facilitate *Toxoplasma* systemic dissemination [8].

After reaching the placenta, it is not completely clear how *Toxoplasma* crosses the placental barrier. Nevertheless, most published data on transmission from mother to fetus are consistent with *Toxoplasma* first infecting maternal leukocytes and subsequently using those as Trojan horses to reach the uterine lining forming the maternal part of the placenta (**decidua**) [14]. Thereafter, *Toxoplasma* invades trophoblasts and subsequently the villous core and fetal vasculature [15,16]. Because *TgWIP* plays a pivotal role in parasite dissemination [3], it is tempting to hypothesize that this effector is also critical for the parasite to reach the placenta and be transmitted to the fetus. It would also be interesting to assess the importance of this gene in the closely related parasite *N. caninum*, as well as in *TgWIP* polymorphisms among different *Toxoplasma* strains that could affect its function.

Immune Response in the Placenta

The maternal–fetal relationship is not simply maternal tolerance of a foreign tissue, but a series of intricate cytokine interactions shaping an appropriate immune regulation. A successful gestation depends on multiple immunologic mechanisms designed to suppress antifetal immunity within the placenta [17]. The expansion of the regulatory T-cell (Treg) subset and a maternal shift toward a Th2 immune response creates a ‘tolerant’ microenvironment during pregnancy by preventing harmful effects from Th1 and Th17 cells [18-20]. The latter are involved in the recruitment of neutrophils to infected tissues, which are good at destroying extracellular parasites but can also cause tissue damage, thus promoting immunopathology [21]. The so-called **Th1/Th2 paradigm** seemed to be a convincing explanation for the challenging question why the fetus is not rejected by the maternal immune system despite the presence of paternal antigens in contact with maternal cells. However, this paradigm appears to be outdated and insufficient to explain the causes of pregnancy loss, because it has been shown that some Th1 cytokines, such as interferon (IFN)- γ or interleukin (IL)-12, may have some protective role during pregnancy, and neither maternal nor fetal production of the anti-inflammatory or regulatory cytokines IL-4 and IL-10 are crucial for the completion of successful allogeneic pregnancies in mice [22,23]. These conflicting findings may be explained by the incorrect consideration of pregnancy as a single homogeneous event that resembles a host-graft model. Rather, gestation should be considered as a dynamic developmental process with different immunological stages, having more similarities to the immune processes taking place during tumor progression and cell metastasis [24]. Indeed, metastatic cell progression resembles the events taking place during gestation: attachment, invasion of tissues, gradual antigen presentation, inflammation, and tolerance induction. Similarly, in the first stages of pregnancy, there is a proinflammatory environment that ensues from implantation and placentation, then there is a shift to an anti-inflammatory state that allows fetal growth, and finally a shift back to a proinflammatory stage that promotes labor and delivery (Box 3). Most studies investigating the gestational immune response have been conducted during the second stage, which is the longest period; hence the generalization that pregnancy is characterized by a Th2 environment [25]. However, pregnancy is both a proinflammatory and anti-inflammatory condition. Thus, its success depends on the ability of the maternal immune response to establish an effective barrier to protect the mother and the fetus against external harms while being permissive at the same time with the nourishment of the developing fetus [24,26]. A failure of this

equilibrium could lead to alterations in the placenta causing fetal damage and abortion without the actual transmission of an invading pathogen [27-29].

In the case of *Toxoplasma* infection, the alteration of the Th1/Th17 vs. Th2/Treg balance in the placenta might play a pivotal role in the occurrence of abortion. Indeed, Tregs are critical in *Toxoplasma* infections during pregnancy because their reduction has been associated with fetal loss, regardless of vertical transmission [30,31]. *Toxoplasma* causes a decrease in Tregs and transforming growth factor (TGF)- β levels at the maternal–fetal interface, thus preventing the control of an exacerbated immune response [32-34]. This decrease in Tregs may be multifactorial, likely caused by **apoptosis** [30], limitation of proliferation [33], and/or differentiation into Th17 cells in the presence of IL-6 [33,36]. The critical role of TGF- β in *Toxoplasma* infection during gestation has been confirmed in pregnant mouse models, where the treatment with TGF- β improves adverse effects by increasing Treg differentiation but not proliferation [37,38]. It is noteworthy that most of these studies were carried out in mice and with the type I RH strain that naturally does not promote an exacerbated inflammatory response. Thus, it is possible that other proinflammatory strains, such as type II, may have a different effect. For instance, type II strains induce IL-6 expression through GRA15, while types I and III strains inhibit IL-6 through ROP16 [39,40].

