Defining the Placental Barrier to *Toxoplasma gondii* Infection

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Toxoplasma gondii is a major source of congenital disease worldwide, but the cellular and molecular factors associated with its vertical transmission are largely unknown. In humans, the placenta forms the key interface between the maternal and fetal compartments and forms the primary barrier that restricts the hematogenous spread of microorganisms. This dissertation describes both the identification of two mechanisms of placental syncytiotrophoblast resistance to *T. gondii* infection and a preliminary understanding of the CCL22 response induced by the *Toxoplasma* dense granule protein GRA28. Collectively, these findings provide new insights into (1) protective role of the syncytiotrophoblast during *T. gondii* infection, (2) interferon-γ independent restriction of *T. gondii* growth, and (3) parasite-directed manipulation of the intercellular communication between the placenta and components of the maternal immune system.

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1.0 Introduction

Toxoplasma gondii is an obligate, intracellular protozoan that is believed to infect all warm-blooded animals, including humans. Based upon serology, it is estimated that one-third of the global human population has been infected, although the degree of seroprevalence varies widely amongst different geographical regions [1]. Most infections with *Toxoplasma* are subclinical, and while infection is considered to be life-long, the otherwise healthy individual experiences no health complications. However, vertical transmission of *Toxoplasma* during pregnancy can be fatal for the developing fetus. This chapter will discuss the immunology of the maternal-fetal interface, key aspects of *Toxoplasma* biology, and congenital toxoplasmosis in order to understand the biological barriers that the parasite must overcome to cause fetal disease as well as the consequences of congenital infection.

1.1 Immune Responses at the Maternal-Fetal Interface

Pregnancy poses an immunological challenge, as a genetically distinct (non-self) fetus must be supported within the pregnant female for the required gestational period. Placentation, or the establishment of the fetally-derived placenta, is a common strategy employed by eutherian mammals to protect the fetus and promote its growth. However, the substantial morphological differences of the placental architecture among species suggest that the process of placentation results from convergent evolution. Although there are considerable similarities in placental function across placental mammals, there are important differences that arise due to species-

specific immunological (and other biological) constraints. This subchapter focuses on the immunological similarities and differences that occur at the maternal-fetal interface in the context of human and mouse pregnancies. Herein will be discussed how the decidua and placenta of these different species form key immunological barriers that sustain maternal tolerance, yet generate innate immune responses that prevent microbial infections.

1.1.1 Introduction

Placentation is a common strategy employed across eutherian mammals to protect and promote fetal growth. Although mice are commonly used to study the maternal-fetal interface within the immunological context of pregnancy, differences exist in placental architecture, gestational period, and mechanisms of maternal tolerance from humans. Throughout this review, we focus on the immunological similarities and differences during human and mouse pregnancies. We define the fetal and maternal components, their interactions, and mechanisms of mediating maternal tolerance in both species. We also examine the role of the placenta as a barrier to maternally-transmitted pathogens and conclude with a discussion on the strengths and weaknesses of commonly used models of the human placenta.

1.1.2 The Maternal-Fetal Interface in Humans and Mice

The maternal-fetal interface is composed of the maternally-derived decidua and the fetally-derived placenta (**Figure 1**). In both mice and humans, the placenta develops from the trophectoderm of the blastocyst. During implantation, invading trophoblasts anchor the blastocyst to the specialized uterine epithelium (the decidua), upon which placentation ensues. Over the

course of pregnancy, the placenta is the sole site for all gas, nutrient, and waste exchange between the fetus and mother.

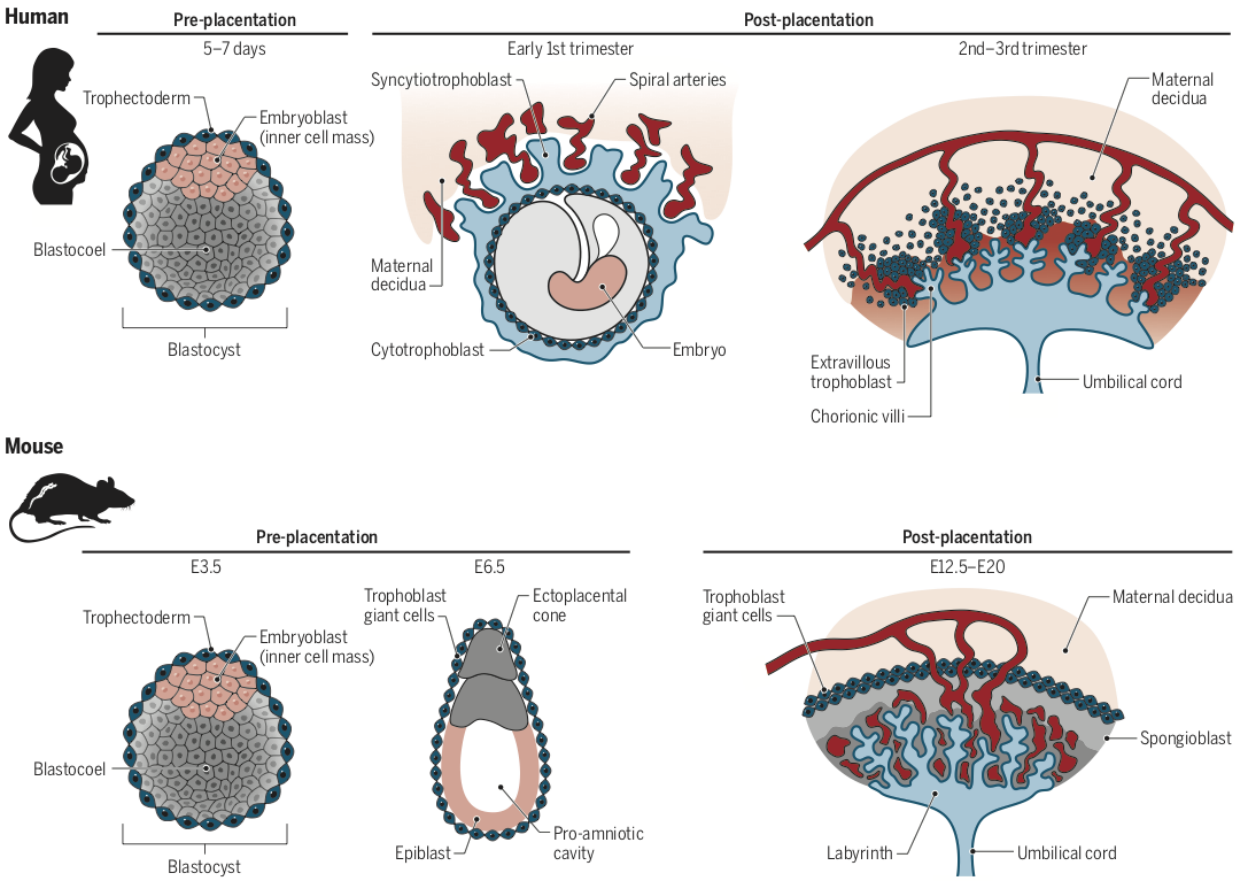


Figure 1 Comparison of Human and Mouse Placentation

Prior to placentation, the blastocysts of humans and mice are similar *(left)*. However, upon implantation placental development progresses differently *(middle and right)*. Following blastocyst implantation, the human syncytiotrophoblast layer burrows into the maternal decidua *(top middle)*. By the third week of gestation, the definitive human placenta is formed and is composed of villous trees. However, at this stage of human pregnancy, the fetal-derived placenta does not directly contact maternal blood. Extravillous trophoblasts anchor the villi to the decidua and are involved in the remodeling of the spiral arteries to flood the intervillous space with maternal blood towards the end of the first trimester of pregnancy *(top right)*. The surface of the villi are covered by the syncytiotrophoblast layer, which directly contacts the maternal blood and facilitates the transport of nutrients, gases, and waste across the placental barrier. Underlying the syncytiotrophoblast layer are mononucleated cytotrophoblasts that can either fuse to replenish the syncytial layer or differentiate into extravillous trophoblasts. In contrast, mouse placental development and organization is different from that in humans. Upon implantation, the trophectoderm differentiates and trophoblast giant cells encapsulate the developing mouse embryo *(bottom middle)*. Halfway through gestation, the definitive

murine placenta is fully formed and functional, where the folded villi form a labyrinth structure that becomes perfused with maternal blood *(bottom right)*. The trophoblast giant cells channel maternal blood from the decidua through the spongiotrophoblast layer (a structure not present in the human placenta) toward the labyrinth zone. In the labyrinth zone, the maternal blood makes contact with the cytotrophoblasts that overlay two separate layers of syncytiotrophoblasts. Graphics generated by A. Kitterman/*Science Immunology*.

1.1.2.1 Decidua Formation

The placenta is embedded within the decidua, the maternal component of the maternal-fetal interface. The decidua only exists during pregnancy and originates from the endometrial lining of the uterus (the endometrium). At the conclusion of pregnancy (parturition), the decidua is shed, to be rebuilt only upon subsequent pregnancy. However, signs of pre-decidualization can be observed within the non-pregnant human endometrium halfway through the luteal phase (around days 23-25 of the menstrual cycle) including: increased prominence of the spiral arterioles, differentiation of endometrial fibroblast-like cells into enlarged and granulated decidual stromal cells, and an influx of leukocytes [2]. It is important to note that timing is critical for pregnancy to occur. Implantation must take place prior to this pre-decidualization, as this thickening of the endometrium is not amenable for implantation.

During decidualization there is both fetally and maternally mediated remodeling of the spiral arteries so that the placenta becomes bathed in maternal blood, which facilitates exchange of nutrients, gases, and waste. After implantation, the endothelial lining of the spiral arteries is eroded (as well as the local decidual stromal cells), creating a fibrinoid wall embedded with invasive fetal placental trophoblasts [3]. Maternal leukocytes, such as natural killer cells and macrophages, have been implicated in this remodeling process (see later section on maternal leukocytes). These concordant efforts of fetal trophoblasts and maternal leukocytes result in the

dilation of the spiral arteries, which decreases the force and maximizes the volume of the maternal blood bathing the placenta [3].

1.1.2.2 Placental Development

In humans, the definitive structure of the placenta is composed of villous trees and is established by the third week of gestation (**Figure 1 and 2**). The structure of the human placenta is composed of both floating and anchoring villi. A single layer of contiguous multinucleated syncytiotrophoblasts (SYN) lines the outermost surface of the human placenta villous trees and acts as the major cellular barrier between the fetal compartment and maternal blood. Underlying the SYN layer are the undifferentiated, mononucleated cytotrophoblasts (CTBs). CTBs are progenitor trophoblast cells and can fuse to replenish the SYN layer or differentiate into mononucleated extravillous trophoblasts (EVTs), which are located at the tips of the anchoring villi. During the first trimester, the human placenta is hemodichorial, with two layers of trophoblasts separating the fetal and maternal bloodstreams (the SYN and CTBs). As the placenta grows, the underlying CTB layer thins and becomes dispersed; thus, the human placenta of the second and third trimesters is essentially hemomonochorial, with only a single layer of SYNs.

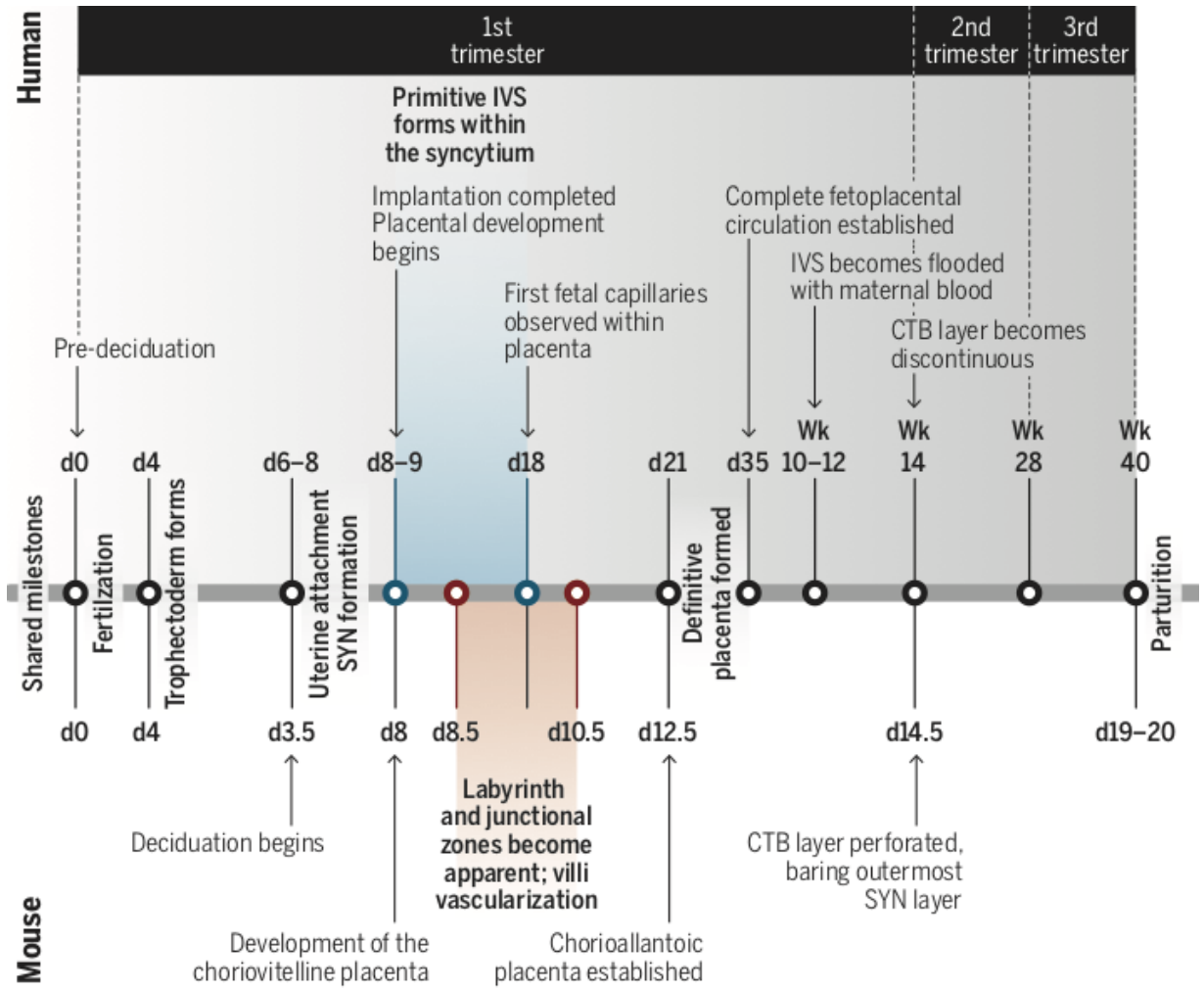


Figure 2 Timeline of Human and Mouse Placentation

The human early blastocyst forms around day 4 (d4) and is marked by the development of the trophectoderm—the first differentiation event in mammalian development. The primitive intervillous space (IVS) forms around d8-9 from the coalescence of vacuoles forming within the syncytiotrophoblast (SYN) mass (creating lacunae). In between the lacunae are columns of SYN (trabeculae), which are invaded by cytotrophoblasts (CTB) around d12 to form nascent villi. Around d15, the CTB invade the decidua (a task previously performed by the SYN for implantation). By d21, the definitive placenta is formed. However, maternal blood does not flood the IVS until weeks 10-12. In contrast, the gestational period of mice lasts just 20 days. Other differences between human and mice include the development of the choriovitelline placenta at d8. This primitive placenta (not formed in human gestation) is composed of the juxtaposition of the yolk sac against the maternal tissues and blood vessels. At d11-12.5, the yolk sac placenta is supplanted by the chorioallantoic (definitive) placenta, and around d14.5 for the mouse, the CTB layer covering the

villi becomes perforated, and maternal blood can now directly contact the outermost SYN layer. Graphics generated by A. Kitterman/*Science Immunology*.

The SYN facilitates the transport of nutrients, gases, and waste across the maternal-fetal interface. The SYN also functions as the main endocrine cell of the placenta, producing human chorionic gonadotropin (hCG) and progesterone, vital hormones that support pregnancy [4]. During the first eight weeks of gestation, the SYN secretion of hCG is required to induce progesterone production by the corpus luteum [5]. Afterwards, the placenta itself becomes the major producer of progesterone [5]. While the mouse also requires progesterone during the course of gestation, its placenta does not synthesize progesterone, and instead continuously throughout pregnancy relies upon the corpus luteum for progesterone [4].

EVTs physically anchor the human placenta to the decidua. The invasive EVTs also are important for remodeling the spiral arteries in the outer third of the myometrium. In the first trimester, EVTs act as a plug for the spiral arteries, thus creating a hypoxic environment by excluding the oxygen-rich maternal blood. During the transition from the first to the second trimester, the EVT plug is eroded and the intervillous space (IVS) becomes flooded with maternal blood (**Figure 2**). Later during gestation, the IVS can fill with as much as 150 mL of maternal blood. The presence of maternal blood in direct contact with the SYN allows for efficient gas, nutrient, and waste exchange, which is maintained throughout the rest of pregnancy. As the direct contact of maternal blood with the placenta does not occur until the end of the first trimester, this event distinguishes the early (first trimester) and later (second and third trimesters) stages of pregnancy.

In the early stage of pregnancy, the IVS is filled with a clear fluid containing uterine gland secretions, which are phagocytosed by the SYN and serve as a nutrient source for the developing

fetus [6]. Uterine glands originate from invaginations of the endometrium and are required for establishing pregnancy. Among their varied secretions are growth factors that regulate placental development, including epidermal growth factor, vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and leukemia inhibitory factor [7].

1.1.2.3 Decidualization and Placentation in the Mouse

Key differences exist between mice and humans during the stages of pregnancy. In particular, the timeframe and establishment of the maternal-fetal interface in each species is distinct. Human gestation takes place over a period of 40 weeks, whereas in mice, it is approximately 3 weeks (**Figure 2**). The timing of decidua formation and placentation also varies. In humans, the uterus is primed for decidualization, independent of fertilization, around menstrual cycle day 23 when the stromal cells near the (now prominent) spiral arteries begin to differentiate into large predecidual cells [2]. However, in mice, spiral artery outgrowth and decidualization does not begin until fertilization and blastocyst attachment to the uterus, respectively [3]. Likewise, the placenta does not have a definitive structure in mice until the mid-point of gestation (around day 10.5-11.5); whereas, the definitive placenta in humans forms far earlier in relative development (around day 21) [4]. Thus, timing is critical to experimental design and interpretation when using the mouse (or any other animal) to model human pregnancy. A timeline highlighting the differences between the human and mouse placentation and the key events that occur throughout pregnancy is shown in **Figure 2**.

Although the hemochorial mouse placenta shares features with the human placenta, several differences exist that impact physiology, immunity, and development (**Figure 1**). Whereas the human placenta is structured as villous trees bathed in maternal blood (after the first trimester), the mouse placenta has a labyrinth structure perfused by maternal blood. In the mouse, the maternal

blood is directed through trophoblast giant cell-lined channels in the spongiotrophoblast layer (a cell type not present in the human placenta) to the chorionic plate and back through the labyrinth zone containing the fetal vasculature [8]. Unlike the anchoring chorionic villi of humans, the mouse chorionic projections are highly interconnected, presenting a maze-like structure through which the maternal blood must pass to leave the placenta. This labyrinth chorionic structure is lined by three layers of trophoblasts: two layers of SYNs overlaid with CTBs. In further contrast to the human placenta, the murine CTBs directly contact the maternal blood. The trophoblast giant cells (large polypoid cells) anchor the mouse placenta to the decidua. Unlike human EVTs, murine giant cells are minimally invasive and do not remodel the maternal spiral arteries. These differences in physiology may impact the placental barrier functionally and the types of strategies utilized to protect the fetus from activation of the maternal immune system, as well as impacting the pathways used by circulating pathogens to access the fetus.

1.1.3 Leukocytes at the Maternal-Fetal Interface

In addition to stromal cells, a remarkably large portion (~40%) of the decidua is composed of maternal leukocytes (**Figure 3**). In the first trimester, decidua basalis (the site of implantation and trophoblast invasion/remodeling), decidual natural killer cells (dNK) comprise the majority (~70%) of immune cells, followed by decidual macrophages (20-25%), and T cells (3-10%) [9, 10]. Maternal leukocytes are present in the decidua throughout pregnancy, although the population frequencies change, with far more dNK and decidual macrophages present at earlier stages of pregnancy than at term [11, 12]. These maternal leukocytes are recruited by chemokine gradients produced by decidual stromal cells and trophoblasts [13, 14], and are typically distinct from their peripherally circulating counterparts in phenotype and function (discussed below). It should be

noted that most of our understanding of maternal leukocytes at the maternal-fetal interface has been determined from mouse studies and correlated to observations in human patient samples. Whereas the majority of the decidual leukocytes are dNKs and decidual macrophages, T cells subsets also have key functions. For more detailed information about leukocytes present at the maternal-fetal interface, the reader is referred to several excellent recent reviews [9, 10, 15].

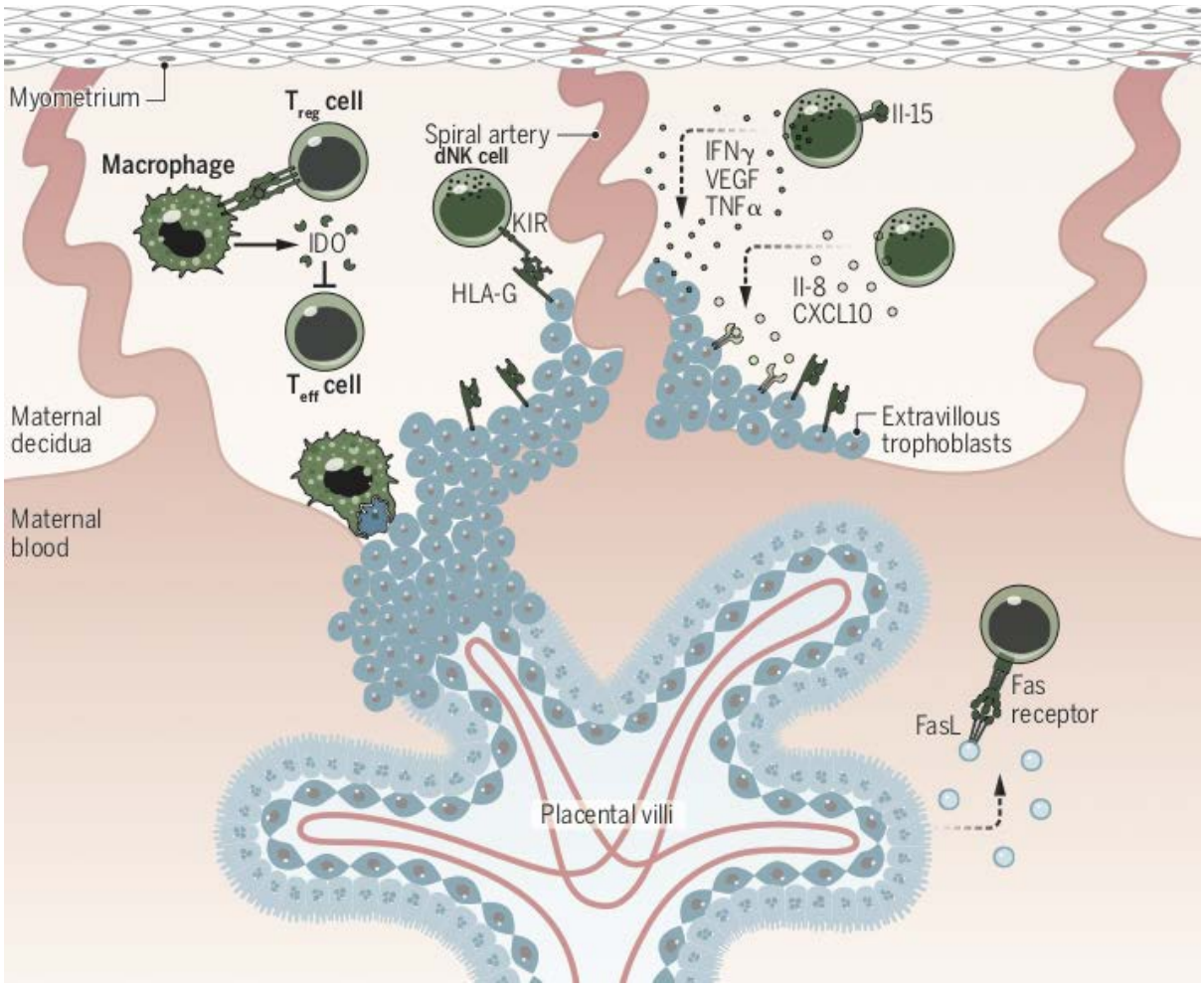


Figure 3 Mechanisms of Maternal Tolerance

The most abundant of the maternal immune cells present in the decidua are decidual natural killer cells (dNKs), which are recruited by various factors released from the decidual stromal cells and placental trophoblasts. The release of uterine IL-15 promotes dNK maturation. The mature dNK cell promotes decidual remodeling and blastocyst implantation through the secretion of cytokines (including IFN_{γ}, VEGF, and TNFα). The release of IL-8 and CXCL10 by dNK also promotes extravillous trophoblast (EVT) invasion. dNK cell cytotoxicity is controlled by the binding of HLA-G (expressed on the EVTs) to the inhibitory receptor KIR2DL4. dMØ prevent maternal intolerance by producing IDO, which hinders T cell activation, and phagocytosing apoptotic trophoblasts. Tregs modulate the activities of both antigen presenting cells and effector T cells. The syncytiotrophoblast also promotes maternal tolerance by secreting exosomes expressing TRAIL and FasL and by the lack of any MHC expression. Graphics generated by A. Kitterman/*Science Immunology*.

1.1.3.1 Decidual Natural Killer Cells

Decidual NK cells are the largest population of maternal leukocytes that accumulate at the maternal-fetal interface, where they contribute to decidualization and implantation. Unlike their circulating counterparts, dNKs produce a vast array of growth factors, angiogenic factors, and cytokines [16]. Through these secretions, they help to remodel the decidua and spiral arteries, promote trophoblast invasion, and increase the availability of maternal blood at the implantation site [16–20]. In the mouse decidua, dilation of the spiral arteries is induced by dNK-secreted type II interferon (IFN-γ [21]. dNK production of IL-8 and CXCL10 has been implicated in promoting EVT migration into the placental bed [16]. An absence of dNKs in murine pregnancies is associated with decreased fetus viability and abnormal formation the decidual structure and spiral arteries at the implantation site [22, 23]. Analogously, in human patients with unexplained infertility, endometrial biopsies have found significantly fewer NK cells than fertile counterparts [24]. Interestingly, a subpopulation of dNKs that exhibit enhanced production of IFN-γ and VEGFα has been identified recently as highly enriched in multigravid women [25]. These Pregnancy Trained dNKs (PTdNKs) are transcriptionally unique from other dNKs, with higher expression of genes related to NK cell activation, growth factors, and immunomodulatory proteins [25]. Using single-cell transcriptomics, another recent study identified three subsets of dNKs in the first trimester decidua, including a highly active subset of dNKs with characteristics similar to the previously described PTdNKs [26]. As improved placentation is seen upon subsequent pregnancies [27], it is interesting to speculate that this subset of dNKs may become enriched upon subsequent pregnancies and boost decidua receptivity.

The dNK cells of pregnancy are phenotypically distinct from the peripherally circulating NK cells, and their specific origins remain enigmatic and may differ between humans and mice

[9]. These cells are highly granulated and distinguished as $CD56^{++}CD16^{-}$ (human) or $CD122^{+}CD3^{-}$ (mouse) [9]. The precursors of dNK cells are speculated to include the uterine hematopoietic stem cells and/or differentiation of thymic or peripheral NK cells [28–30], with the final differentiation into mature dNK cells driven, at least in part, by uterine IL-15 production [31]. The size of the NK cell population in the uterus is regulated by endocrine signaling. Following the human menstrual cycle, there is a cyclic enrichment of NK cells in the uterus post-ovulation that is sustained by pregnancy. In contrast, the population of NK cells in the mouse uterus does not accumulate until blastocyst implantation [9]. It should be noted that in the murine model of artificial decidualization, placement of an inert bead in the uterus of hormone-stimulated mice can stimulate local uterine NK to differentiate into dNK cells [32]. However, these cells are not as functionally active as those derived in the presence of a conceptus [32].

1.1.3.2 Decidual Macrophages

Decidual macrophages are the primary antigen presenting cells (APCs) at the maternal-fetal interface in early pregnancy [10]. Like uterine NK cells, levels of uterine macrophages rise and fall with the menstrual cycle and then increase upon fertilization [33, 34]. Phenotypically, decidual macrophages are believed to exist as regulatory/homeostatic, anti-inflammatory cells of an M2-like phenotype [10]. The phenotype of decidual macrophages is believed to be influenced by trophoblasts, which secrete macrophage colony-stimulating factor (M-CSF) and IL-10 [35]. Human decidual macrophages are $CD163^{+}CD206^{+}$DC-$SIGN^{+}$ and predominantly express IL-10, CCL2, and CCL18 [36–41].

Decidual macrophages have many functions during pregnancy. Like dNK cells, they aid in remodeling of the spiral arteries and trophoblast invasion [42, 43], and localize to sites of disruption near the spiral arteries [44]. *D*ecidual macrophages *in vitro* produce VEGF and matrix

metalloproteinase 9 (MMP9), which may promote angiogenesis and tissue remodeling [39, 45, 46]. Decidual macrophages are proposed to perform “cleanup” functions by phagocytosing apoptotic trophoblasts, which prevents activation of pro-inflammatory pathways in the decidua [47–50]. These cells also produce indoleamine 2, 3-dioxygenase (IDO), which catabolizes tryptophan and hinders T cell activation [51, 52]. Decidual macrophages also may have a more canonical antimicrobial role in protecting the fetus against infections, as suggested by the surface expression of pattern recognition receptors CD163 (hemoglobin scavenger receptor), CD206 (mannose receptor), and CD209 (DC-SIGN) [53].

1.1.3.3 Regulatory T cells

The importance of regulatory T cells (Tregs) in pregnancy has become increasingly apparent. Observational experiments with human samples demonstrate the presence of Tregs in the human decidua, and cases of human infertility, recurrent spontaneous abortions, and other pregnancy complications have been inversely correlated with Treg frequencies or functionality [54–57]. In mice, fetal specific Tregs are recruited to and induced at the maternal-fetal interface where they confer tolerance to fetal antigens and help maintain a homeostatic environment conducive to fetal survival. Fetal specific Tregs are capable of persisting beyond parturition, while maintaining their functionality [58]. Upon subsequent pregnancy with the same paternal background, the expansion of these cells correlates with decreased fetal resorption [58]. Intriguingly, the origins of these fetal specific Tregs may be linked to the *in utero* exposure of non-inherited maternal antigens (NIMA). A multigenerational mouse study found pregnancies to be more successful when the sire has overlapping allogenicity with the maternal grandmother [59]. Thus, *in utero* exposure to NIMA may expand a female’s Treg repertoire and explain the presence of maternal Tregs specific for fetal (non-self) antigen [59].

1.1.4 Maternal Tolerance

Maternal tolerance, which permits a mother to carry the fetus to term despite the presence of foreign fetal antigen, is a poorly understood phenomenon that seems to defy some of the basic tenets of immunology. For a successful pregnancy, maternal tolerance must be established, and failure of maternal tolerance is correlated with pre-eclampsia and miscarriage [60–62]. In general, tolerance is mediated by the restriction and modulation of leukocytes that permeate the maternal-fetal interface. Although there is an abundance of NK cells in the decidua, the numbers of DCs and effector T cells are low. As has been demonstrated in the mouse decidua, this may be due to the absence of local lymphatic vasculature in the endometrium [63, 64] and epigenetic silencing of T cell chemoattractants in decidual stromal cells [65]. As for leukocytes that do gain access to the maternal-fetal interface, intercellular communication between the resident decidual leukocytes, stromal cells, and trophoblasts can alter the functional profile of leukocytes and promote regulatory phenotypes. For example, first trimester human placental explants produce G-CSF, IL-10, and TGF-β, which are known to promote differentiation of peripherally circulating monocytes and T cells into M2 MØ and Tregs, respectively [35]. Apoptosis also is employed to mediate immune privilege. The SYN secretes exosomes expressing TRAIL and Fas ligand on their surface, which are capable of binding to their cognate death receptors on leukocytes to trigger apoptosis [66].

Maternal tolerance also may occur through species-specific mechanisms. In humans, placental EVTs express HLA-G, a non-classical MHC molecule, for which there is no homolog in the mouse genome. Unlike the canonical class I MHC molecules, of which exist thousands of allelic variations that serve to distinguish self from non-self, there are only 16 protein variants of HLA-G [67]. Uniquely expressed by EVTs, HLA-G binds to dNK inhibitory receptors KIR2DL4 [68] and LILRB [69] to protect the trophoblasts from NK-mediated cytolysis [70]. Likewise, the

membrane-localized regulator of complement, *Crry*, is an example of a rodent-specific mechanism which protects the mouse placenta from the deposition and activation of circulating maternal complement, and its expression is required for fetal survival [71]. As Crry is rodent-specific, it remains to be determined whether the human placenta also expresses such complement regulatory proteins that inhibit complement deposition and activation. Such disparities in mediating maternal tolerance between mice and man may be due to differences in the degree of placental invasiveness and length of gestational period. Likewise, different mechanisms could have arisen independently to handle the same problem of maternal immune responses that antagonize fetal viability.

1.1.5 Intrinsic Immune Responses of the Placenta

Once the IVS fills with maternal blood, the placenta is continuously exposed to any and all pathogens circulating systemically in the maternal circulation. As such, the placenta possesses several intrinsic defenses to protect the fetus from infection (**Figure 4**).

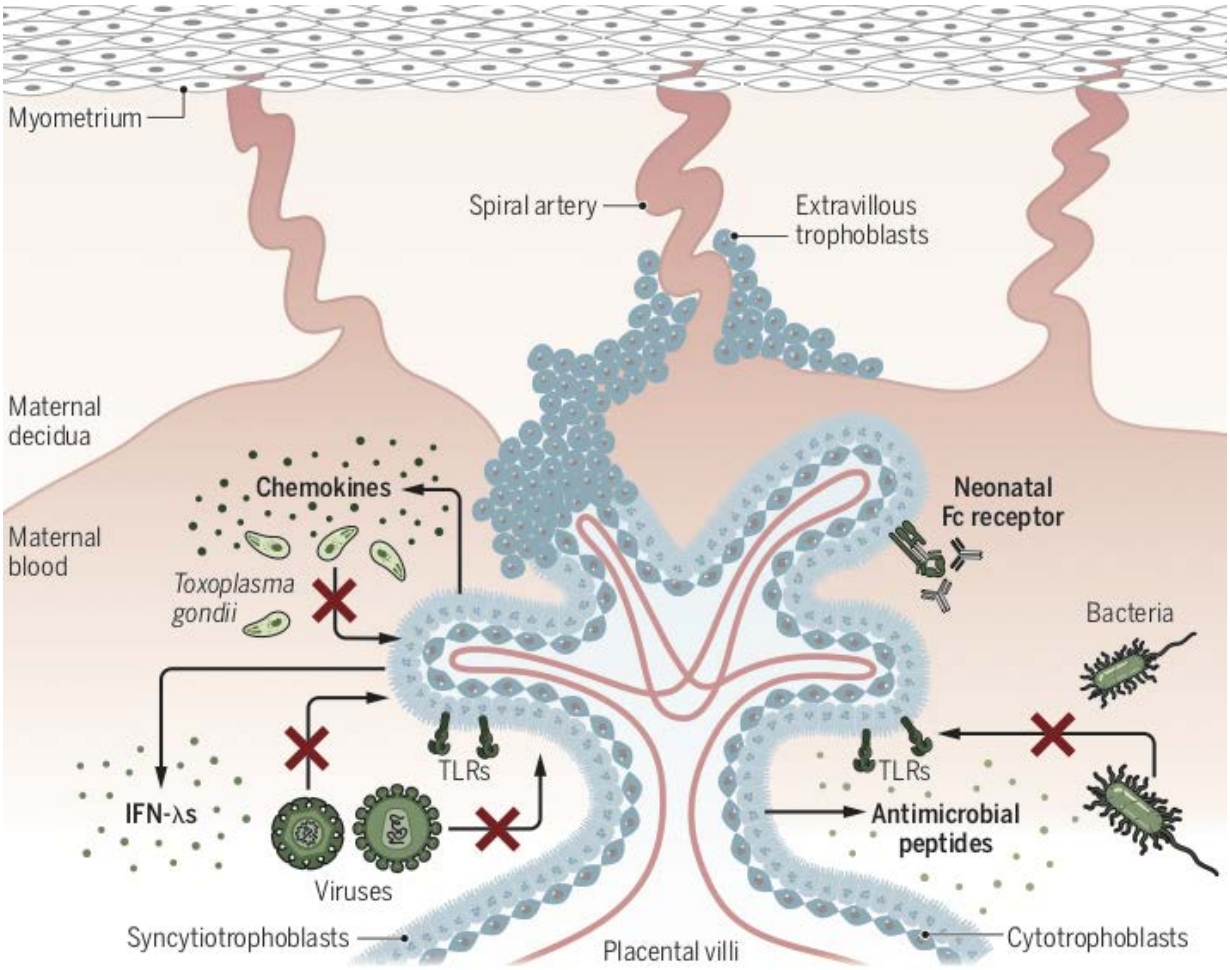


Figure 4 Mechanisms of Placental Immune Defense

The placenta has a number of innate immune mechanisms to protect the fetus from congenital infections of all types, including the expression of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), the constitutive expression of type III interferons (IFN-λ), and the release of antimicrobial peptides. Inoculation of placental trophoblasts with the parasite *Toxoplasma gondii* induces the secretion of chemokines, including the potent Th2 and Treg chemoattractant CCL22, suggesting that parasite infection alters or signals to maternal-derived immune cells. Furthermore, SYN expression of the neonatal Fc receptor also suggests a protective role for maternal IgG within the fetal compartment through the development of passive immunity. Graphics generated by A. Kitterman/*Science Immunology*.

1.1.5.1 Structure and Location

The architecture of the human placenta allows it to present its strongest cellular defense, the SYN layer, on its outermost surface. The SYN forms a single continuous cell, and thus lacks cellular junctions that can be exploited by pathogens or modulated by inflammatory signals. This contrasts with the mouse, which is arranged with the two layers of SYN buried beneath the CTBs contacting the maternal bloodstream. Another physical property that confers microbial resistance to the SYN is the dense cytoskeletal network that creates a dense brush-border formed at the apical surface. This brush border provides a vast surface area for nutrient and gas exchange between the maternal and fetal compartments, but also protects from direct microbial invasion, in part due to the dense underlying actin network. For example, SYN are highly resistant to infection by *Listeria monocytogenes* [72], but become more permissive upon pharmacological disruption of the actin cytoskeleton [73]. In addition, SYN restrict *Toxoplasma gondii* entry, most likely via a unique plasma membrane composition not amenable to parasite attachment [74, 75].