Regardless, it seems clear that *Toxoplasma* infection during pregnancy puts the maternal immune system in a quandary. The strong Th1 response associated with the production of IFN- γ and tumor necrosis factor (TNF)- α after infection with *Toxoplasma* is a double-edged sword. Although these cytokines are needed for the control of the pathogen, they can also be deleterious for the maintenance of gestation, leading to enhanced transmission and fetal damage [32,41]. For example, IFN- γ together with either TNF- α or IL-1 β upregulates adhesion receptors such as **intercellular adhesion molecule 1 (ICAM1)** on trophoblasts, leading to increased adhesion of *Toxoplasma*-infected maternal monocytes to these cells and thus facilitating crossing of the placental barrier by the parasite [40]. In addition, the macrophage migration inhibitory factor (MIF) can induce the expression of ICAM1 on the surface of trophoblasts [43,44]. *Toxoplasma* also produces a homologue of this cytokine (*Tg*MIF) that facilitates immune evasion and intracellular parasite multiplication. This factor stimulates antiapoptotic pathways through phosphorylation of extracellular signal-regulated kinases, induction of IL-8 secretion, and increase of host cell proliferation and differentiation, globally favoring intracellular parasite multiplication [45,46]. In fact, a recent study described an association between high IL-8 production and parasite transmission to the fetus in pregnant women [41]. This could be explained by an increased immune cell attraction to the placenta, which would in turn favor parasite replication within trophoblastic cells and dissemination to the fetus. IFN- γ and other Th1-produced cytokines can also induce vascular damage (congestion, thrombosis, and/or hemorrhage), leading to a reduced nutrient/oxygen transport in the placenta [47] that may cause embryo resorption or fetal damage without actual vertical transmission. Furthermore, although the precise role of the Treg chemoattractant CC chemokine ligand 22 (CCL22) during *Toxoplasma* infection is not well understood, it has been shown to be highly upregulated in infected human **syncytiotrophoblasts** in a manner that is dependent on the *Toxoplasma* c-Myc regulation

(MYR) protein 1 (MYR1) [48]. This secreted protein is localized at the PV membrane and participates in the translocation of *Toxoplasma* GRA effector molecules to the host cell cytoplasm [49]. Furthermore, very recently, it has been shown that GRA28, a secreted *Toxoplasma* effector, through MYR1, regulates the secretion of CCL22 in murine and human cells, including syncytiotrophoblasts [50]. Because CCL22 is known to be constitutively expressed during pregnancy and has been found to be increased in miscarriage cases [51], it is possible that the increased secretion of this chemokine by infected trophoblasts may contribute to the abortion in *Toxoplasma* infections.

Outcome of *Toxoplasma* Infection during Gestation

T. gondii can be vertically transmitted from mother to fetus, leading to miscarriages, stillbirths, and other adverse outcomes, depending on the stage of gestation at which infection takes place [20]. There is an inverse relation between gestation period and fetal death rate. Vertical transmission in the first trimester is infrequent, although it usually triggers fetal death and abortion because the immune system of the fetus is still underdeveloped. In the second trimester, the rate of vertical transmission increases, and fetuses are commonly born prematurely or with severe conditions, such as hydrocephalus, microcephaly, or mental retardation. In the third period of gestation, fetal infection is likely to occur, and although most babies are asymptomatic at birth, they may develop neurological, ophthalmological, or auditory diseases months or years later [14,52]. These observations were reported mainly from pregnant women, and although similar outcomes have been described in other animals, it is possible that a different scenario might occur in more susceptible or resistant animals. In pregnant ewes, two different clinical presentations of toxoplasmosis have been observed: late, classical abortion produced approximately 1 month after infection and early abortion produced during the acute phase of the disease (within the first 2 weeks after infection). Moreover, apart from the time of gestation, the outcome of infection during pregnancy greatly depends on the parasite strain and the host species. In the following sections, these aspects are covered individually.

Late Classical vs. Early Abortion

Typically, pregnant ewes abort 1 month after *Toxoplasma* infection. The underlying mechanisms are attributed to the tissue damage caused by the multiplication of the parasite in the placenta and, after crossing the placental barrier, in the fetus, ultimately leading to fetal death [15,28]. However, aside from this late 'classical' abortion, another type, named 'early abortion,' has also been described [53,54]. The mechanism of early abortions during the acute phase of the disease is not well understood but has been associated with hypoxic damage to the fetus without the direct implication of the parasite; hence, these abortions were also described as 'sterile.' These early abortions occurred within the first 2 weeks after oral infection with M4 type II oocysts (Box 1), regardless of the time of gestation and surprisingly even with doses as low as 50 oocysts [53-55]. Nevertheless, early abortions were more frequently found in ewes infected in the second trimester of gestation, whereas late abortions were more common after infection with lower doses and at day 40 (first trimester) or 120 (third trimester) of gestation. In addition, placentas from ewes suffering early abortions showed lesions compatible with ischemic necrosis and thrombosis and had