1.1.5.2 Secreted Antiviral Factors

The placenta secretes antiviral molecules that broadly function to restrict viral infections. The SYN layer constitutively secretes type III IFNs (IFN-λ) and vesicle-enclosed primate-specific placental microRNAs (C19MC, chromosome 19 microRNA cluster) that restrict viral infections in autocrine and paracrine manners [76, 77]. Whereas C19MC miRNAs are unique to primates, and it is unclear whether mice express an analogous inhibitory miRNA, the murine placenta also uses type III IFNs to protect against viral infections. Mid-gestation murine fetuses lacking IFN-λ signaling are more permissive to Zika virus (ZIKV) infection and vertical transmission [78]. Likewise, injection of pregnant dams with pegylated IFN-λ restricted vertical transmission of ZIKV [78, 79].

1.1.5.3 Transport of Passive Immunity

The human placenta also can actively transport protective antibodies to the fetus via expression of the IgG receptors neonatal FcRn and FcγRIII on the surface of the SYN layer. This transplacental passage of maternal humoral immunity in humans begins at week 16 of gestation and increases during the course of pregnancy, such that at term, the fetus has a greater serum concentration of maternally-derived IgG than the mother [80]. The murine chorionic placenta does not transport IgG as efficiently; instead mice acquire the bulk of maternal antibodies via FcRn expression on yolk sac-derived cells and after birth via suckling [81, 82].

1.1.5.4 Intracellular Defenses

In addition to these processes, the placenta can directly initiate innate defenses aimed at suppressing microbial infections and/or alerting the maternal immune system to infection. Placental trophoblasts recognize pathogens via toll-like receptors and RIG-I-like receptors, which trigger the induction of antimicrobial signaling pathways [83, 84]. Trophoblasts also exhibit high rates of basal autophagy, which can serve as a pan-antimicrobial strategy to restrict the replication of diverse intracellular pathogens [76, 85].

1.1.6 Modeling the Maternal-Fetal Interface for Congenital Infection Studies

Despite the formidable barrier presented by the placenta, some pathogens are capable of overcoming these placental defenses and induce devastating consequences to the developing fetus. These pathogens are collectively referred to as TORCH pathogens with the acronym referring to *Toxoplasma*, Other (Zika virus, *Listeria monocytogenes*, *Treponema pallidum*, varicella zoster virus (VZV), human immunodeficiency virus (HIV), and others), rubella virus, cytomegalovirus

(HCMV), and herpes simplex virus. To understand how these pathogens cause fetal disease, it is important to consider routes of entry to the fetal compartment and methods of evading the intrinsic defenses and barriers of the maternal-fetal interface. Several different models have been employed to study this evasion, including: immortalized trophoblast cell lines, primary trophoblast cultures, human tissue explants, and *in vivo* models. Each model has advantages and limitations, which must be understood to interpret and extrapolate the conclusions to human pregnancy.

1.1.6.1 *In Vitro* Models to Study Human Placental Functions

BeWo, JEG-3, and JAR cells are commonly used tractable trophoblast cell lines derived from choriocarcinomas. Although these cell lines all express trophoblast markers, they do not spontaneously fuse to form syncytia, and thus they are more suited to model either CTBs or EVTs and do not recapitulate the biology of the SYN layer. Accordingly, these cell lines do not recapitulate the microbial resistance phenotypes observed in primary trophoblasts or placental explants [74, 76, 86]. BeWo cells can be compelled to syncytialize by treatment with agents that increase cAMP levels to enhance the expression of endogenous retrovirus fusion proteins responsible for CTB fusion [87], however, the elevation of intracellular cAMP levels can produce other phenotypes and these cells still remain susceptible to microbial infection [74]. In comparison, JEG-3 cells grown in a three-dimensional bioreactor-based system co-cultured with human endothelial cells spontaneously fuse to form SYNs and are able to recapitulate resistance to *T. gondii* and viral infections, as well as the constitutive release of type III IFN-λ [86, 88]. Primary human trophoblasts are excellent cellular models of CTBs and SYNs. However, the lack of efficient means to genetically manipulate these cells, coupled with their limited (usually 3-5 day) lifespan post-isolation, limits their utility. *Ex vivo* placental explants maintain the unique morphological structure of the placenta as well as the multicellular complexity and can be isolated

at all stages of pregnancy [89]. However, procurement of placentas from early to mid-gestation is complicated by increasingly restrictive government regulations. Indeed, several countries and states within the United States have illegitimized medical research using fetal-derived tissue, which includes the placenta, and other localities have limited access to tissues obtained from elective terminations.

1.1.6.2 *In Vitro* Models to Study Human Decidual Functions

While the above-mentioned models can provide valuable information regarding specific trophoblast-pathogen interactions and trophoblast-intrinsic immunity, they lack the maternal component. As with trophoblast models, there are a variety of different endometrial epithelial cell lines available for studies; with particular lines better suited as models for particular regions of the endometrium (such as glandular vs luminal models) [90]. Primary stromal cells can be obtained and are capable decidualizing in culture. Co-cultures of endometrial stromal cells and trophoblasts are used to model implantation [91, 92]. These models have limitations, including the exclusion of maternal immune cells that also comprise up to 40% of the decidua. Studies using *ex vivo* decidual explants are able to model the multicellular composition (including dNKs and decidual macrophages) and three-dimensional structure better, and recent congenital transmission studies using this model have provided insight into the decidual innate immune response and mechanisms of viral transmission [93, 94].

1.1.6.3 Animal Models

Animal models are necessary to understand the dynamic immunological complexities of maternal-fetal tolerance, inflammation at the maternal-fetal interface, and the disruption of tolerance associated with congenital infections. Although a number of studies on placenta biology

have come from experiments in mice, other animal models also have provided insights. Commonly used *in vivo* models include nonhuman primates (NHPs), sheep, and rodents [95]. As might be expected, NHPs are good models for human pregnancy, as there are many common characteristics including a hemochorial placenta, singleton pregnancies, and a long gestation period comparable to human pregnancy [95]. However, these models are ethically challenging, may be difficult to access or generate for some researchers, and are costly. Sheep also are commonly used to study placental vasculature since their villous trees are shaped similarly to that of humans [95]. However, placentation is different in sheep; in particular, the depth of implantation is minimal (with no trophoblast invasion through the endometrial epithelium), and there is a greater degree of separation between the fetal and maternal vasculature (epitheliochorial placenta). Rodents, and specifically mice, are the most commonly used animal models. Among rodents, the guinea pig shares more similarities with human pregnancy than the mouse, including: the source and levels of progesterone produced, deep trophoblast invasion, and long gestation (around 3-times the length of mouse gestation).

Nonetheless, the most commonly used animal model for studying congenital transmission is the mouse, because it facilitates the use of many valuable immunological tools and techniques, has a short gestation period and large litter size, which enables a robust sample size, and is relatively inexpensive. Murine models are particularly advantageous in the context of genetic deficiencies (on the maternal or fetal side of the interface) and results from these animal studies can be complemented with data from human models. The use of the mouse model for congenital infections has elucidated some of the mechanisms by which pathogens cause congenital disease. Experiments on *Listeria*-induced fetal wastage have demonstrated the importance of maintaining maternal tolerance toward the fetus; where promoting the accumulation of fetal-specific CD8+ T

cells in the decidua causes fetal resorption, and most of the damage to the fetus is likely the result of a loss of maternal tolerance, rather than the maternal response necessary to control the bacterial invasion [96]. A similar phenomenon has been described in *Salmonella*-induced placental inflammation; where the host response upsets the balance of maternal tolerance and leads to fetal loss [97].

However, when studying congenital infections, it is important to consider that many TORCH pathogens are species-specific, and mice may lack susceptibility. Each model must be interpreted carefully, keeping in mind its limitations. One example is the use of a mouse pathogen analogous to the human TORCH pathogen; such as the use of the mouse cytomegalovirus (MCMV) as a substitute for HCMV. It is important to note, though, that MCMV cannot cross the placental barrier (unlike HCMV) [98]. Another approach to overcome the barrier of host-specificity is the use of immunocompromised mice, such as studies on Zika virus that use mice lacking the receptor to type I interferon [78, 99, 100]. Likewise, there also exists variability in both susceptibility and immune response between inbred strains of laboratory mice, as has been shown with *Listeria* [101]. Notwithstanding these issues, mice are still a highly useful tool and have provided much insight on the complexity of the maternal immune response during congenital transmission.

1.1.7 Summary of Immune Responses at the Maternal-Fetal Interface

Placentation is a common strategy employed by eutherian mammals to generate a conduit and barrier between the maternal and fetal environments. The variety of strategies employed to support placentation across the breadth of placental mammals is particularly interesting. Humans and mice both rely upon hemochorial placentas, but the structure and tissue organizations are

distinct. While the composition of the decidua is similar between mouse and human, the timing and mediators of decidualization are disparate. Through millions of years of evolution, each species has optimized the complex immunological equilibrium that is required to sustain healthy pregnancy. Therefore, both the advantages and limitations of various models should be considered when extrapolating data to human congenital transmission and disease. Although no model is perfect, biological insights can be obtained through the continued use and development of *in vitro*, *ex vivo*, and *in vivo* models. Collectively, these systems will provide new paradigms for one of the most unique aspects of human biology and, potentially, strategies to protect the developing fetus from potentially devastating congenital disease.

1.2 *Toxoplasma gondii* Biology

Toxoplasma gondii was initially discovered in 1908 by two independent research groups, Nicolle and Manceaux in Tunisia and Splendore in Brazil [102]. Both groups originally believed it to be a species of *Leishmania*, however in Nicolle and Manceaux soon realized it to be a novel protozoan and bequeathed it the name *Toxoplasma gondii*: *toxo* referring to the arc or bow-shape of the parasite, *plasma* meaning shape, and *gondii* for the hamster-like rodent in which it was initially found [102]. As *Toxoplasma gondii* is the only species within its genus, it is frequently referred to simply as *Toxoplasma*.

Today, *Toxoplasma* is classified as a member of the phylum Apicomplexa. Characterized by their polarized cell structure consisting of an intricate apical arrangement of specialized organelles, all apicomplexans are single-celled, obligate intracellular parasites. In addition to *Toxoplasma*, other Apicomplexans of note for either notoriously causing disease in humans or for

their high synteny with *Toxoplasma* (useful for comparative genomic studies) are the malarial parasites in the genus *Plasmodium* and the cattle parasite *Neospora caninum*, respectively.

1.2.1 The Parasitic Life Cycle of *Toxoplasma*

Toxoplasma is a fascinating parasite, capable of infecting any nucleated cell and possessing a vast range of hosts that includes humans and other warm-blooded animals [102, 103]. This phenomenon is attributed to the parasite synthesizing and injecting its own receptor, RON2, into target cells. The secreted RON2 localizes to the host cell plasma membrane where it projects outward and binds to AMA1 on the parasite's surface. This interaction then allows the parasite to create an invagination of the host plasma membrane and concurrently invade [104, 105]. The parasite relies upon asexual replication for propagation in most warm-blooded animals (intermediate hosts), while sexual replication is restricted to the gut epithelium of felids (the definitive host) for as-yet-unknown reasons [106–110]. *Toxoplasma* is capable of clonally maintaining its population for infinite generations solely within intermediate hosts, or bypassing the intermediate host to continuously infect definitive hosts (**Figure 5**). Thus, there are several routes by which the *Toxoplasma* may be transmitted to a new host via its three infectious life-forms: the sporozoite that matures within oocysts shed into the environment, the fast-replicating tachyzoite, and the slow-growing bradyzoite found in tissue cysts.

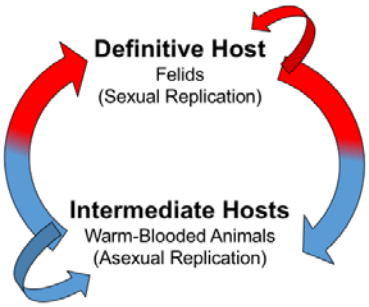


Figure 5 The Parasitic Lifecycle of *Toxoplasma*

The definitive host for *Toxoplasma* is the felid. Specifically within the gut of the felid, the parasite is able to sexually replicate. Progeny from sexual replication is then shed into the environment where they can be inadvertantly consumed by various intermediate hosts. During infection of intermediate hosts (which is composed of a wide range of warm-blooded animals), the parasite asexually replicates and is transmitted between intermediate hosts and back to the definitive host via carnivorism.

The sporozoite, or progeny of sexual replication, is enclosed within the oocyst and shed from an infected felid into the environment. There, it can be inadvertently consumed by another felid (to possibly repeat the sexual life cycle) or by an intermediate host (upon which it will enter the asexual life cycle). Oocysts must be matured before they are infectious, and this process occurs 1 to 5 days post cat excretion [111], however, these oocysts can persist as infectious entities in the environment for as long as a year post deposit [112]. A single oocyst contains 2 sporocysts, that mature to form 4 sporozoites each [111]. Upon entering the digestive tract of the intermediate host, these sporozoites invade the gut epithelium and subsequently become tachyzoites.

The tachyzoite is the fast (asexual) replicating lifeform. Physically characterized by its curved, arc shape, it is around 5µm by 2µm in dimension and follows a well-defined lytic cycle (detailed in the next section). This lifeform is the easiest to study and maintain in culture *in vitro* as it asexually replicates every 6 to 8 hours and can be maintained in this manner indefinitely

[113]. This quick-replicating, invasive form disseminates throughout the host's body during the acute phase of infection. Meanwhile, the host immune system begins to mount a response to control the parasitemia, and this immunological pressure promotes conversion to the slow-growing bradyzoite form [114–116].

Bradyzoites are the slow-growing, persistent lifeform of the parasite that form tissue cysts [111]. In immunocompetent animal studies, bradyzoite conversion is typically seen a week post-infection [117], and the resultant tissue cysts are primarily located in muscle and central nervous tissues [111]. They range in size from 10μm-100μm and contain anywhere from two to over a thousand parasites [111]. *In vitro* studies have found that bradyzoite conversion can be induced by environmental stressors, such as alkaline pH [118, 119], arginine depletion [120], and heat shock [118], but bradyzoite development can also be spontaneous [121] and is particularly favored within certain cell types, like neurons and muscle cells [122–124]. These tissue cysts are refractory to drug treatment and assumed to reside within the host for the remainder of the host's lifetime [114, 117]. The tissue cyst wall also allows the bradyzoites to survive ingestion and to infect the gut epithelium of its new host [111]: either the cat, where sexual replication can proceed or another intermediate host to resume asexual replication. However, that is not to say that the bradyzoite is incapable of converting back into the virulent tachyzoite within the same host. Rather, clinical observations and *in vivo* animal studies suggest the bradyzoite is capable of sensing and taking advantage of signs of decreased immunological pressure from the host: for example, it is common for AIDS patients to suffer from relapsing toxoplasmosis [125, 126], as it is known that interferon-γ (IFNγ)-secreting T cells are required to suppress and maintain chronic infection [127]. Likewise, chronically infected mice experience parasite reactivation following T cell depletion [127] and/or IFNγ suppression [128–130].

1.2.2 The Tachyzoite's Lytic Cycle

During primary infection, *T. gondii* actively replicates and invades various tissues as part of its lytic cycle; eventually, though, the host immune system is able to control the infection and confine the parasite to intracellular cysts. The tachyzoite's lytic cycle is a well-orchestrated process of attachment, invasion, parasitophorous vacuole (PV) formation, replication, and egress (**Figure 6**). Moreover, this process is very fast: a tachyzoite can firmly attach, penetrate, and establish a PV in less than 30 seconds [131].

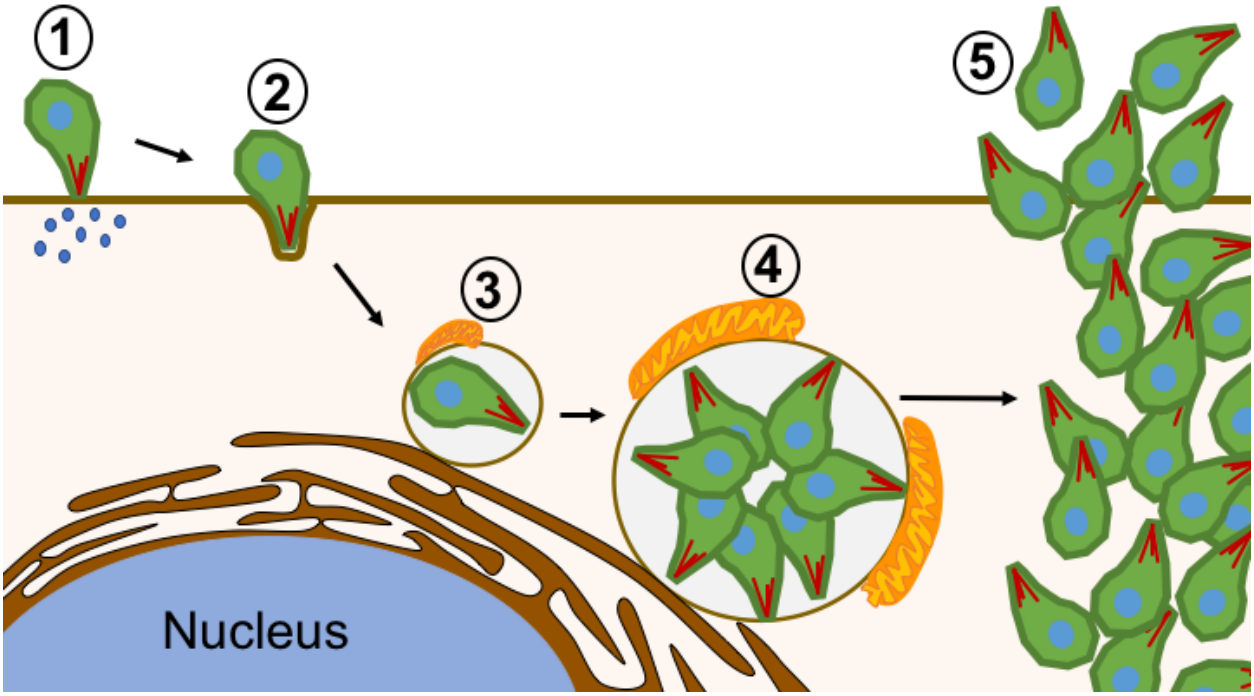


Figure 6 The Tachyzoite's Lytic Cycle

The tachyzoite's lytic cycle begins with (1) attachment onto the host cell surface and a primary secretion event. (2) The tachyzoite then invades the host cell by creating an invagination of the the host plasma membrane. (3) The resulting parasitophorous vacuole associates with host endoplasmic reticulum and mitochondria. (4) The tachyzoite asexually replicates via endodyogeny within the parasitophorous vacuole. (5) Evenutally, the parasites will lyse out of both the parasitophorous vacuole and the host cell; ready to begin the lytic cycle again.

The attachment process begins when the extracellular tachyzoite glides to its new host cell and makes surface contact via non-specific interactions between parasite surface antigens and sulfated proteoglycans at the host cell surface [132]. Various effector molecules are secreted into the host cell and help to form a tighter connection and the moving junction (the mobile interface of the host cell and actively invading parasite during the invasion process). Key effector molecules that make up the moving junction are the parasite's apical membrane antigen 1 (AMA1) and a complex of rhoptry neck (RON) proteins [133]. Anchored in the parasite's plasma membrane, AMA1 interacts with the RON complex embedded within the host cell membrane.

As the parasite invades, it forces its way through the moving junction into a newly formed invagination of the host cell membrane [134]. In the process of creating this PV from the host cell's plasma membrane, the moving junction also acts as a molecular sieve to exclude embedded host proteins [135]. During infection, the PV closely associates with the host ER and mitochondria [136]. Throughout this process, the PV is nonfusogenic and does not acidify; thus serving as a protective barrier throughout infection [137].