higher expression levels of the proinflammatory cytokine TNF- α and the adhesion molecules vascular cell adhesion molecule 1 and ICAM1. Furthermore, ischemic lesions (leukomalacia) were found in the brain of some fetuses, and *Toxoplasma* DNA was detected in only half of the placentas and in none of the fetuses from the early abortions. Therefore, it seems likely that the acute inflammatory response and vascular lesions found in the placenta caused a disruption in nutrient or oxygen transport, consequently producing lethal hypoxic damage to the fetus [56].

Toxoplasma infections have been associated with spontaneous abortions in pregnant women; hence, it is likely that the abortions early after infection observed in sheep can also happen in humans. However, very few case–control studies have compared *Toxoplasma* seropositivity between women who had spontaneous abortions and women with normal pregnancies [57,58]. Likewise, the cause of most abortions in sheep and goats is unknown, although it is possible that early abortions during the acute phase of the disease are underdiagnosed in the field because there are no detectable antibodies/parasites when it occurs [53]. Thus, further studies are needed to shed light on these early abortions.

Influence of the Host

Very little is known about the correlation between *Toxoplasma* strain virulence in mice compared with other animal species and humans. In fact, some studies have shown that the virulence observed in mice by a certain strain cannot always be extrapolated to other animals. For instance, in a recent study, the type II ME49 strain was compared in pregnant mouse and sheep models, being more virulent in the former [59]. This discrepancy is probably due to differences in the physiology of gestation, placentation, or immune response, among others. This inconsistency between murine and ruminant models is also observed with *N. caninum*, the closely related apicomplexan parasite causing abortion in cattle [60]. In humans, limited data have been obtained so far from clinical studies on pregnant women infected with *Toxoplasma* [58]. This is mostly due to the relatively low number of human cases and the limitations of the study of processes taking place in the placenta, because these can only be assessed at the end of pregnancy, and the kinetics are difficult to elucidate [20]. In this section, we focus on different animal models that have been used to study abortion and congenital toxoplasmosis. Although animal models are highly valuable to gain further knowledge of *Toxoplasma*-induced abortion in humans, in veterinary research, the results are also directly relevant to the disease in the host of interest. In general, most farm animals, including pigs, horses, sheep, and goats, are susceptible to abortion at different degrees, although sheep and goats are especially vulnerable [61]. By contrast, cows are very resistant to *Toxoplasma* infection, and abortions caused by this parasite are considered a rare event [61]; instead, another apicomplexan parasite, *N. caninum*, is responsible for a considerable part of cattle reproductive failure, similar to the effect that *Toxoplasma* has in sheep [62].

Despite the marked differences in the placenta and gestation, the mouse is the animal model most frequently used to study congenital toxoplasmosis. These differences include the time of gestation and the morphology and histological structure of the placenta, among others. For instance, rodents possess a discoidal **hemochorial** placenta, and the gestation period is 3

weeks, while ruminants have a **cotyledonary synepitheliochorial** placenta and longer gestations (5 months in small ruminants such as sheep or goat and 9 months in cattle) [63]. The availability of numerous transgenic and knockout mouse strains offers a valuable advantage to address specific questions [20,29]. While there are indeed many advantages in using this experimental model, care should be taken in extrapolating results from mice to other species, because their immune response to *Toxoplasma* is different from that of other hosts. Rats, like humans and other primates, do not display clinical signs after *Toxoplasma* infection; hence, they could serve as a better model of congenital toxoplasmosis [64]. However, although the placenta of rodents and humans have in common their hemochorial structure [63], the short duration of murine gestation restricts the value of this model [27]. Halfway between the rat and mouse, guinea pig sensitivity to *Toxoplasma* is medium, and it has a gestation time of approximately 2 months, long enough to enable comparative studies with different inoculation times. Thus, the pregnant guinea pig model could be a good alternative, although experiments based on this model are very scarce [65,66]. Moreover, in murine species, *Toxoplasma* is primarily detected by endosomal **Toll-like receptors (TLRs) 11/12**, which bind to the parasite actin-binding protein profilin [67]. Given that infection-induced inflammation is likely a major influence on parasite transmission to the fetus or spontaneous abortion, investigating the role of TLRs in abortion seems important. However, inflammation in rodents is mainly dominated by TLR11/12 activation, while larger animals, including humans, lack TLR11/12 [68]. Therefore, other TLRs, such as TLRs 2, 4, and 9, which have been shown to play an indirect role in immunity to oral *Toxoplasma* infection in rodents [69], could also be involved in abortion. Sheep have been used to investigate congenital toxoplasmosis because, similarly to humans, they do not develop clinical signs beyond transitory fever and are susceptible to *Toxoplasma*-induced abortion. In addition, sheep do not possess TLR11/12 [68]; therefore, this model provides a useful tool to study *Toxoplasma* congenital infection and abortion in close relation to humans. Indeed, as mentioned in the previous text, early abortions described in sheep might also happen in humans; thus, the sheep pregnant model could be used to help understand the pathogenesis of these abortions. Moreover, sheep are of interest to study in their own right because abortion due to congenital toxoplasmosis poses a major economic loss to the sheep industry worldwide [70].