Within its PV, the successful tachyzoite invader can now commence with its asexual replication via endodyogeny [138–140]. Endodyogeny is an asexual replicative process by which two daughter cells are assembled within a mother cell. Ironically for a parasite, the daughter cells essentially consume the mother cell from the inside out (**Figure 7**). The anterior end of the daughter cells is synthesized first (inner membrane complex), and as replication proceeds, the maternal nucleus and organelles are split between the two nascent daughter cells. Upon completion of this internal partitioning of the mother cell contents, the progenitor cell's inner membrane complex dissolves and the remaining maternal plasma membrane is cleaved in two, beginning at the anterior pole. Consequently, the daughter cells maintain a tenuous connection via maternal

residue at the posterior pole; this tether gives a cluster of synchronous, replicating tachyzoites its characteristic arrangement in a rosette pattern (**Figure 7**) [139, 141]. *In vitro*, the tachyzoite undergoes endodyogeny every 6 to 8 hours [113]. After 6 or 7 rounds of replication the parasites egress [113], causing lysis of the host cell [142]. Thus, one parasite can produce 64 to 128 tachyzoites during infection of a single cell. Egress can be triggered by a variety of intrinsic and extrinsic triggers, such as the pH of the PV dropping below a certain threshold or activation of the extrinsic host cell death pathway [133]. The egressing tachyzoites will then disseminate from the site of infection to invade other tissues.

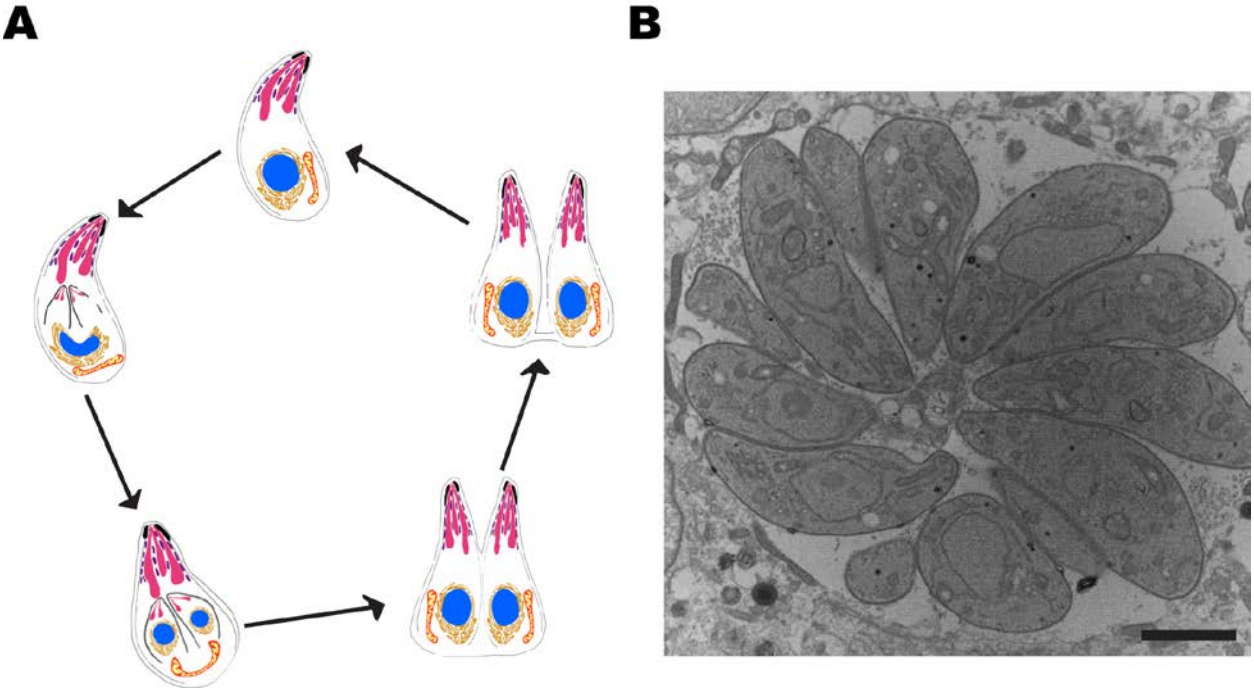


Figure 7 Endodyogeny

Asexual replication of *Toxoplasma*. (A) Illustration of endodyogeny. First the inner membrane complex of the daughter cells forms within the mother cell. Organelles are divided between the two developing daughter cells. The process is completed as a furrow forms between the daughter cells, dividing the plasma membrane. (B) Repeated, synchronized endodyogeny results in the formation of a rosette of parasites with leftover maternal residue at the center.

1.2.3 Host-Parasite Interactions

In order to successfully infect and propagate, *Toxoplasma* secretes various proteins into the host cell. Secreted proteins are organized into specialized secretory organelles that release their contents in an orderly fashion during invasion and intracellular replication. Proteins secreted after parasite invasion must also cross the PV membrane in order to gain access to the host cytoplasm and nucleus.

1.2.3.1 Specialized Secretory Organelles in *Toxoplasma*

The ultrastructure of the parasite is illustrated in **Figure 8**. Notably, *Toxoplasma* possesses three kinds of specialized secretory organelles: micronemes ("little threads"), rhoptries ("club-shaped"), and dense granules (named for their electron-dense appearance in transmission electron micrographs). These specialized secretory organelles are discharged in a particular order during infection. Most proteins secreted via one of these organelles follow a simple naming convention in accordance to their organelle of origin: microneme proteins are designated as "MICx", rhoptry neck proteins as "RONx", rhoptry bulb as "ROPx", and dense granules as "GRAx"; "x" representing a number in its order of identification [143]. In the tachyzoite life stage, the micronemes and rhoptries are positioned near the conoid; in contrast, the dense granules have a scattered distributed within the cell.

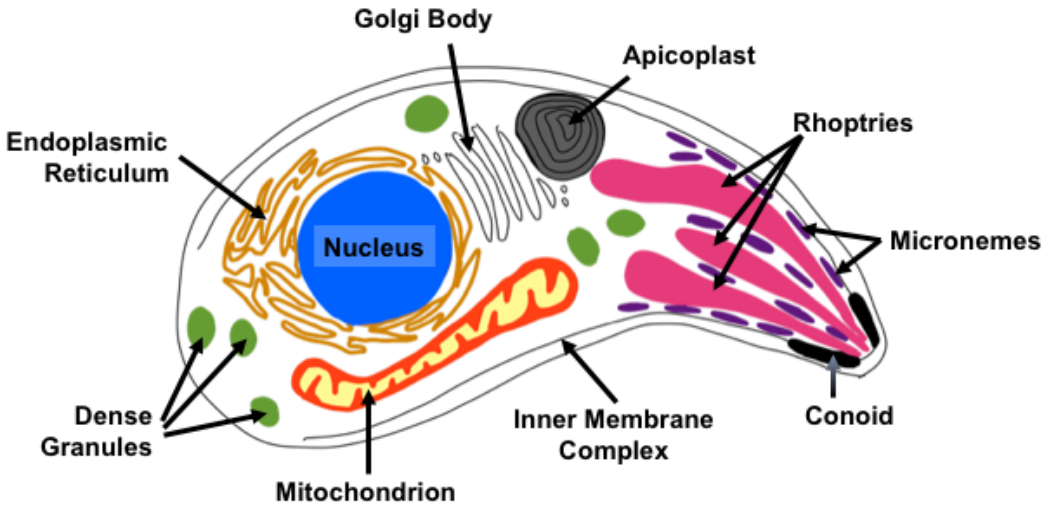


Figure 8 The Tachyzoite Ultrastructure

The *Toxoplasma* tachyzoite possesses three types of specialized secretory vesicles: micronemes, rhoptries, and dense granules. Like other eukaryotic cells, the tachyzoite has a nucleus, endoplasmic reticulum, and Golgi body. There is also a single long mitochondrion. The apicoplast is a non-photosynthetic plastid common amongst organisms in the phyllum Apicomplexa. Along most of the inner periphery of the tachyzoite is the inner membrane complex; which consists of membrane sacs flattened into sheets.

Secretion is temporally regulated in a cascade that is initiated upon the parasite's attachment to a host cell [144]. During the first, transient attachment, microneme contents are discharged through the conoid, releasing microneme adhesions that assist with firm attachment and creation of the moving junction. Also contributing to the moving junction is the contents of the rhoptry neck (the RON complex), which are discharged around the same time. Next to be secreted is the cargo of the rhoptry bulbs, initializing the formation of the PV. Infections performed in the presence of the cytochalasin D (an actin inhibitor that prevents parasite invasion, but not attachment and initial injections) illustrated that these early secreted proteins are capable of forming small vacuoles (evacuoles) possessing properties similar to that of the real PV: tightly

associating with the endoplasmic reticulum and mitochondria while non-fusogenic with the host's endosomes nor lysosomes [145]. After invasion and formation of the parasitophorous vacuole, the dense granules begin to release their cargo and will continue to do so throughout the rest of the infection [144]. Unlike the other secretory organelles, the dense granules do not secrete their contents near the conoid [146]. Instead, they penetrate the inner membrane complex to fuse with the outer membrane. GRA proteins will then be targeted to the PV vacuolar network, associated with the PV membrane, or exported out of the PV into the host cell.

1.2.3.2 Exporting Proteins Beyond the PV Membrane

While the PV membrane forms a seemingly impenetrable barrier segregating the host cell's cytoplasmic contents from the replicating tachyzoites, an increasing number of *Toxoplasma* dense granule proteins have been identified that transverse this barrier and even enter the host cell nucleus [147–151]. At first, GRA17 and GRA23 were suspected to form this protein export machinery due to their shared homology with known *Plasmodium* PTEX (*Plasmodium* translocon of exported proteins) components, however, this function was not found to be conserved in *Toxoplasma* [152]. Instead, these two proteins only facilitate the translocation of small molecules (<3 kDa) across the PV membrane. Recently, the MYR family of proteins have been identified as the true components of the *Toxoplasma* translocon for protein exportation [153, 154]. MYR1, MYR2, and MYR3 are localized to the PV membrane and deletion of either one has been shown to disrupt protein export [153, 154]. Furthermore export into the host cytoplasm is dependent upon the translocating protein's structure, or rather its absence thereof [154–156].

Unlike its relative *Plasmodium* which has the ability to unfold highly structured proteins in order to secrete them beyond the PV membrane [157], the *Toxoplasma* PV translocon (of which the MYR family is believed to be a part) relies upon the unstructured nature of the secreted proteins

themselves [154–156]. Thus, only disordered proteins may be funneled through *Toxoplasma* PV membrane. This scheme for secreting factors into the host cytoplasm decreases the energy expenditure, and it may explain the observation that many GRA proteins are intrinsically disordered and lack obvious catalytic domains [156, 158]. Investigation of GRA16 also suggests that there could be an additional advantage to these disordered GRA proteins [147]. GRA16 was one of the first dense granule proteins found to not only cross the PV membrane but also enter the host cell nucleus, where it interacts with several host factors to regulate the p53 pathway and hijack metabolism and cell cycle progression [147]. These activities are mediated by the presence of short linear motifs (SLiMs) of no more than 11- and no less than 3-contiguous amino acids long [159, 160]. SLiMs may serve as nuclear localization signals, protease cleavage sites, docking sites, etc. Their short size is evolutionarily advantageous as they may arise (or disappear) with a single point mutation [161–163]. Thus, *Toxoplasma* gains several benefits from utilizing a translocon whose substrates are intrinsically disordered.

1.2.3.3 The IFNγ Response and Parasite Countermeasures

While Toxoplasma can productively infect practically any nucleated cell in vitro [142], IFNγ-primed tissue cultures are capable of significantly resisting infection [164–168]. This dependence on IFNγ to control T. gondii is also seen at the organismal level, where the host immune system relies upon the production of IFNγ to control and limit the primary parasitemia [128, 169, 170] and control chronic infection [129, 130, 171, 172]. Herein is summarized the pathways of host recognition of Toxoplasma and the induction of IFNγ, the anti-parasite responses stimulated by IFNγ, and the known mechanisms by which the parasite is capable of evading this response.

Initial Detection of **Toxoplasma** *Infection*

The robust IFNγ response to *Toxoplasma* infection can be considered to begin with the DC, whereby sensing of the parasite by its innate immune sensors leads to the induction of IL-12. In turn, IL-12 secretion amplifies IFNγ production by NK and T cells [173–175]. While IL-12 can be produced by several other kinds of immune cells, including macrophages, monocytes, and neutrophils, the initial secretion of IL-12 by the DC is ultimately critical for coordinating the amplifying cascade that culminates in high IFNγ production and the confinement of the parasites to quasi-dormant tissue cysts in the mouse model [176–181] (**Figure 9**).

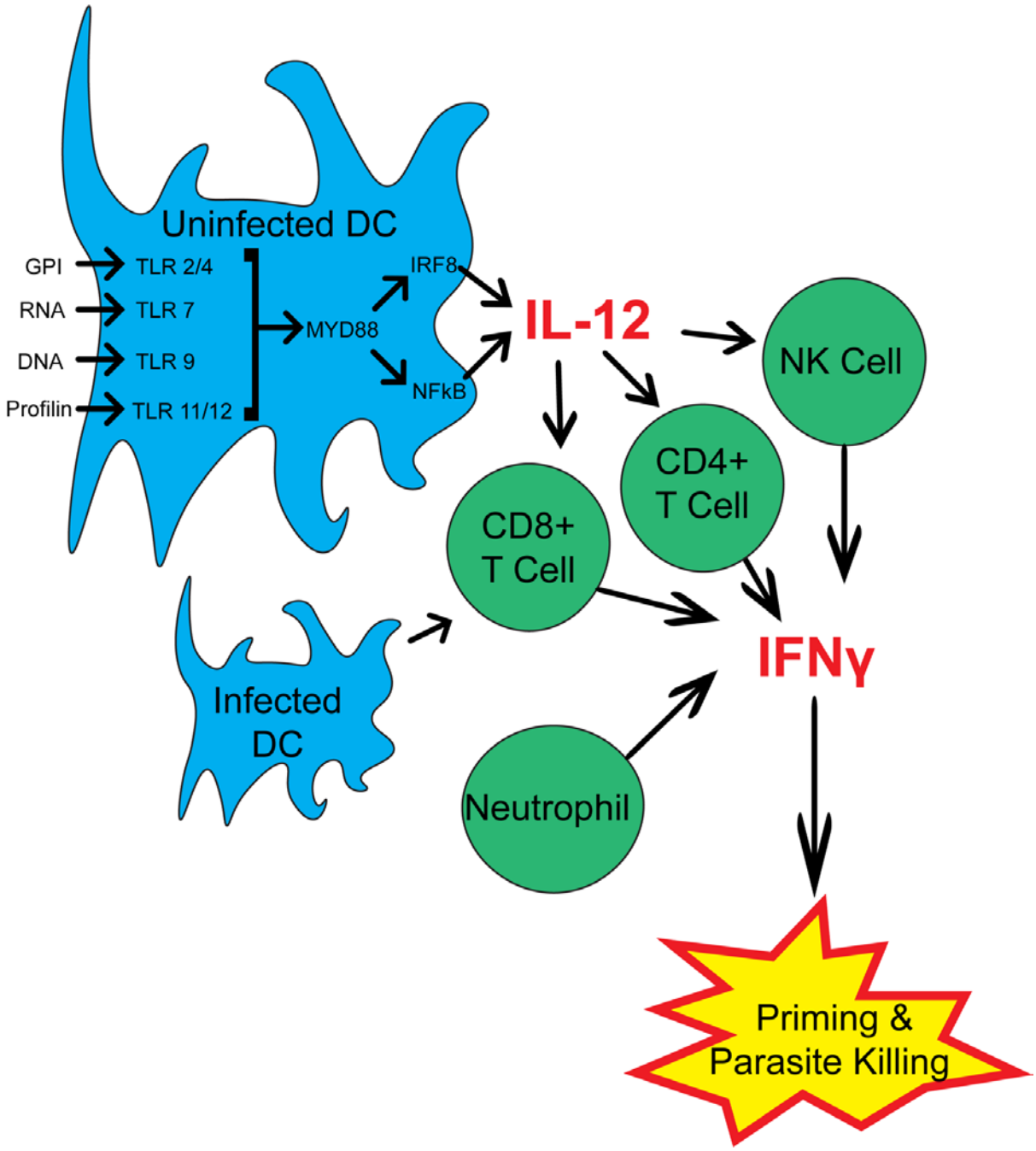


Figure 9 Induction of IL-12 & Interferon-γ

TLR activation of uninfected DCs leads to the induction of IL-12. IL-12 then further promotes IFNγ production by NK and T cells. Neutrophils also produce IFNγ, but it is through an as-yet-unknown IL-12 and MYD88 independent mechanism. Infected DCs activate T cells. As a result of IFNγ production uninfected cells become primed and able to kill subsequent parasite invaders.

To date, only two innate sensors haven been found to directly and specifically recognize *Toxoplasma*: Toll-like receptors 11 and 12 (TLR11, TLR12) [182–186]. Homodimers of TLR12 or heterodimers of TLR11-TLR12 are located in the endolysosome where they bind *Toxoplasma* profilin and signal through the adaptor protein MYD88 to promote IL-12 induction. Profilin is an actin-binding protein essential for the parasite's motility and host cell invasion process. As profilin is regularly shed from the parasite into the environment [182, 183] and can be endocytosed by host cells, this allows the DC to detect the parasite without physical contact. Mice with DCs deficient in TLR signaling exhibit low IL-12 production, and as a consequence, IFNγ production is reduced; resulting in an increased parasite burden [187].

However, it is important to note that primates, birds, and cats do not express any homologs of TLR11 and TLR12 [188]. In the human genome, *TLR11* is a pseudogene and no homolog of *TLR12* exists. Furthermore, primary human monocytes and DCs can only induce IL-12 when in direct contact with live parasites; in contrast, their murine counterparts that can easily be stimulated with heat-killed or soluble extracts from *Toxoplasma* tachyzoites [189–191]. Yet, in these other species, the primary parasitemia is still effectively controlled. This suggests that various species have evolved their own mechanisms to recognize *Toxoplasma* infection [188].

Other TLRs, including TLR9 and TLR2, have been implicated in eliciting an immune response upon *Toxoplasma* infection, however they are hypothesized to only indirectly or nonspecifically recognize the parasite. For example, data suggests that TLR9 (which binds unmethylated DNA in the endolysosome) may become activated in response to commensal gut bacteria affected by the presence of *Toxoplasma* [186, 192–196]. These studies have found the role of TLR9 to be highly dependent upon the route of infection (oral versus intraperitoneal injection). [194, 197–199]. TLR2 has been proposed to nonspecifically recognize

glycophosphatidylinositol (GPI) anchors [200–203]. GPI is a posttranslational modification of a protein's C-terminus that allows it to bind to the plasma membrane upon secretion, and is commonly found on the surface of various protozoans [200, 201, 204]. However, like TLR9, studies have found discrepancies in TLR2's role depending upon oral or intraperitoneal injection routes of infection [194, 203, 205]. Furthermore, TLR2 activation does not majorly affect IL-12 production, the main driver of downstream IFNγ [206].

Besides TLRs, the components of inflammasome activation have also been implicated in the early detection and control of *Toxoplasma*: the $P2X_7$ receptor and NALP1. $P2X_7R$ is a membrane ion channel found on the cell surface of monocytes and macrophages and activated by extracellular ATP [207]. $P2X_7R$ in particular plays a large role in formation of the inflammasome, induction of apoptosis, and elimination of intracellular pathogens via phago-lysosomal fusion [208–212]. Studies have found that both human and mouse macrophages are capable of killing intracellular *Toxoplasma* upon $P2X_7R$ activation, mediated by reactive oxygen species (ROS) and enhanced targeting of the PV for lysosomal fusion [213, 214]. Human studies even suggest natural single nucleotide polymorphisms (SNPs) that render $P2X_7R$ functionality defective may make those individuals more susceptible to both acquired and congenital toxoplasmosis [214, 215]. Another innate sensor that may be triggered by *Toxoplasma* is the NOD-like receptor NALP1. Located in the cytoplasm, activation of NALP1 results in inflammasome assembly, secretion of IL-1beta and IL-18, and activation of the inflammatory cell death pathway pyroptosis. While the exact mechanism by which NALP1 is activated by *Toxoplasma* is not known [216], it may play an important role in IFNγ induction. The importance of NALP1 was first recognized in a screen for susceptibility markers for congenital toxoplasmosis, where some allelic variants are more closely associated with congenital toxoplasmosis [217]. *In vitro* experiments where NALP1 was knocked-

down found parasite infection of these monocytes could no longer elicit IL-1beta, IL-18, and IL-12 upregulation [217].

Anti-Parasite Responses Induced by IFNγ

Animal and *in vitro* studies have found IFNγ to be essential for controlling parasitemia and host survival [169, 179, 181, 218, 219], however the exact mechanisms that mediate control are still not fully understood. *In vitro* experiments have found IFNγ pre-stimulation makes the host cell inhospitable toward the intracellular parasite, either by attacking the protective PV, starving the parasite of essential molecules, or by diffusing across the PV to cause direct damage to the parasite itself. Importantly, the toxoplasmacidal effects of IFNγ are completely dependent upon priming before infection [220].

As mentioned previously, the PV is carefully formed upon invasion: while derived from the host cell membrane, the moving junction sieves-out and prevents the incorporation of a majority of host membrane proteins that could act as receptors for innate immune pathways. However, priming with IFNγ can stimulate expression of genes and noncanonical pathways that protect the host cell from the intruding parasite by attacking that carefully crafted PV. In murine cells, IFNγ induces expression of immunity-related GTPases (IRGs) and p67 guanylate-binding proteins (GBPs). These proteins are loaded onto the PV with the aid of several core autophagy proteins (such as the Atg5-Atg12-Atg16L1 E3 ubiquitin ligase complex) to disrupt the PV membrane [221–223]. The exposed parasites can then be quickly cleared from the cell cytoplasm. However, while the mouse genome has encoded 21 members of the IRG family and 13 GBPs, humans only have a single IRG homolog and 7 GBPs [224, 225]. Furthermore, *in vitro* studies have found most of the human GBPs to be nonessential for controlling *Toxoplasma* replication in

IFNγ-activated HAP1 cells [226]. Yet, a similar strategy may still be used by human cells; for example, Selleck *et al* found several core autophagy proteins to play important roles in controlling *Toxoplasma* via a noncanonical autophagy pathway [168]. Early components that label targets for autophagy (such as ubiquitin, p62, and LC3) are recruited to the PV and encased by multiple membranes, but never fuse with late endosomes or lysosomes. This suggests that while human cells may be capable of similarly targeting the PV, other mechanisms may also be at play.

Another strategy IFNγ-primed cells employ is starving the parasites of essential molecules. The first such mechanism was identified in the 1980s by Pfefferkorn and colleagues [164, 227]. Spurred by the observation that higher doses of IFNγ were required to inhibit *Toxoplasma* replication when human fibroblasts were grown in Dulbecco's media compared to Eagle's media, they ultimately found IFNγ induces expression of indoleamine 2,3-dioxygenase (IDO). IDO degrades tryptophan to *N*-formylkynurenine, thus starving *Toxoplasma* of the essential amino acid. Another target for parasite starvation is iron; a study performed in primary IFNγ-activated rat enterocytes found *Toxoplasma* replication to be dependent upon the level of intracellular iron available [228].

Reactive oxygen and nitrogen derivatives are the third mechanism by which IFNγ stimulation can restrict *Toxoplasma* infection, specifically in macrophages. These molecules can easily diffuse across the PV to directly cause damage to the parasite in a non-specific manner. Upon phagocytosis of *Toxoplasma*, monocytes and macrophages respond with respiratory burst activity that is associated with mass killing of parasites within 6 hours [229]. In contrast, monocytes collected from individuals with chronic granulomatous disease (and thus oxidatively deficient) exhibited markedly less toxoplasmacidal activity [229]. Likewise, reactive nitrogen species have also been implicated in macrophage-mediated killing of *Toxoplasma*.

Toxoplasmacidal activity of IFNγ and LPS-activated macrophages has been demonstrated *in vitro* to be abrogated by the addition of a competitive inhibitor or L-arginine [230], and *in vivo* deletion of inducible nitric oxide synthase in the mouse model was found to be essential for controlling persistent, chronic *Toxoplasma* infection [231]. However, the production of reactive nitrogen species consumes the intracellular pool of arginine—an amino acid essential for *Toxoplasma* replication [120]. Arginine starvation also acts as trigger for tissue cyst formation and bradyzoite differentiation [120].

Parasite Manipulation and Evasion of the Host Immune Response

It is interesting to note that all known mechanisms by which a cell can autonomously kill intracellular *Toxoplasma* require stimulation with IFNγ prior to infection. Post-invasion, the parasite is capable of effectively modulating the host cell's responses in order to promote its own propagation and parasitic life cycle, including interactions with the host immune system. To persist within the ecosystem, it is advantageous for the parasite to both disseminate throughout the body of the host and elicit a strong host immune response. Rather than completely clearing infection, the antagonism of IFNγ ultimately confines the parasites to slow-growing tissue cysts in the skeletal muscle and brain. These pockets of parasites may then persist throughout the lifetime of the host. Thus, the infected host becomes the vector by which the parasite can pass onto its next host, via carnivorism. Toward this goal, *Toxoplasma* secretes various effector proteins into the host cell during invasion and intracellular residency. These effector proteins can manipulate the host cell by directly inhibiting innate cellular defenses, interfering with IFNγ receptor signaling, and manipulating interactions between immune cells.

Obstruction of host IRGs' ability to puncture the PV is a most notable example of the parasite's capacity to directly inhibit key intrinsic cellular defenses. ROP18, a serine/threonine

kinase, is the primary virulence factor *in vitro* [232, 233]. By phosphorylating several key IRG proteins, it is able to prevent their oligomerization and subsequent loading onto the PV membrane [234, 235]. ROP5 is a pseudokinase that allosterically enhances the function of ROP18 and is also capable of independently preventing IRG oligomerization [236, 237]. Interestingly, while ROP18 has a strong phenotype *in vitro*, its absence only moderately decreases virulence *in vivo* [233]; while on the other hand, ROP5 actually has a very strong *in vivo* phenotype [238]. It should be noted, though, that these virulence traits are strain-specific, as not all strains of *Toxoplasma* possess the virulent alleles of these genes [232, 233, 238, 239].

In addition to counteracting innate cellular defenses, *Toxoplasma* is also capable of making host cells unresponsive to IFNγ receptor signaling [240–242]; thus cells treated with IFNγ post-infection do not exhibit any parasite growth restrictions. The dense granule protein TgIST (*T. gondii* inhibitor of STAT transcription) is a striking example of how the parasite is able to override the host cell's normal response to IFNγ [149, 150]. Normally, upon binding of IFNγ with its receptor, STAT1 is phosphorylated, dimerizes, and translocates into the host cell nucleus. There, STAT1 will bind the promoter regions of interferon-stimulated genes (ISGs) and stimulate their induction [243]. However, TgIST interrupts this chain of events by binding both nuclear STAT1 and the nucleosome-remodeling and deacetylase (NuRD) complex, resulting in an altered chromatin environment non-conducive for ISG induction [149, 150]. Strikingly, ectopic expression of TgIST alone is sufficient to prevent ISG induction in this manner [149], however, the particular mechanism by which this nucleosome alteration is not yet understood [156].

A third method by which *Toxoplasma* can manipulate and co-opt the host immune response is by GRA-influenced cytokine induction. Initially assumed to be limited to the PV, an increasing number of dense granule proteins (GRAs) have been shown to exert effects beyond the PV and

even translocate across the PV membrane to enter the host cell nucleus [147–151]. GRA15 was the first dense granule protein reported to exert activity beyond the PV to which it is bound: in particular, the variant encoded in the type II genome is known to activate the p65 subunit of NF-kB and induce IL-12 [244]. While IL-12 induction may seem counterintuitive for the individual tachyzoite, the downstream production of IFNγ can be seen as a way for the parasite to control its own virulence, promote persistent infection, and increase the probability of transmission to the next host through carnivorism. To aid in parasite dissemination, another dense granule protein, GRA6, recruits monocytes and neutrophils via secretion of chemoattractants CXCL2 and CCL2. The exact mechanism by which GRA6 exerts this effect despite being restricted to the PV is not completely known, but involves the activation of NFAT4 by direct interaction of GRA6 with the calcineurin activator calcium-modulating ligand [245]. Meanwhile, GRA24 is able to promote both the induction of IL-12 as well as CCL2 via the p38alpha MAPK pathway [148]. Thus, by activating the host immune system and specifically recruiting particular lymphocytes, *Toxoplasma* may hijack these lymphocytes in order to better disseminate throughout the host [246–248].

1.3 Congenital Toxoplasmosis

The first recorded case of human toxoplasmosis was in 1939 [249]. A 3-day old infant presented with seizures, respiratory complications, spinal cord injury, and macular lesions and subsequently died by day 31. Brain lesions containing a crescent-shaped protozoan were found upon autopsy, and samples of these lesions were capable of causing similar lesions following intracerebral inoculation of rabbits and infant mice [249]. Ultimately, the infant was determined

to have died from toxoplasmic encephalomyelitis, a disease today recognized to have been congenital toxoplasmosis (CT).

The rates of CT incidence vary across the globe, with an estimated 5-23 cases per 10,000 live births in Brazil [250], 2.9 cases per 10,000 live births in France [251], and 0.5 cases per 10,000 live births in the United States (likely an underestimate, as there is no routine, nationwide screening of CT in the United States) [252]. Vertical transmission of *Toxoplasma* can have devastating consequences: from miscarriage or still-birth to birth defects, such as developing blindness due to ocular toxoplasmosis or neurological defects, with the degree of disease severity varying according to the stage of pregnancy transmission occurred. Infection during the first trimester often results in miscarriage and at the third trimester, loss-of-vision and mental disabilities in surviving children. Infants with severe clinical signs of infection may present with hydrocephalus, chorioretinitis, and intracranial calcifications [253]. However, 75% newborns with CT are asymptomatic at birth, and may not develop deafness, epilepsy, or symptomatic ocular toxoplasmosis (most commonly typified as chorioretinitis) until months, years, or decades later [253]. This delay in presentation can inadvertently cause postponement of treatment and potentially increase the risk and severity of disease manifestation. Case-in-point, several longitudinal studies have found correlations between pre-natal and early post-natal treatment and improved prognosis years later [254, 255].

1.3.1 Diagnosis

Cases of CT are typically diagnosed by serology and PCR [256, 257]. Risk of CT is determined by monitoring maternal seroconversion. When a previously *Toxoplasma* seronegative individual later tests positive, this indicates a current or recent exposure to the parasite:

Toxoplasma-positive IgM is indicative of an infection within the past several months, while IgG specific to *Toxoplasma* can be detected in the serum 1-2 weeks post infection and will be sustained indefinitely. Seroconversion during pregnancy thus serves as a risk marker for the possibility of *in utero* transmission to the fetus. Fetal infection can be confirmed by indirect and direct methods. Post-birth, the infant can be tested for the presence of *Toxoplasma*-specific antibodies or for the parasite directly by PCR of bodily fluids. PCR can also be used prior to birth on samples of amniotic fluid or fetal blood collected from cordocentesis, although these procedures carry their own risks to fetal safety [258]. As will be discussed later, in the event that a woman tested seropositive for *Toxoplasma* prior to pregnancy, transmission to the fetus is considered unlikely due to cross-protection [257].

1.3.2 Treatment

The standard treatment for CT is a combination of pyrimethamine and sulfadiazine (P/S), supplemented with folinic acid [256, 259]. This drug is given to infected infants as well as pregnant women beyond the first trimester. Pyrimethamine and sulfadiazine are competitive inhibitors of key enzymes in *Toxoplasma*'s folate metabolism; while folinic acid is commonly given to ameliorate the side effects of pyrimethamine on the patient. This drug combination is the most effective and standard treatment for toxoplasmosis, but cannot be given to women in the first trimester of pregnancy for risk of teratogenic side effects on the developing fetus [259]. In these cases, spiramycin is the drug of choice as it is considered nonharmful to the fetus and primarily concentrates in the placenta [259, 260]. The specific mechanism of action for spiramycin is not known, but it is hypothesized that it inhibits protein synthesis by the apicoplast [261]. Current consensus holds that due to its propensity to concentrate at the maternal-fetal interface, spiramycin

treatment during pregnancy functions more as a fetal prophylactic by preventing placental infection [262]. If there is still concern of fetal infection, after the first trimester, spiramycin may be supplemented or replaced with the standard P/S regimen [263].

It is important to note that diagnosis, treatment, and prognosis all depend upon how soon the maternal seroconversion is detected. Numerous patient studies have found that the sooner treatment can be given, the better the potential prevention of CT or at least lessening of disease severity [253, 259, 263]. The importance of time in diagnosis and treatment may at least partially, if not completely, explain the differences in CT disease severity amongst patients in France versus the United States [264]. In France, a pregnant woman is routinely tested for seroconversion throughout the pregnancy [264]. Historically, France has had high seroprevalence among women of childbearing age (an estimated 83% of French women in 1965), and while seroprevalence has decreased (to 37% in a 2010 estimate), CT is still a national concern [251, 265, 266]. In contrast, seroprevalence amongst women in the United States is estimated to be only 9.1%, according to a 2009-2010 survey [267], and routine testing for seroconversion is not standard [264]. Many cases of CT in the United States are not detected until later into the infection, when an abnormal fetal ultrasound is presented or even not until post-birth [264, 268]. Thus, timing of treatment for CT in the United States is more delayed, usually not given until after obvious fetal infection. Differences in disease severity can be seen in comparing follow-up studies of French [269–271] and American CT patients [272, 273], where a far higher percentage of American children will develop additional ocular lesions later in life, albeit still far better prognoses than seen in earlier studies with no treatments [273, 274].

1.3.3 Transmission

Classically, CT occurs as a result of *in utero* transmission of *Toxoplasma* during primary maternal infection, marked by maternal seroconversion. During pregnancy, the immunologically naïve mother may orally inoculate herself with *Toxoplasma*-contaminated food. Unwashed raw vegetables and fruits may be contaminated with highly infectious, sporulated oocysts shed into the environment by the cat. Consumption of undercooked meat is also a risk, as it can harbor *Toxoplasma* tissue cysts [257]. In most cases, if an expectant mother has previously been infected two or more months prior to conception, the primed immune system is able to prevent productive infection upon secondary exposure and there is little risk of transmission to the fetus [257, 259, 260]. Whereas if the pregnant woman had not been previously infected, the parasite is able to disseminate un-checked, during which it may encounter the fetal compartment either upon further dissemination within the pregnant uterus or hematogenously as free tachyzoites or via infected leukocytes [74, 75, 275].

While most cases of CT are linked to maternal seroconversion during pregnancy, there have also been rare reports of congenital transmission from women who were seropositive before pregnancy. Typically in such cases, the women are severely immunocompromised, such as with HIV, and experience reactivation of latent *Toxoplasma* that can lead to congenital transmission [276–278]. Interestingly, there are also reports of CT of an acquired secondary *Toxoplasma* infection from otherwise healthy, immunocompetent women due to highly virulent atypical strains [279–283]. An increasing number of reports have found the atypical strains of *Toxoplasma,* predominant in South America, to exhibit increased virulence resulting in more severe infections [250, 284, 285] with no cross-protection from primary infection with classical *Toxoplasma* strains seen in animal models [286, 287].

1.3.4 *Toxoplasma* Genetic Diversity and Associations with Human Infections

The *Toxoplasma* genome is haploid and consists of 65 x 10^6 base pairs arranged amongst 14 linear chromosomes [288]. Most parasite populations are clonal with very little genetic variation, and these parasites can be phylogenetically organized into three lineages: type I, type II, and type III [289]. Differences in mouse virulence between the three classical *Toxoplasma* types has been used to map and identify virulence alleles: where type I is considered the most virulent, LD_{100}=1 parasite; type II is of intermediate virulence, LD_{50}=10^2-10^4 parasites; and type III is of least virulence, LD_{50}=10^5-10^6 parasites [233, 290]. Atypical parasites are unrelated to these lineages and possess a combination of various alleles typically assigned to the three clonal lineages, as well as unique polymorphisms [291, 292]. Different atypical strains have been associated with high and low virulence, and extreme genetic variations makes mouse virulence difficult to predict [291]. Furthermore, virulence in mice does not directly correlate to virulence in other species, including other rodents [293].

Amongst human toxoplasmosis cases, type II is the predominant lineage found in circulation within Europe, present in 64.1% of cases; while 12.5% and 9.4% of European infections are caused by types III and I, respectively [294]. Classically, North American parasites were believed to be highly clonal and similar to Europe, however, recent studies have found an increasing number of atypical strains: 43.9% atypical, 43.9% type II, and 12.2% type III [295]. In Central and South America, there are no predominant genotypes apparent and the majority of these parasites are classified as atypical strains [294]. In Africa, the degree of diversity is dependent upon region: North and East Africa are more similar to Europe (predominately type II and III), while tropical Africa displays more diversity and a predominance of atypical strains akin to that which is found in South America [296].

1.3.5 The Placenta as a Barrier to *Toxoplasma* Transmission

The fetus's first line of defense against vertical infection is the placenta. As previously discussed, the placenta is a formidable barrier that performs a myriad of duties, including protecting the fetus from any maternal pathogens. These functions are all orchestrated by the placental trophoblasts, which line the villous trees and directly contact the maternal tissues. Interestingly, recent studies with PHT cells and placental villous explants have found SYN to pose a formidable barrier against a range of infectious agents—including *Toxoplasma* [74, 75, 86]. However, congenital transmission does occur, and as the pregnancy progresses, there is an increased risk of transplacental transmission. During the first trimester, the probability of vertical transmission is 10-15%, but later in pregnancy, this risk increases to 30% during the second trimester, and 60% at the third trimester [259].

Based on the published data and current understanding of placental biology, several hypotheses could explain this phenomenon [297]. First, there is the correlation between increasing risk of transmission and increased placental contact with maternal blood [259]. Furthermore, *Toxoplasma*-infected monocytes have been shown to adhere more tightly to trophoblasts *in vitro* [275]. This close contact may increase the probability of hematogenous spread during maternal parasitemia. A second hypothesis proposes EVTs anchoring the placental villous trees to be the gateway for parasite dissemination into susceptible fetal tissues. In support of this idea, EVTs from first trimester explants have been found to be susceptible to *Toxoplasma* and may provide a route of entry into the villous core (and from there, the fetus itself) [75]. Third, damage to the *Toxoplasma*-resistant SYN layer exposes the susceptible underlying cells [74, 75]. As the placenta continues to grow throughout pregnancy, the late pregnancy placenta may present a larger target: with increased surface area, there may also be more opportunities for SYN damage that the parasite

can exploit. Despite its vital position at the maternal-fetal interface, the biology of placenta-*Toxoplasma* interactions is not well understood, and more research is required to explain how *Toxoplasma* is able to overcome the placental barrier.