Influence of the Strain

Several epidemiological studies have sought a connection between parasite strain and congenital infection in humans, but definitive conclusions have been difficult to reach (Box 2). Likewise, very little information is available in other animal species [61]. Hence, it is unknown whether infections with certain strains induce more abortion or differ in transplacental transmission, although it seems likely that the *Toxoplasma* strain type is an important factor in the outcome of gestation. Nonetheless, heterogeneous results have been described in pregnant models even within the same type II strain. For example, the Pru strain induced a decrease in fertility if infection occurred during the last two periods of gestation, regardless of host species [27,66,71,72]. In addition, apart from the genetic background, the *in vitro* passage number has also been proposed to exert an important influence in the parasite phenotype and biological characteristics, typically enhancing virulence in mice. This fact has been widely studied in more virulent type I strains such as RH and GT1 [73]

and recently in the less virulent type II ME49 strain [59]. Furthermore, inhibition of host cell death is another important trait that varies between strains, which could determine the outcome of infection. For instance, it is possible that more virulent strains are better at inhibiting apoptosis, because it has been shown that BeWo choriocarcinoma cells are more likely to undergo apoptosis upon infection with ME49 than with RH [74]. Finally, the ability to subvert the host immune response is one of the best studied strain-specific *Toxoplasma* traits. For example, in mice, ROP5/ROP18 prevents the IFN- γ -induced **immunity-related GTPase (IRG)** coating of the PV membrane, thereby limiting parasite clearance [75]. However, what determines parasite survival and avoidance of the immune response in other species that do not possess IRGs is currently unclear. Herein, we focus on genes that are directly implicated in the induction of a potentially damaging proinflammatory response that might influence the outcome of infection.

It is well known that type II strains induce a much stronger proinflammatory response than type I and type III strains [1]. Quantitative trait locus analysis performed in F1 progenies identified the *Toxoplasma* genes that determine most of the strain differences in the modulation of the inflammatory response: the secreted proteins ROP16 [76] and GRA15 [77]. On the one hand, through the **nuclear factor- κ B (NF- κ B)** pathway, type II GRA15 (GRA15_{II}) induces the production of inflammatory cytokines, such as IL-12 and IL-1 β , and elicits **classically activated (M1)** macrophages that are responsible for Th1-dominant immunity [39,78,79]. On the other hand, type I/III ROP16 (ROP6_{I/III}) phosphorylates **signal transducer and activator of transcription (STAT) 3/6**, driving macrophages toward an **alternatively activated (M2)** state that is associated with increased IL-10 production and a Th2 polarized response [40,80]. In addition, ROP16 can inhibit GRA15-mediated activation of NF- κ B [40], providing a safe niche for parasite replication. Moreover, although all three clonal lineages express an active form of GRA24, the GRA24-mediated activation of p38 mitogen-activated protein kinase only has a significant effect on the classical activation of macrophages (M1) when combined with type II GRA15-induced NF- κ B activation, resulting in a potent inflammatory response [81,82]. Besides type II strains, we have shown that Cougar (type XI) and other closely related type XII strains (Ari, WTD3, or B41) induce a stronger proinflammatory response than other clonal strains by stimulating a strong secretion of IFN- β 1 in both murine bone marrow-derived macrophages and human fibroblasts [83].