1.4 Conclusions

Globally, 70% of women of childbearing age are immunologically naive for *Toxoplasma* and at risk for primary infection. While the prevalence of *Toxoplasma* infections within specific regions can widely vary, risk of infection during pregnancy is a serious concern, especially in this era of increasing globalization. In countries that do not practice active surveillance for potential vertical transmission, congenital toxoplasmosis can be particularly devastating as a seemingly healthy pregnancy can result in miscarriage or birth defects. The placenta is the fetus' first line of defense against vertically transmitted *Toxoplasma*. However, as the pregnancy progresses, the parasite is increasingly able to surpass this barrier. More research is needed to understand both the mechanisms that promote the placenta's barrier function, as well as strategies employed by *Toxoplasma* to overcome this barrier and cause fetal damage. The data presented in the subsequent chapters seeks to address some of this gap in our knowledge of *Toxoplasma* infections during pregnancy. In Chapter 2, I define the barrier functionality of the SYN to *T. gondii* infection, while Chapter 3 is focused on understanding how the parasite elicits a strong immunomodulatory response. Specifically, I use CCL22 induction as a model to understand one of the unique responses induced during *T. gondii* infection of primary trophoblasts, a response that cannot be modeled in trophoblast cell lines.

2.0 Human Placental Syncytiotrophoblasts Restrict *Toxoplasma gondii* Attachment and Replication and Respond to Infection by Producing Immunomodulatory Chemokines

Toxoplasma gondii is a major source of congenital disease worldwide, but the cellular and molecular factors associated with its vertical transmission are largely unknown. In humans, the placenta forms the key interface between the maternal and fetal compartments and forms the primary barrier that restricts the hematogenous spread of microorganisms. Here, we utilized primary human trophoblast (PHT) cells isolated from full-term placentas and human mid-gestation chorionic villous explants to determine the mechanisms by which human trophoblasts restrict and respond to *T. gondii* infection. We show that placental syncytiotrophoblasts, multinucleated cells that are in direct contact with maternal blood, restrict *T. gondii* infection at two distinct stages of the parasite lytic cycle—at the time of attachment and also during intracellular replication. Utilizing comparative RNAseq transcriptional profiling, we also show that human placental trophoblasts from both the second and third trimesters respond uniquely to *T. gondii* infection when compared to trophoblast cell lines, typified by the up-regulation of several immunity-related genes. One of the most differentially induced genes was the chemokine CCL22, which relies on the secretion of a parasite effector(s) either during or after invasion for its induction. Collectively, our findings provide new insights into the mechanisms by which the human placenta restricts the vertical transmission of *T. gondii* at early and late stages of human pregnancy and demonstrate the existence of at least two interferon-independent pathways that restrict *T. gondii* access to the fetal compartment.

2.1 Introduction

Toxoplasma gondii is a major source of congenital disease, with ~200,000 global cases of congenital toxoplasmosis reported each year [298]. In the majority of instances (~80%), *in utero* infections by *T. gondii* result in a range of severe birth defects, including ocular disease and developmental delays, and can also result in fetal death [299]. However, despite the clear impact of *T. gondii* infections on fetal health, the mechanisms by which the parasite is transmitted from the maternal bloodstream into the fetal compartment are largely unknown.

In eutherian organisms, the placenta serves as the sole source of gas, nutrient, and waste exchange between the maternal and fetal compartments and acts as a key barrier to restrict fetal infections. At the forefront of these defenses is the syncytiotrophoblast (SYN), a multinucleated cell layer that comprises the outermost layer of the human placenta and which is in direct contact with maternal blood. Subjacent to the SYN layer are cytotrophoblasts (CYTs), mononucleated and proliferative cells that fuse to replenish the SYN layer throughout pregnancy. Together, these trophoblast layers form a primary barrier to the passage of pathogens that may infect the fetus by the hematogenous route.

In general, the pathways that exist in the human placenta to limit the vertical transmission of microbes are poorly defined. Our previous studies in primary human trophoblast (PHT) cells have identified at least two potent antiviral pathways that restrict viral replication in trophoblasts [300, 301]. However, these pathways do not appear to be relevant during infection with non-viral pathogens, including *T. gondii* [77]. While studies in placental explants suggest that the SYN layer is not permissive to *T. gondii* infection [75], the mechanistic basis for SYN resistance is incompletely understood, as is whether the SYN layer mounts any innate defense in response to parasite exposure. Moreover, while placental explant models are useful in their recapitulation of

placental structure, they are limited in their capacity to dissect trophoblast cell type-specific pathways that might exist to limit *T. gondii* infection.

In this study, we interrogated the trophoblast cell-type specificity of *T. gondii* infection utilizing PHT cells isolated from full-term placentas and identified two cellular mechanisms that mediate SYN-specific resistance to *T. gondii* infection. In addition to discovering that SYNs restrict *T. gondii* attachment and replication while CYTs do not, we also identified cell signaling pathways uniquely induced by parasite infection in PHT cells, which included the induction of several immunity-related genes. We show that the majority of transcriptional changes in PHT cells is specific to the human pathogen *T. gondii* and does not occur in response to infection with the closely related parasite *Neospora caninum* (which is not known to cause disease in humans). Moreover, we show that the production of one such uniquely induced immunity-related gene, the T regulatory (T-reg) chemoattractant CCL22, is dependent on host cell invasion and the secretion of *T. gondii* effectors into the host cell. To expand our findings to earlier in human pregnancy, when the fetus is likely to face the more severe consequences of congenital *T. gondii* infections, we also utilized a mid-gestation chorionic villous explant model and show that second trimester SYNs also resist *T. gondii* attachment and induce CCL22 in response to infection whereas the fetal-derived amnion and chorion are permissive to infection and do not induce CCL22. Taken together, we have identified previously unknown intrinsic features in primary human placental cells from both the second and third trimesters of pregnancy that limit *T. gondii* infectivity at the level of attachment and replication, and provide details on both host- and parasite-specific transcriptional responses of placental cells to infection.

2.2 Results

2.2.1 Syncytiotrophoblasts Isolated from Full-Term Placentas Resist *T. gondii* Infection

We found that PHT cells isolated from full term placentas exhibited reduced susceptibility to *T. gondii* infection when compared to primary human foreskin fibroblast (HFF) cells (**Figure 10A, 10B**). These data are consistent with our previous work demonstrating that PHT cells exhibit reduced susceptibility to infection by the three major types of *T. gondii* in North America and Europe compared to non-placental cells [86]. Importantly, human trophoblast cell lines (including BeWo, HTR8, and JEG-3 cells) were unable to recapitulate this restrictive phenotype and were permissive to parasite infection (**Figure 10C, 10E**). In addition, this phenotype was specific to PHT cultures as primary placental fibroblasts were as permissive to infection as HFF cells (**Figure 10D**).

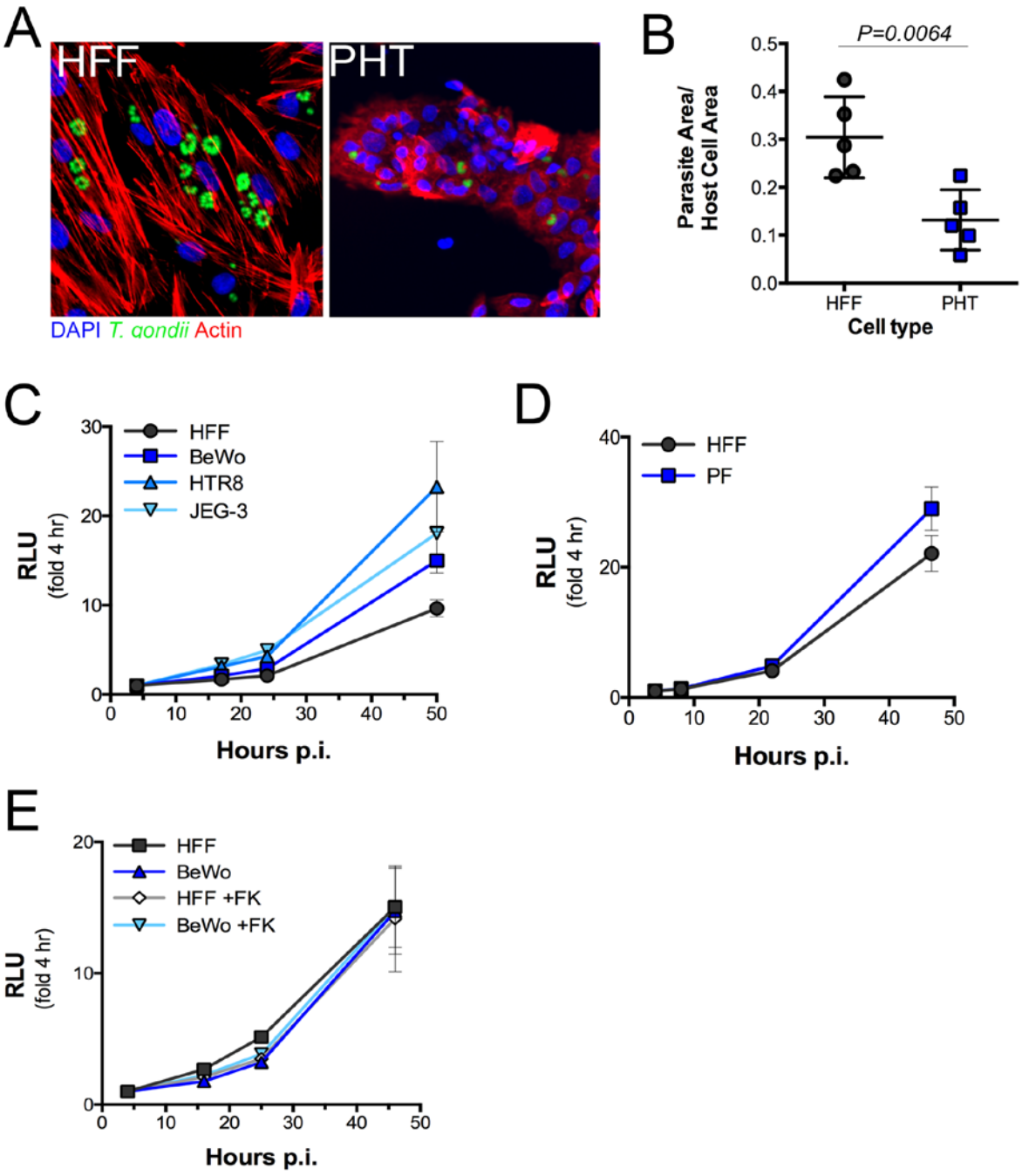


Figure 10 Trophoblast Cell Lines Do Not Recapitulate PHT Resistance to *T. gondii* Infection

(A, B) Immunofluorescence microscopy of HFF and PHT cultures inoculated with T. gondii RH strain (green) at MOI=2 for 24 h. (A) Representative images of HFF (left) and PHT (right) cultures. Actin is shown in red; DAPI in blue. (B) Ratio of parasite to host cell areas based on immunofluorescence of five fields of view per culture; single PHT preparation. P=0.0064 based on 2-tailed T-ˇtest. (C, D) Growth curves of *T. gondii* CEP strain at MOI=0.5 in the

indicated cell types, as measured by luciferase expression by parasites. Growth over time is indicated in relative light units (RLU) as normalized to expression at 4 hpi; and represented by the mean of three samples plus standard deviation. (C) *T. gondii* growth in three different trophoblast cell lines (BeWo, HTR8, and JEG-3) as compared to HFF cells. (D) Comparison of *T. gondii* growth in primary cultures of HFF and PF (placental fibroblasts). (E) *T. gondii* (CEP) growth in HFF and BeWo cultures +/- 10 μM forskolin pretreatment at MOI=0.5 as measured by luciferase expression by parasites. Growth over time is indicated in relative light units (RLU) as normalized to expression at 4hpi and represented by the mean of three samples plus standard deviation. At least two biological replicates were performed.

PHT cells isolated from full-term placentas spontaneously fuse to form SYNs during their culture period (~72hrs), with some retaining a mononuclear CYT phenotype. Therefore, to determine whether the lack of PHT cell infection occurred in a cell-type specific manner, we infected PHT cells with YFP-tagged *T. gondii* (RH strain) and quantified parasite growth specifically in CYTs versus SYNs. These studies revealed dramatic differences in the susceptibility of SYNs and CYTs to *T. gondii* infection—whereas CYTs were permissive to infection, SYNs were highly resistant (**Figure 11A, left**). Furthermore, we observed that parasites within SYNs replicated to a lesser degree, as indicated by a highly significant reduction in total cell area occupied by parasites (**Figure 11A**). Since *T. gondii* replicates within a parasitophorous vacuole (PV) generated at the time of invasion by each individual parasite, the number of parasites within a PV can serve as an indicator of parasite growth and replication. In contrast to the PVs in CYTs, those in SYNs most often contained 1-2 parasites (**Figure 11A, right**). Importantly, fusion of BeWo cells with forskolin, which induces syncytin-mediated fusion [86], was not sufficient to confer resistance to *T. gondii* infection (**Figure 10E**), supporting that this phenomenon is specific to primary cells and does not rely on fusion alone.

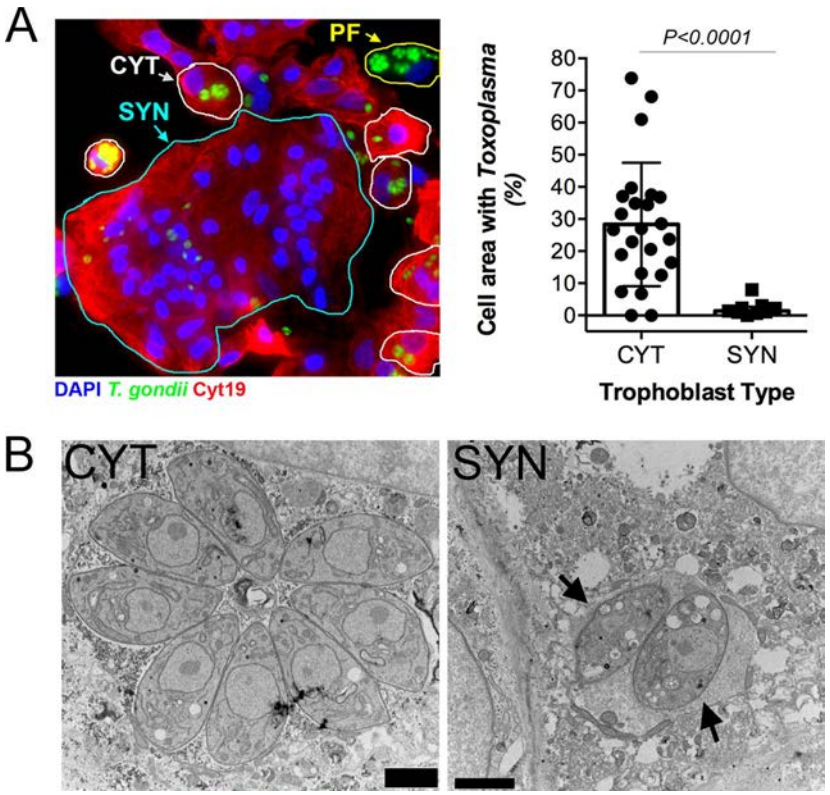


Figure 11 Placental Syncytiotrophoblasts Resist *T. gondii* Infection

(A) Left, immunofluorescence microscopy of PHT cells inoculated with *T. gondii* RH strain (green) for ~24h. Cytokeratin-19 is shown in red; DAPI in blue. SYN (outlined in cyan), CYT (outlined in yellow), and placental fibroblast (PF, outlined in white). PF cells are distinguished by the lack of cytokeratin-19 (red). Right, percentage of cell area occupied by *T. gondii*, as compared between CYT and SYN. N_{CYT}=24, N_{SYN}=9, from one preparation of PHT cells. 2-tailed T-test *P*<0.0001 with Welch correction for unequal variances. (B), Transmission electron microscopy of PHT cells infected with *T. gondii* (RH) for ~23hpi. Mononucleated CYT at left and SYN, identified by its more than two nuclei at right. Arrows indicate the parasites in the SYN. Scale bar is 2 μm.

Transmission electron microscopy (TEM) revealed that whereas parasite growth and PV morphology were normal in mononucleated cells within the preparation (which are likely CYTs but could also be rare contaminating placental fibroblasts), SYN-internalized parasites were found within PVs containing host cell cytoplasmic contents indicative of a loss of vacuole integrity (**Figure 11B**). Moreover, the parasites within these PVs contained more vacuoles of minimal

electron density and poorly defined organelles (**Figure 11B**). This phenotype is reminiscent of drug-induced death that we observed previously after treatment with a benzodioxole-containing compound [302], indicating that SYNs have potent Toxoplasmacidal activity. Taken together, these data implicate SYNs as an innately resistant cellular barrier to *T. gondii* infection and suggest that unlike other cultured cells, these cells potently resist *T. gondii* infection.

2.2.2 SYN-Mediated Resistance to *T. gondii* Infection Occurs at Two Stages of the Parasite Lytic Cycle

Our data suggest that SYNs restrict *T. gondii* infection at a stage of intracellular parasite growth. The primary mechanisms for cell-autonomous immunity to *T. gondii* are driven by the effector cytokine interferon γ (IFNγ). However, we found that uninfected and *T. gondii*-infected PHT cells had low levels of IFNγ transcript (**Figure 12A**) and that culture supernatants were devoid of secreted IFNγ protein (**Figure 12A, 2B**). Importantly, while the expression of GBP1 and GBP2 as well as other innate immunity-related factors (e.g., NOS1, 2 and IDO) have comparatively higher transcript levels in PHT cells (**Figure 12A**), none of these well-characterized IFNγ-driven host effector proteins were uniquely expressed in PHT cells (**Figure 12A**). These findings suggest that the innate resistance of SYNs is not dependent on basal expression of IFNγ or its stimulation by infection. To explore other related mechanisms, we performed immunofluorescence microscopy for markers of autophagy- and lysosomal-mediated degradation pathways given the high level of basal autophagy we previously observed in PHT cells [301]. We found that there was no association between SQSTM/p62 or the lysosomal associated component LAMP2 and internalized parasites at either early or later stages of infection (**Figure 12C** and **Appendix A, Figure 22**). These findings are consistent with our TEM-based microscopic studies,

in which we did not observe the association between double membraned autophagosomes or lysosomes with internalized parasites (**Figure 11B**).

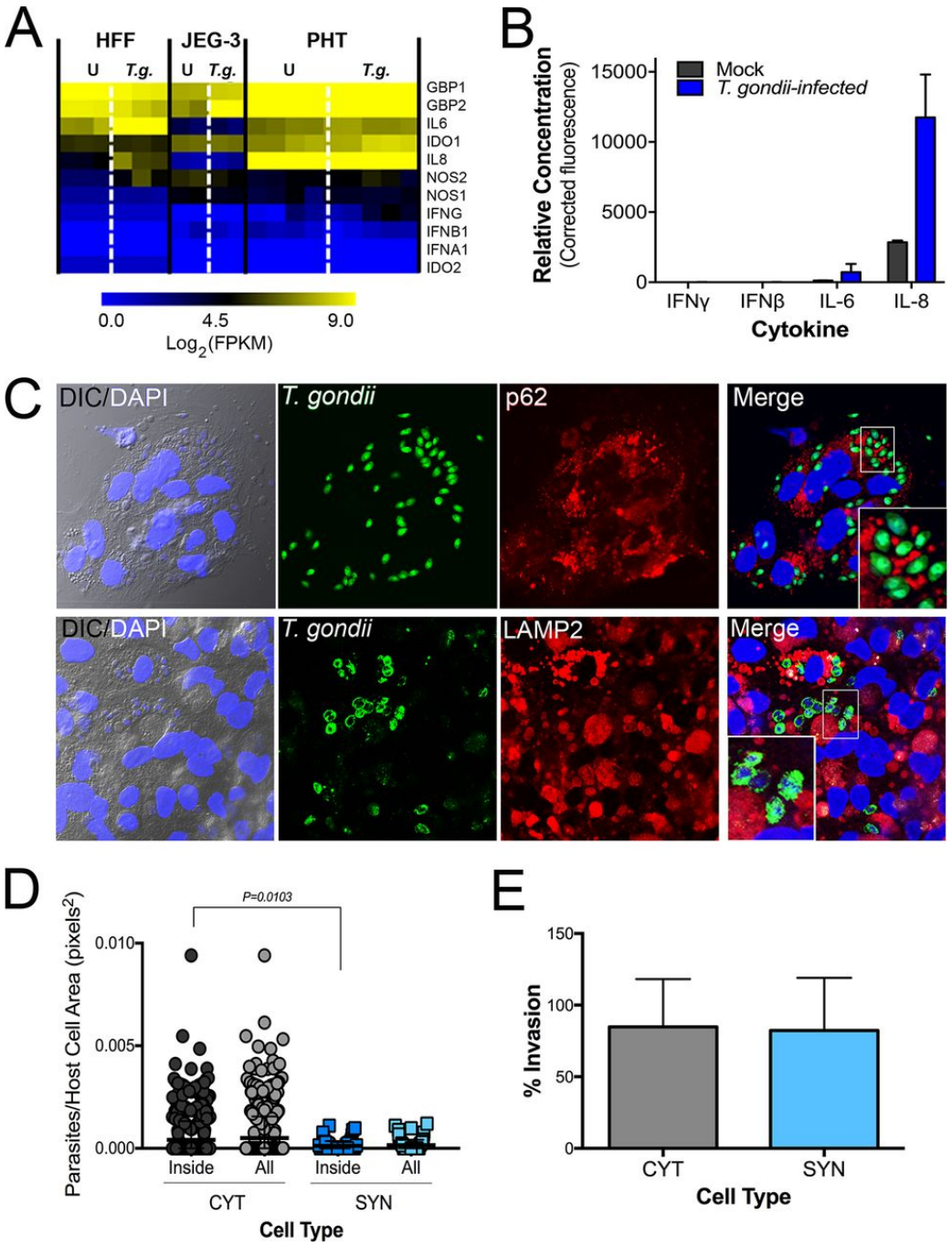


Figure 12 SYN-Mediated Restriction Of T. gondii Infection Is Not The Result Of Autophagy, Lysosomal Degradation, Or Inability To Invade.