Because *Toxoplasma*-induced placental inflammation is associated with early abortions, it is likely that *Toxoplasma* effectors inducing a proinflammatory response play an important role in the pathology of such cases (Figure 1, Key Figure). In this sense, some studies have tested the effect of different type Chinese 1 (ToxoDB 9) strains on adverse pregnancy outcomes in mouse models [29,34]. These strains carry a mixture of the GRA15_{II} and ROP16_{I/III} effectors that is different from those of the archetypal types commonly found in Europe and North America [84], therefore having an opposing inflammation-inducing feature. It is possible that other parasite effectors might be responsible for the biased immunity in the placenta; however, these studies support the notion that GRA15 and ROP16 may play an important role in the pathogenesis of abortion. Indeed, an ROP16-knockout version of the WH3 strain (GRA15_{II}/ rop16_{I/III}) induced increased apoptosis *in vitro* when infecting

trophoblasts and higher resorption rates *in vivo* than the wild-type strain. Moreover, the increased IFN- γ , TNF- α , IL-17, and IL-12, together with the decreased IL-4, TGF- β , and IL-10 levels observed in the WH3 *rop16* strain mimicked the typical type II proinflammatory response and confirmed the critical role of GRA15 in the induction of immune-induced pathogenicity in the placenta [29]. Finally, an RH *rop16* strain, which lacks an active form of GRA15, was assessed in pregnant mice. Compared with the wild-type strain, infection with the RH *rop16* strain produced higher resorption rates and promoted an M1 phenotype in macrophages from placental homogenates, with increased TNF- α and IL-12 levels and decreased TGF- β [85].

Taken together, these studies strongly suggest that in a dominant GRA15_{II} background, as it naturally happens in type II strains, a subversion of the immune tolerance, attributed to an M1/Th1/Th17 biased response, takes place at the maternal–fetal interface and leads to fetal death. Nevertheless, because these studies were only conducted in mouse models, further studies in more relevant animals, such as pregnant ewes, are needed to confirm its importance in humans. Furthermore, it is likely that, in addition to GRA15 and ROP16, other *Toxoplasma* genes may contribute to the pathology of the adverse immunity imbalance in pregnant animals. For instance, only type I or III, but not type II, GRA6 induces the expression of the host transcription factor nuclear factor of activated T cells 4 [86], a factor that recruits immune cells through the local induction of chemokines such as CXC chemokine ligand 2 (CXCL2) and CCL2 [87], while only type II GRA25 is able to dampen the expression of the chemokines CCL2 and CXCL1 [88].

Concluding Remarks

Although recent advancements have contributed to a better understanding of the role that certain *Toxoplasma* effectors exert on the outcome of infection during gestation, many questions remain to be addressed (see Outstanding Questions). It is likely that different *Toxoplasma* genes can predispose toward early vs. late abortions. For example, on the one hand, *Toxoplasma* gene products that induce host inflammation could lead to the elimination of the parasite but with the risk of early abortions. On the other hand, *Toxoplasma* virulence factors involved in evasion of the host immune response and dissemination will likely predispose toward late (classical) abortions caused by the vertical transmission and fetal damage caused by replicating parasites. Different polymorphisms or combinations of active forms of such factors could give each particular strain a specific phenotype that could predict the most likely outcome of gestation. In this sense, serotyping techniques may assist researchers to perform further investigations to associate particular strains with abortions [89]. Likewise, because unexplained abortions are often not further investigated, serotyping in these cases would allow investigators to determine if specific *Toxoplasma* strains are involved. Finally, further studies are needed to elucidate the pathological mechanisms involved in the outcome of gestation and the effect that particular parasite factors and strains have in abortion and vertical transmission.

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Glossary

Alternatively activated (M2)

M2 macrophages decrease inflammation by secreting anti-inflammatory and regulatory cytokines, allowing tissue repair. These macrophages are less efficient in killing intracellular pathogens.

Apoptosis

a form of programmed cell death characterized by changes such as blebbing, cell shrinkage, or nuclear fragmentation, among others.

Classically activated (M1)

M1 macrophages are generally good at killing intracellular pathogens, but their secretion of proinflammatory cytokines can cause pathology through an excessive activation of NK and Th17 cells and induction of proapoptotic pathways.

Cotyledonary synepitheliochorial

type of placenta observed in ruminants that consists in multiple discrete areas of attachment to the endometrium (placentomes) formed by a fetal portion (cotyledon) and a maternal contact site (caruncle).

Decidua

modified endometrial layer forming the maternal part of the placenta in close contact with trophoblasts. It protects the embryo from external insults and provides nutritional support.

Hemochorial

type of placenta present in rodents, high-order primates, and humans where maternal blood is in direct contact with the fetal chorion. It is characterized by a limited cellular barrier and extensive intrauterine trophoblast invasion.

Immunity-related GTPase (IRG)

family of IFN-inducible GTPases that have been implicated in resistance to intracellular pathogens. In mice infected with *Toxoplasma*, IRGs mediate the destruction of the intracellular PV.