(A) Heat-map of innate immune effector gene expression as determined by RNAseq of uninfected and *T. gondii*-infected HFF, JEG-3, and PHT cells. (B) PHT cytokine secretion as detected by luminex assay of media from mock and *T. gondii*-infected PHT cells. Data shown is from one of two PHT preparations. (C) Immunofluorescence microscopy of PHT cells infected with *T. gondii* (RH) (green) at 8hpi. *(Top)* Infection with YFP-RH at MOI=10; p62

staining is shown in red. *(Bottom)* Infection with RH (anti-GRA2, green) at MOI=2; lysosome-associated membrane protein 2 (LAMP2) is shown in red; DAPI in blue. (D, E) Quantification of inside/outside staining of *T. gondii* (YFP-RH) infected PHT cells at 2hpi, MOI=1 in one PHT cell preparation. Using DIC/DAPI images, cells were classified as mononucleated (CYT) or multinucleated (SYN). (D) To compare the attachment efficiency between cell types, the number of internalized parasites per 2D cell area was calculated for 813 cells (N_{CYT}=729, N_{SYN}=84) and the resulting distributions were compared using the Kolmogrov-Smirnoff test. The comparison used was: CYT_{inside} vs SYN_{inside} (*P*=0.010). (E) Comparison of percent invaded parasites of all parasites-associated cells by cell type ((N_{CYT}=176, N_{SYN}=34).

In addition to the intracellular control of parasite replication, it is possible that SYNs are protected from infection by defects in parasite attachment and/or invasion. To quantify parasite attachment and invasion, we performed a two-step immunofluorescence-based invasion assay to distinguish extracellular from intracellular parasites (as in [303] and others). PHT cells were exposed to *T. gondii* (RH-YFP) for 2 hrs, at which point monolayers were washed to remove unbound parasites, cells were fixed, and attached parasites were identified using an antibody against surface antigen-1 (SAG1) in the absence of cell permeabilization, followed by detection using a secondary antibody conjugated to Alexa Fluor 594. Samples were then permeabilized and incubated again with anti-SAG1 antibody, which was detected with a secondary antibody conjugated to Alexa Fluor-633. Using this approach, extracellular parasites exhibited fluorescence in all channels (YFP, 594, and 633) whereas intracellular parasites exhibited fluorescence in only two (YFP and 633). Using differential contrast imaging (DIC) and automated image analysis, we quantified the extent of attached and internalized parasites in CYTs versus SYNs, which were easily distinguishable using DIC based upon the number, size, and clustering of their nuclei. Using this approach, we found that there were significantly fewer parasites overall (i.e., uninvaded and invaded) that were associated with SYNs compared to CYTs (normalized for cell area; p=0.010;

Figure 12D). However, the percentage of invasion events (of all total parasite associations) was nearly identical between SYNs and CYTs, demonstrating that while there is a significant defect in parasite attachment to and/or association with SYNs, there is no obvious impediment to invasion (**Figure 12E**). While we do not know the stage of attachment at which *T. gondii* tachyzoites are arrested when associating with SYNs compared to CYTs, these data point to a defect in *T. gondii* attachment as a primary mediator of SYN resistance to infection in addition to their ability to potently resist parasite replication when the parasites do successfully invade.

2.2.3 PHT Cells Have a Unique Response to *T. gondii* Infection Characterized by the Induction of Immunity-Related Transcription Factors and Chemokines

Given the dramatic differences in infectivity and growth of *T. gondii* in PHT cells, we infected PHT cells or the choriocarcinoma JEG-3 cell line with *T. gondii* and compared their transcriptional responses to infection using RNAseq. Following infection for 24 h, we identified 401 transcripts of significantly different abundance ($P<0.01$; Fold-change>4) in infected PHT cells, and 106 transcripts of different abundance in infected JEG-3 cells (**Figure 13A and 13B**). To identify which transcripts were uniquely induced in PHT cells compared to JEG-3 cells and another primary cell line, we compared these data to a recently published RNAseq dataset from *T. gondii*-infected primary HFF cells [304]. While we identified 858 host cell transcripts that were of different abundance in *T. gondii*-infected HFFs, there was a significant lack of overlap between infection-altered transcripts in HFF and PHT cells (**Figure 13B**). Cluster analysis of all genes induced in *T. gondii*-infected PHT cells revealed multiple categories of genes specifically induced in these cells and not in either HFFs or JEG-3 cells. While some genes were induced uniquely in PHT cells and were expressed poorly in other cell types, (**Figure 13A**, cluster “a”), others were of

high abundance only after infection in PHT cells, but constitutively expressed in other cell lines/types (**Figure 13A**, cluster "b"). Focusing on genes uniquely induced in PHT cells compared to other cell types ("Cluster 1", **Figure 13C**), multiple immunity-related transcription factors (e.g., IRF4, EGR4), chemokines (CCL22, CCL17, CCL20, CCL1) and the chemokine receptor CCR7 were all significantly induced by *T. gondii* infection. We confirmed this cell-type specific induction of a subset of the "Cluster 1" genes via RT-qPCR (**Figure 13D**). Of note, we found that CCL22, a chemokine known to be expressed constitutively during pregnancy [305, 306] and that has also been found to increase during miscarriage [305], was induced by >400 fold in infected PHT cells based on RNAseq and confirmatory RT-qPCR (**Figure 13C and 13D**). Subsequent experiments with heat-killed *T. gondii* failed to induce CCL22 secretion from PHT cells (**Figure 13E**), indicating that production of this chemokine requires live parasites and suggesting that the CCL22 response requires parasite invasion. Additional experiments treating PHT cultures with either neutralizing antibody to CCL22 or recombinant CCL22 showed no difference in the infection status of SYNs (**Appendix A, Figure 23**), providing support for the fact that CCL22 is not responsible for the reduced parasite attachment to or replication within SYNs compared to CYTs. This finding is consistent with the role of CCL22 as a chemoattractant rather than an effector cytokine like IFNγ.

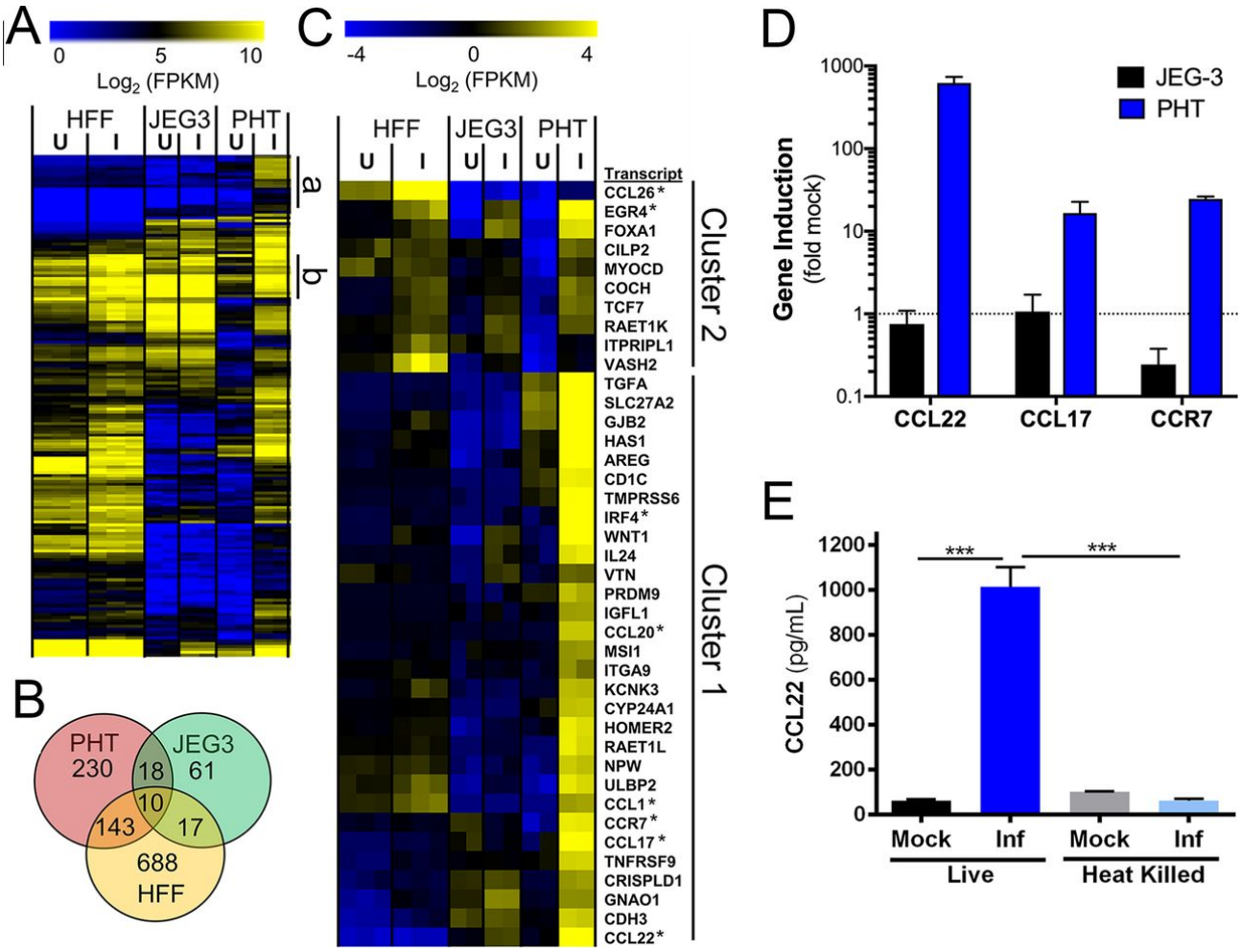


Figure 13 PHT cells infected with *Toxoplasma gondii* have a unique transcriptional response to infection

(A) Heat map of all genes with significantly higher transcript abundance in *T. gondii*-infected (I) PHT cells than in mock-infected (U) cells ($P < 0.01$; fold change, >4). (B) Genes with significantly higher abundance ($P < 0.01$; fold difference, ≥4) in PHT cells, HFFs, and JEG-3 cells. (C) Hierarchically clustered heat map of 40 genes induced in infected PHT cells. Cluster 1 contains genes that are induced in other cell types, while cluster 2 consists primarily of transcripts induced by *T. gondii* infection only in PHT cells (27/30). Transcription factors and chemokines and their receptors are indicated with asterisks. (D) qPCR validation of three genes specifically induced in *T. gondii*-infected PHT cells as identified by RNA-seq. Data shown consist of three technical replicates from an independent PHT cell preparation. (E) Induction of CCL22 secretion in PHT cells requires live parasites. Parasites were incubated at 23°C or 65°C for 1 h prior to being used to infect PHT cells. Data shown consist of two technical replicates from one PHT cell preparation. ***, $P < 0.001$ following one-way analysis of variance and multiple-comparison post hoc tests.

2.2.4 Infection of PHT Cells with *Neospora caninum* Does Not Induce the Production of Immunomodulatory Chemokines

Host transcriptional responses to infection with *T. gondii* have been shown in a variety of cell types to be specific for *T. gondii* and are not associated with infection by one of its apicomplexan relatives, *Neospora caninum* [148, 153, 307]. Unlike *T. gondii*, *N. caninum* is not a human pathogen, but causes significant mortality in cattle and dogs and is associated with congenital disease in these animals [308, 309]. To determine the specificity of the host response to *T. gondii* infection in PHT cells, we infected cells with *T. gondii* (RH-YFP) or *N. caninum* (NC-1-dsRED [310]) and compared the cellular responses to infection using RNAseq. We found that *N. caninum* failed to significantly induce any of the chemokine/chemokine receptor genes that were induced by infection with *T. gondii* of PHT cells (**Figure 14A**; asterisks indicate focus chemokine genes). The remarkable lack of differential transcript abundance in *N. caninum*-infected PHT cells compared to matched *T. gondii* infected PHTs was further illustrated by MA plot (**Figure 14B**). In PHT cells, we identified 206 genes that were significantly induced by *T. gondii* infection ($P<0.05$; fold-induction>2), and only 10 genes that were significantly induced after *N. caninum* infection (**Figure 14C**). Consistent with this, infection with *N. caninum* had no effect on CCL22 levels and co-infections with *N. caninum* and *T. gondii* showed that there was also no synergistic or additive effect (**Figure 14D**). Importantly, despite the significant differences in gene induction, we found that PHT cells were similarly susceptible to *N. caninum* infection, with CYTs being readily invaded and supportive of parasite growth, while SYNs exhibited reduced invasion by and growth restriction of *N. caninum* in a fashion very similar to that observed for *T. gondii* (**Figure 14E**).

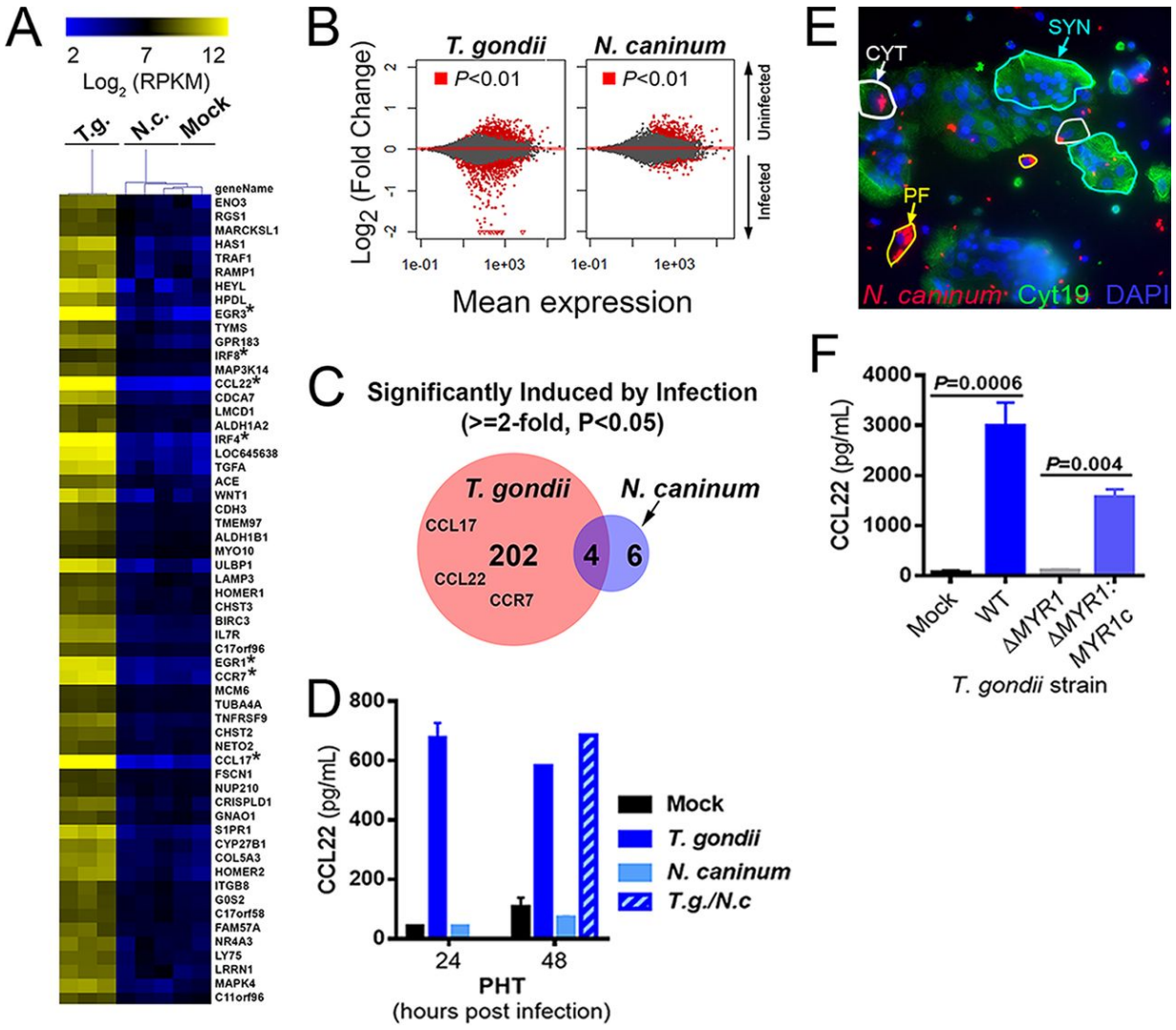


Figure 14 Infection-modulated gene expression is specific to *T. gondii* and requires parasite effectors

(A) Heat map of 59 genes induced by at least 1.8-fold in PHT cells ($P < 0.01$) after infection with *T. gondii* (T.g.) RH strain (multiplicity of infection of 3). Raw count data were converted to normalized RPKM using DESeq2. Data are also shown for *N. caninum* (N.c.)-infected ($n = 3$) and mock-infected ($n = 2$) PHTs, and mean centered data were clustered by sample and gene using the Euclidean distance (implemented in MeViewer [TM4 microarray suite]; see Materials and Methods). Asterisks denote genes that are very highly induced in *T. gondii*-infected PHT cells. (B) MA plots of PHTs comparing gene expression profiles in mock- and parasite-infected cells for all 23,735 queried genes. Genes of higher abundance in uninfected cells are indicated by positive changes, and those of higher abundance in infected cells are indicated by negative changes. (C) In *T. gondii*-infected PHTs, 206 genes were found to be of higher abundance than in mock-infected cells, while only 10 such genes were found in *N. caninum*-infected PHTs, consistent

with the MA plots in panel B above. (D) Results from ELISA showing induction of CCL22 secretion in PHTs infected with *T. gondii* but not *N. caninum*. Host cells were infected with a multiplicity of infection of 2 (for each parasite species), and supernatants were harvested at the indicated time points. n = 2 to 3 wells for all treatments except for 48-h *T. gondii* and *T. gondii*/*N. caninum* (n = 1); single PHT cell preparation. (E) PHTs were infected with NC-1-dsRED *N. caninum* (multiplicity of infection of 3) for 24 h and stained with cytokeratin 19 antibodies and DAPI. Similarly to *T. gondii*, *N. caninum* grew efficiently in PFs (yellow outlines) and CYTs (white outlines) and poorly or not at all in SYNs (blue outlines). (F) CCL22 induction in PHT cells requires MYR1. PHTs were infected with either wild-type (WT) RH-YFP *T. gondii*, RHΔMYR1, or RHΔMYR1 complemented with a hemagglutinin-tagged copy of MYR1 (RHΔMYR1c). n = 3 for each *T. gondii* strain. Data shown are from one PHT cell preparation.