Integrin

transmembrane receptor that, after binding to diverse ligands, facilitates cell adhesive interactions with other cells and with the extracellular matrix by cytoskeleton reorganization.

Intercellular adhesion molecule 1 (ICAM1)

also known as CD54, ICAM1 is a cell surface glycoprotein expressed on endothelial and immune cells that plays an important role in transendothelial migration of immune cells. It is a ligand for *Toxoplasma* MIC2.

Nuclear factor- κ B (NF- κ B)

a family of inducible transcription factors that play a key role in regulating genes implicated in the immune response. Some important members are RelA (p65), RelB, c-Rel, NF κ B1 (p50), and NF- κ B2 (p52).

Podosomes

actin-rich conical structures present on the plasma membrane of many cells. Through recruited matrix metalloproteinases, podosomes degrade the extracellular matrix by proteolysis, playing an important role in cell migration.

Signal transducer and activator of transcription (STAT)

these transcription factors regulate several cellular processes, including immune activation and cell growth/differentiation.

Syncytiotrophoblasts

multinucleated cells that form the outermost layer of the hemochorial placenta, comprising the primary barrier to avoid vertical transmission. They are extremely resistant to *Toxoplasma* infection.

Synepitheliochorial

type of epitheliochorial placenta present in ruminants where the uterine epithelium is invaded by multinucleated cell masses (syncytia).

Th1/Th2 paradigm

This paradigm supports the idea that the fetus acts as an allograft to the mother and that a predominant anti-inflammatory Th2 response is needed to maintain pregnancy, with increases in proinflammatory Th1 cells/cytokines resulting in abortion.

Toll-like receptors (TLRs)

type of pattern recognition receptors that, upon recognition of pathogen-associated molecular patterns, activate signal transduction pathways culminating in the expression of cytokines.

Trojan horse

Mechanism used by intracellular pathogens whereby infected cells act as carriers or vehicles to help the pathogen disseminate and cross biological barriers without being detected.

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Box 1.**Toxoplasmosis and *Toxoplasma* Life Cycle**

The protozoan parasite *Toxoplasma gondii* is the causative agent of toxoplasmosis, a worldwide zoonotic disease that can affect virtually all mammals and birds, including approximately one-third of humans. Although the majority of infected healthy individuals are asymptomatic, *Toxoplasma* infection can cause neonatal mortality, abortions, and a wide variety of neurological symptoms, especially in immunocompromised or congenitally infected individuals [90]. A range of variables, including individual susceptibility, strain virulence, and infective dose, have been proposed as key factors in the development of one or the other of these clinical forms. Infection can occur after ingestion of oocysts shed in the feces of an infected cat (definitive host) contaminating food or water, or tissue cysts present in meat of infected animals (intermediate hosts). Upon ingestion, excysted parasites infect intestinal epithelial cells, where they differentiate into fast-replicating tachyzoites, the life cycle stage responsible for proliferation, dissemination, and clinical symptoms. After eliciting an efficient immune response, tachyzoites escape by infecting long-lived cells such as neurons and myocytes, where they convert into the slow-dividing bradyzoite stage and form tissue cysts, which normally remain dormant throughout the lifetime of the host [2]. The ingestion of undercooked meat is considered an important source of *Toxoplasma* infection for humans and a risk factor in pregnant women, representing a significant public health hazard. Likewise, oocysts shed by cats are highly stable in the environment and extremely resistant to inactivation procedures; hence, they are exceptionally infectious (a single oocyst is able to produce the infection), playing a pivotal role in the spread of toxoplasmosis. This is important not only for humans but also for livestock; as a matter of fact, toxoplasmosis causes important economic losses in the livestock sector related to reproductive failure, mainly in sheep and goats [61].

Box 2.***Toxoplasma* Strain Diversity**

In Europe and North America, strains belonging to four different *Toxoplasma* clonal lineages (types I, II, III, and XII) are commonly isolated in animals and humans, with most infections caused by type II strains [91]. By contrast, the majority of characterized isolates from South America are genetically distinct from the North American/European strains and have been referred to as atypical strains [92]. Although type II strains have frequently been associated with congenital infections, type II strains are also the prevailing strains in Europe and North America, where most of such studies have been performed [14]. Nevertheless, other studies have observed an association between type XII strains, which are the most dominant non-type II strains in North America, and severe congenital toxoplasmosis cases [93]. Furthermore, nonclonal *Toxoplasma* strains (i.e., not belonging to type I, II, III, or XII) are associated with disease and abortion in South America (e.g., [94,95]). However, multiple factors might be exerting a strong influence, such as the presence of regionally prevalent strains and a bias against asymptomatic infections from which fewer samples are normally available.

Box 3.**Variations in the Inflammatory Profile during Gestation**

During the early stages of gestation when implantation and placentation take place, there is a mixture of invading, dying, and repairing cells, and thus a strong proinflammatory response is required to secure the adequate repair of the uterine epithelium and the removal of cellular debris [26]. This response at the implantation site is characterized by the presence of IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, CXCL1, CCL4, osteopontin, and TNF- [96]. Following implantation and placentation, the longest period of pregnancy ensues when fetal development takes place. During this period, any proinflammatory response triggered by insults, such as infections, can lead to miscarriage [24]. Several immune cells, including macrophages, NK cells, and Treg cells, contribute to the establishment of an anti-inflammatory microenvironment [97]. Among them, Treg cells promote immune tolerance by secreting IL-10 and TGF- β 1 by dampening IFN- γ and other Th1/Th17 inflammatory cytokines, and by acting as an IL-2 sponge to deprive Th1 cells from their activation/proliferation [29,98]. One of the key cytokines in the maintenance of a balanced Th1/Th2 environment in the placenta is TGF- β [38,99]. However, when exposed to both IL-6 and TGF- β , CD4⁺ T cells develop instead into Th17, the proinflammatory IL-17-producing cells [33,100]. Treg and Th17 cells have opposed effects on inflammation and thus immunologic tolerance. In fact, a low Treg/Th17 rate has been associated with unexplained miscarriage, pre-eclampsia, and recurrent spontaneous abortions in humans [101-103]. However, because they are proinflammatory in nature, these Th17 cells contribute to protecting the maternal-fetal interface from external pathogens. Like T cells, macrophages have also been described to contribute to normal and pathological pregnancies. Most of the decidual macrophages are characterized by an immunosuppressive phenotype (M2), supporting fetal-maternal immune tolerance [104]. However, the macrophage profile varies along gestation: There is a slight inflammatory profile (M1) for implantation, then it switches primarily to anti-inflammatory to avoid rejection and finally at parturition switches again to proinflammatory [105]. Moreover, an M1/M2 dysregulation has been observed during adverse pregnancy outcomes, and upon contact with pathogens, pattern recognition receptors, such as TLRs, may alter decidual macrophage polarity, shifting the immunosuppressive M2 dominance to an inflammatory M1 phenotype [104]. Another important factor, particularly in *Toxoplasma* infection, could be the role of indoleamine 2,3-dioxygenase (IDO). IDO is an immunosuppressive enzyme produced primarily by Tregs and alternatively activated macrophages (M2) that locally depletes L-tryptophan needed for T-cell proliferation and promotes more Treg activity [105]. IDO is also expressed by maternal uterine mucosa and fetal syncytiotrophoblast, thereby controlling local inflammation. However, because *Toxoplasma* is a natural auxotroph for L-tryptophan, it acts as a barrier for parasite growth. To prevent this, *Toxoplasma* secretes TgIST, an effector that can effectively block IDO induction and promote parasite growth [85], potentially leading to abortion.

Highlights

Different *Toxoplasma* strains may predispose livestock and humans to early (acute) or late (classical) abortions, depending on the induction of inflammation vs. evasion of the immune response, respectively.

Dense granule proteins and rhoptry bulb proteins, parasite effectors implicated in the balance between evasion of the immune response and induction of a proinflammatory response, likely play a critical role in abortion.

Uncontrolled multiplication of the parasite in the placenta and fetus is associated with late classical abortions.

Severe inflammation at the maternal–fetal interface is associated with acute abortions without direct implication of the parasite. Instead, fetal death is produced by a disruption in nutrient or oxygen transport due to the vascular lesions formed in the placenta, causing lethal hypoxic damage to the fetus.

Outstanding Questions

What molecular mechanisms are implicated in the induction of early acute abortions?

What parasite factors are critical in the production of late classical abortions?

Is *TgWIP* a critical effector in parasite dissemination to the placenta and vertical transmission?

Which *Toxoplasma* strain types predispose to early/acute and late/classical abortions?

Are *Toxoplasma*-induced early acute abortions underdiagnosed in livestock and pregnant women on account of the absence of anti-*Toxoplasma* antibodies and parasite detection in the placenta/fetus at the time of abortion?

What components of the immune response make cows much more resistant than sheep/goats to *Toxoplasma*-induced abortions?

Why are these components not efficient in controlling *Neospora caninum*?

Why is abortion in successive gestations common in *Neospora* but not in *Toxoplasma* infections?

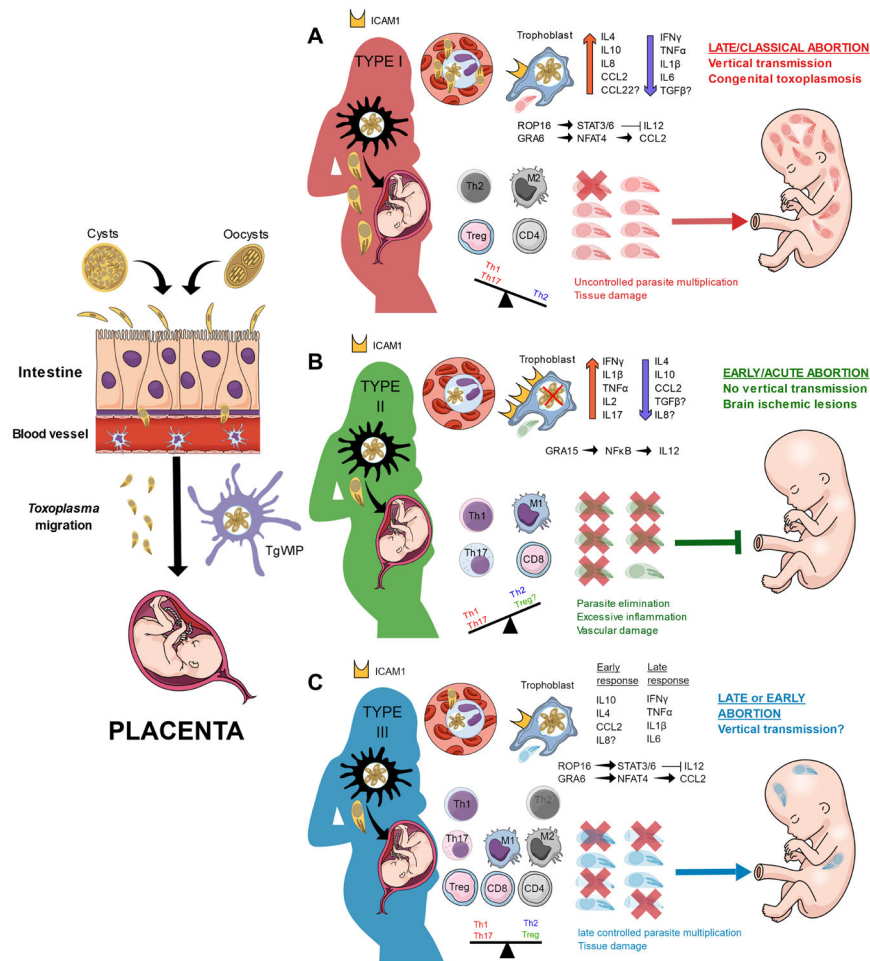


Figure 1. Key Figure. Immune Response to *Toxoplasma* Infection during Gestation

After oral infection, *Toxoplasma* disseminates either as extracellular parasites or by a Trojan horse mechanism and reaches the placenta. (A) In type I strains, rhostry bulb protein ROP16_I induces sustained activation of signal transducer and activator of transcription (STAT3/6), dampening the production of interleukin (IL)-12, IL-1 β , and IL-6, while dense granule protein GRA6_{I/III} induces the secretion of CC chemokine ligand 2 (CCL2). In the absence of IL-12, an anti-inflammatory profile predominates with M2 macrophages and Th2, CD4, and regulatory T cells (Treg). As a consequence, the uncontrolled parasite multiplication produces tissue damage, which in turn facilitates the invasion of the fetus. The most likely outcomes are late abortion and/or congenital infection during early or late gestation, respectively. (B) Type II strains produce a strong inflammatory environment in the placenta due to the early secretion of IL-12 induced by GRA15. This stimulates the secretion of proinflammatory cytokines [interferon (IFN)- γ , IL-1 β , and tumor necrosis factor (TNF)- α], which in turn upregulates the expression of intercellular adhesion molecule 1 (ICAM1) on trophoblasts, facilitating the adhesion of infected cells. The intense proinflammatory response eliminates most of the parasites but produces extensive tissue damage that compromises nutrient/oxygen transport to the fetus. Hence, the most likely outcome is an early, 'sterile' abortion without vertical transmission. (C) Similar to type I, ROP16_{III} limits the initial production of IL-12, delaying the induction of antiparasitic mechanisms and

establishing an initial anti-inflammatory response. However, type III strains are not effective at avoiding intracellular killing, and a proinflammatory immune response is eventually mounted when IL-12 is produced by dendritic cells. However, because the control of the parasite is delayed, some parasites can cross the placental barrier and reach the fetus. Therefore, both early and late abortion with or without vertical transmission are possible, depending on the stage of gestation.