2.2.5 CCL22 Induction in PHT Cells Requires the *Toxoplasma gondii* Dense Granule Protein MYR1

Given that CCL22 induction in PHT cells required live parasites and was not induced by *N. caninum*, we reasoned that this induction likely resulted from a *T. gondii*-specific parasite effector that would be secreted after host cell invasion. *T. gondii* MYR1 is a recently identified dense granule protein that is required for the export/secretion of multiple dense granule effectors (including GRA24, GRA25 and GRA16; [147, 148, 311]) and was discovered based on its role in mediating *T. gondii*-specific activation of the transcription factor c-Myc [153]. When we infected PHT cells with *T. gondii* lacking MYR1 (RH:Δ*MYR1*) and complemented control parasites (RH:Δ*MYR1*:*MYR1c*), we found that CCL22 production by PHT cells was entirely dependent upon MYR1 (**Figure 14F**), which provides strong evidence that *T. gondii* CCL22 induction in PHT cells is driven by (a) MYR1-dependent secreted effector(s).

2.2.6 Second Trimester Human Placental Villi Resist *T. gondii* infection and Induce CCL22 in Response to Infection

Because PHT cells are isolated from term placentas, we next determined whether SYNs from earlier in human pregnancy also resist *T. gondii* infection and similarly induce immunity-related genes. To do this, we utilized second trimester chorionic villous explants, which retain the morphology of human placental villi, including a layer of cytokeratin-19 positive SYNs covering the villi surfaces (**Figure 15A**). Consistent with our findings in PHT cells, and the work of others utilizing first trimester explants [75], we found that second trimester SYNs were resistant to *T. gondii* infection, even when infected with very high numbers of parasites (10^7) (**Figure 15B, left panel**). This resistance appears to be primarily at the level of parasite attachment as we detected very few internalized parasites in placental villi and most parasites detected appeared to be extracellular (**Figure 15B, left panel, white arrows**). Importantly, unlike placental villi, we found that fetal membrane (amnion and chorion) and maternal decidua supported *T. gondii* replication (**Figure 15B, middle and right panels**), highlighting the specific resistance of placental villi. In addition, consistent with the work of others utilizing first trimester explants [75], we found that CYTs subjacent to the SYN were permissive to *T. gondii* only when the SYN layer was breached (**Figure 15C**). These data show that the SYN layer also forms a barrier to *T. gondii* vertical transmission in mid-gestation.

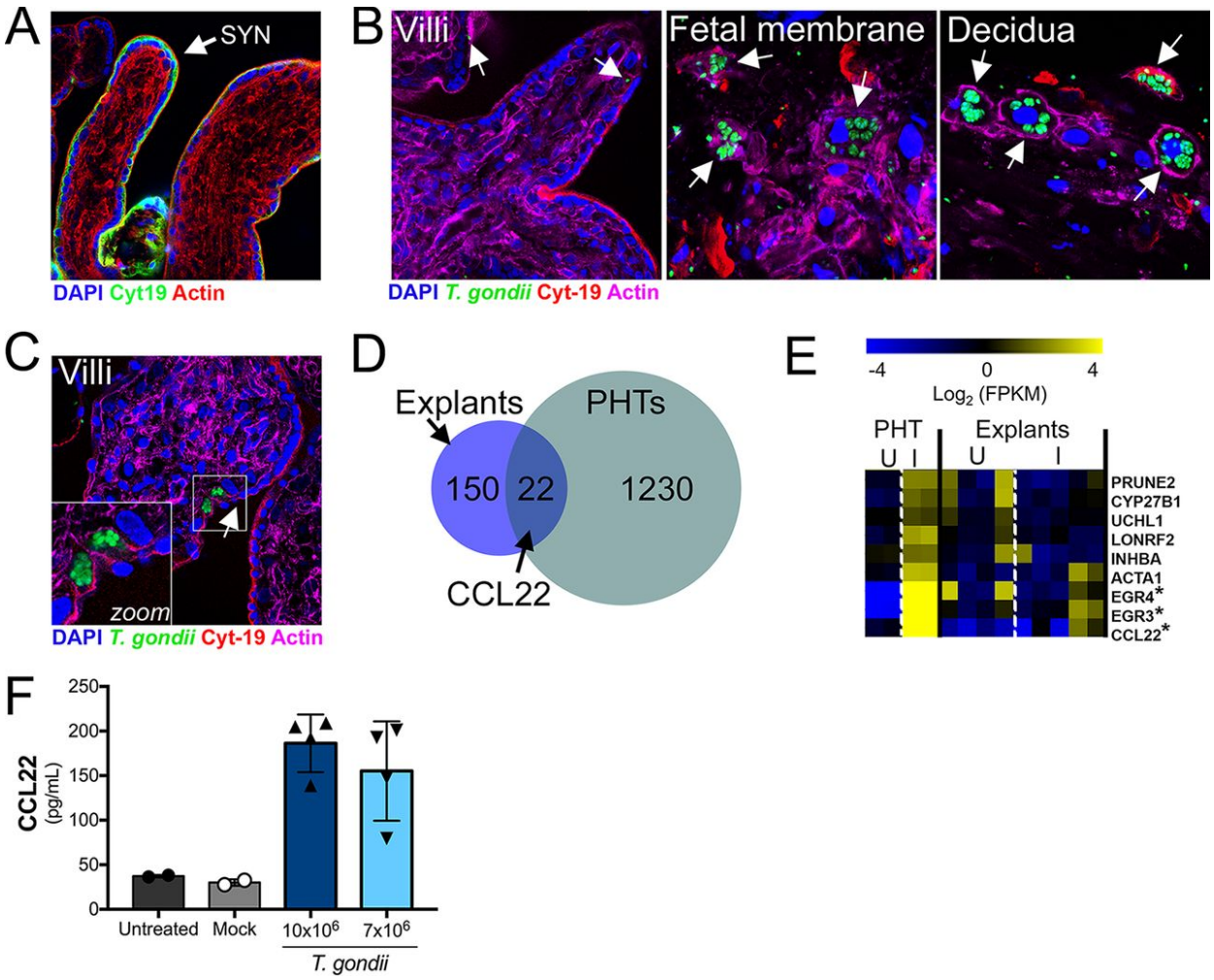


Figure 15 Chorionic villous explants from the second trimester are resistant to *T. gondii* infection and induce similar cytokine profiles in response to *T. gondii* as do full-term PHT cells

(A) Immunofluorescence microscopy of uninfected second-trimester placental villous explant with SYN localization indicated. Cytokeratin 19 in green; actin in red; DAPI in blue. (B) Immunofluorescence microscopy of midgestation villi (left panel), fetal membrane (middle panel), or decidua (right panel) inoculated with 10^7 *T. gondii* cells (RH strain) for 24 h. *T. gondii* in green; cytokeratin 19 in red; actin in magenta; DAPI in blue. White arrows denote single parasites in isolated villi and PVs in fetal membrane and decidua. (C) Immunofluorescence microscopy of midgestation villi inoculated with 10^7 *T. gondii* cells (RH strain) for 24 h. *T. gondii* in green; cytokeratin 19 in red; actin in magenta; DAPI in blue. Zoomed image from white box denotes PVs in CYTs located beneath a tear in the SYN (white arrow). (D and E) RNA-seq analysis of *T. gondii*-infected villous explants. Venn diagram (D) and heat map (E) showing a subset of genes that are similarly induced in one of the *T. gondii*-infected villous explants (explant

5) and PHTs. Genes were selected if they were at least 2-fold induced compared to mock infection and the value was significant using a DESeq2-determined adjusted *P* value of <0.05. Explant data in heat map are from 3 genetically distinct placenta preparations, while PHT data shown are the same as in Fig. 3. I, infected; U, uninfected (mock); FPKM, fragments per kilobase per million. Asterisks denote genes similarly induced in both explant 5 and PHT cells. (F) ELISA showing *T. gondii* (RH strain)-mediated enhancement of CCL22 secretion from villous explants at 24 h postinfection.

Next, we profiled the transcriptional changes induced by *T. gondii* infection of second trimester chorionic villi using RNAseq to determine whether they responded to parasite infection similarly to PHT cells from late gestation. We found that 172 transcripts were differentially expressed in response to *T. gondii* infection of villous explants, with 22 of these transcripts also being differentially expressed in response to infection of PHT cells, which included EGR3, EGR4 and CCL22 (**Figure 15D, 5E**). We confirmed that CCL22 was induced at the protein level by ELISA in supernatants from *T. gondii*-infected second trimester villi (**Figure 15F**). These data suggest that CCL22 is specifically induced by the human placenta in response to *T. gondii* infection at both early and late stages of pregnancy.

2.3 Discussion

T. gondii infections present a worldwide threat to pregnant women, and there is an urgent need to develop novel treatment regimens to block congenital transmission of *T. gondii* and other pathogens that pose risks to the developing fetus. Our data presented here point to a direct role for SYN-intrinsic pathways in the protection of the fetus from *T. gondii* infection, making the SYN a highly unique cell type given that a diverse array of cells studied to date are susceptible to *T. gondii*

infection and replication. A particularly remarkable feature of the PHT cell culture model is that there are both permissive and resistant cells in very close proximity, indicating that cell-autonomous features determine the fate of *T. gondii* upon interaction with CYTs and SYNs. Our studies in both human primary full-term and second trimester SYNs suggest that these cells evade *T. gondii* infection at two critical stages of the parasite lytic cycle—at the point of parasite association with the host cell and during intracellular growth. Moreover, by comparing cell-type specific transcriptional profiles from *T. gondii* infected primary trophoblasts and placental tissue with those of other cell types, we identified unique sets of genes induced by *T. gondii* infection in the human placenta, including the induction of CCL22 that requires the presence of parasite-encoded MYR1. Collectively, our data provide significant advances in our understanding of how the human placenta controls, responds to, and is manipulated by, *T. gondii* infection.

We found that the first point of SYN-mediated restriction of *T. gondii* infection occurred at the level of parasite association and/or attachment, which we observed both in SYNs isolated from full-term placentas and from mid-gestation chorionic villi. These findings are consistent with the previous work of others suggesting that attachment might be reduced in first trimester SYNs [75], although this was not directly tested. Our attachment and invasion data from PHT cells provide direct evidence that SYNs naturally restrict parasite attachment but are susceptible to invasion once parasite attachment occurs, which appears to be a rare event. It remains unclear which point of the attachment process is altered when *T. gondii* associates with SYNs, but a likely stage is during the early phase of gliding motility prior to the second phase of attachment, (which is mediated by the secretion of microneme and rhoptry organelles [145]). One possibility is that when parasites encounter SYNs they glide less efficiently than on CYTs or placental fibroblasts, which ultimately results in significantly reduced “full” attachment (mediated by microneme and

rhoptry secretions). Differences in membrane biochemistry in SYNs versus CYTs and fibroblasts could underlie these important differences in early parasite association. Differences in glycosaminoglycan (GAG) content between CYTs and SYNs could also explain these differences given the known importance of GAGs in parasite gliding motility and attachment [312]. However, once secondary attachment occurs invasion seems to proceed normally, indicating that whatever membrane/surface differences there are between CYTs and SYNs, they do not impact the phase of invasion that requires organelle discharge.

In mid-gestation chorionic villi, the poor association/attachment phenotype was even more profound than that observed in PHT cells, with little to no parasite association with the villi observed. These findings suggest that in addition to biochemical surface differences between SYNs and CYTs, the morphology of the SYN layer itself may directly impact parasite association and attachment. This could be influenced by morphologic differences in this model, including the positive membrane curvature associated with the significant branching of the placental villous trees, which might impact lipid, protein and/or carbohydrate composition. In addition, the apical surfaces of SYNs associated with placental explants may be more differentiated than PHT cells, which might impact parasite attachment through the presence of a highly dense brush border in the explant model. Consistent with this, we previously observed very little *T. gondii* association in a bead-based three-dimensional cell line model of human SYNs, which also induces significant membrane curvature and allows for the formation of a well-differentiated brush border [86].

For parasites that attach to the SYN layer, our data suggest a second level of resistance to infection that occurs post-invasion. Importantly, in contrast to other cells types, our data show that this resistance is not mediated by IFNγ, which is not basally expressed in PHT cells or induced by *T. gondii* infection. Furthermore, we did not find any evidence for autophagy- or lysosomal-

mediated degradation pathways in the intracellular restriction of parasite replication. To date, all known "cell-autonomous" mechanisms of parasite killing in human cells rely on previous stimulation with IFNγ. For example, IFNγ can induce a variety of downstream effector mechanisms depending on the cell type, including tryptophan starvation in HFFs [220] and decoration of the vacuole with guanylate binding proteins [313, 314], ubiquitin or other markers for autophagy including LC3B [314, 315]. Ultimately, these pathways would lead to lysosomal fusion with the parasite-containing vacuole and parasite destruction, which we did not detect in infected SYNs at any timepoint tested. However, our data conclusively show that parasites that invade this specific trophoblast cell type are able to form what functional vacuoles, but are ultimately destroyed in a fashion reminiscent of some Toxoplasmacidal drugs [302, 316]. Hallmarks of parasite killing are vacuolation of the parasites, breakdown of the vacuolar tubulovesicular network, and lack of integrity of the parasitophorous vacuolar membrane and leakage of host cytoplasmic contents into the lumen of the compromised vacuole. These data place PHT cells, and specifically the subpopulation of fused SYNs, into a rare class of cells that not only restrict the growth of *T. gondii* after invasion in the absence of any external stimuli, but actively destroy invaded parasites. While we did not compare them head to head with SYNs, neutrophils restrict *T. gondii* growth after invasion but are possibly less Toxoplasmacidal compared to SYNs given that neutrophils have been implicated in the spread of *T. gondii* throughout the intestine in a murine model [317]. Head-to-head comparisons between SYNs and innate immune cell types like neutrophils will help to illuminate what potential killing mechanisms might be shared, or not, between these cell types. Moreover, determining the differences between SYNs and CYTs would require the development of new methods to separate them for downstream analyses including RNAseq and quantitative proteomics.

In addition to resisting *T. gondii* infection, our data show that PHT cells robustly induce the chemokine CCL22 in response to infection by a Myr-1 dependent effector secretion mechanism. We do not know which cell types within the PHT preparation produce CCL22 after exposure to *T. gondii*, but given the dependence upon successful invasion for this response, a good candidate cell type is the CYT rather than the SYN, although cell-specific analyses are required to address this directly. Importantly, the major inflammatory responses induced in PHT cells by *T. gondii* infection are not induced (or induced much more poorly) after infection with *N. caninum*, a near relative of *T. gondii* that does not successfully infect humans or rodents, suggesting that it may be a host and/or parasite adaptation that may impact disease outcome.

The precise role of CCL22 in human pregnancy is unknown, but maternal cells express CCL22 at low levels throughout pregnancy, with increased levels associated with miscarriage [305]. Moreover, the induction of chemokines, including CCL22 and CCL17, are associated with preterm birth in humans [318] and in small animal models [319]. The precise role played by CCL22 during *T. gondii* vertical transmission remains to be determined. However, exposure of PHT cells to recombinant CCL22 or CCL22 neutralizing antibody had no impact on parasite replication (**Appendix A, Figure 23**), supporting the idea that it has no direct anti-parasitic activity. A likely scenario is that the induction of CCL22, and other immunity-related genes, is aimed at alerting the maternal immune system to placental infection, where it could play any number of roles in mediating the dialogue between maternal and fetal tissues, such as enhancing immune cell-mediated protection at the maternal-fetal interface, or in terminating the pregnancy should levels reach a specific threshold. Given that CCL22 levels are elevated in maternal serum during healthy, infection-free pregnancies [305, 306], CCL22 and its recruitment of regulatory T cells may also play a role in immune tolerance throughout gestation, which is modulated in

response to infection. This increased T-reg recruitment may even promote *T. gondii* infection, as was seen in *Listeria* and *Salmonella* infections in the pregnant mouse model [320].

Our findings provide important insights into the molecular and cellular pathways utilized by human SYNs at both late and mid stages of gestation to restrict *T. gondii* access to the fetal compartment. In addition, by characterizing the immunological pathways induced by *T. gondii* infection of SYNs, our findings have uncovered potentially novel biomarkers of infection severity that might have important roles in shaping the maternal systemic immune response. These findings provide an example of the signaling crosstalk that exists between the maternal and fetal compartments and the mechanisms by which this signaling is impacted by parasite-associated effectors. Taken together, these findings provide important insights into *T. gondii*-induced congenital disease that could lead to the design of novel therapeutics aimed at reducing congenital toxoplasmosis.

2.4 Materials and Methods

2.4.1 Cell Culture

All cell and tissue cultures were incubated at 37°C and 5% CO_2 and all media were supplemented with 10% FBS and 50 μg/mL penicillin/streptomycin. JAR and HTR8 cells were grown in RPMI-1640 media (HyClone); BeWo cells in F-12K (Corning); and JEG-3 cells in Eagle's Minimum Essential Medium (EMEM; Lonza). To induce fusion of BeWo cells, cells were treated with 10 μM forskolin for 24 hours, then washed with PBS before infection. Primary human trophoblast (PHT) cells were isolated from healthy, term-pregnancies, and were cultured as

described previously [300, 301]. PHT cells were cultured for ~48h prior to infection to allow for SYN formation. Primary placental fibroblasts were isolated and cultured as described previously [321].

2.4.2 Mid-gestation Placental Explants

Human placental tissue from less than 24 weeks gestation was obtained from the University of Pittsburgh Health Sciences Tissue Bank through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. Chorionic villi, fetal membrane, and decidua were dissected and cultured in DMEM/F12 (1:1) supplemented with 10% FBS, penicillin/streptomycin, and amphotericin B. For *T. gondii* infections, isolated tissue was infected immediately following isolation with $2.5x10^4$-$1x10^7$ parasites for ~24hrs. For imaging, tissue was fixed in 4% paraformaldehyde and imaging performed as detailed below.

2.4.3 Parasites

Type I (RH) and type III (CEP) *T. gondii* and *N. caninum* (NC-1) tachyzoites were used for this study. All parasites were maintained by continual passage in human foreskin fibroblast (HFF) cultures incubated at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 50 μg/mL penicillin/streptomycin, and 2mM glutamine. The YFP-RH was a gift from David Roos, and the RH-*MYR1*-KO and RH-*MYR1*-KO/complemented parasites were gifted by John Boothroyd. For infections, infected monolayers were scraped and syringe-lysed to release the tachyzoites. These parasites were then pelleted at 800 x *g* for 10 minutes, resuspended in fresh media, filtered through

a 5 μm filter, and counted to determine the appropriate dilution for infection. Mock inoculum was produced by filtering out the tachyzoites with a 0.2 μm filter.

Parasite growth curves were generated by luciferase assay (Promega) using luciferase-expressing CEP parasites. Briefly, at each time-point, samples were lysed using the passive lysis buffer (Promega) and stored at -20°C until at least 8h past the last time-point collection. Samples were then thawed and incubated with substrate, and fluorescence was measured.

2.4.4 RT-qPCR and RNAseq

RNA was isolated from cultures using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and the associated DNase digestion set (Sigma). Both a NanoDrop and an Agilent bioanalyzer were used to determine sample quality. Sequencing libraries were prepared from 0.2-0.9 μg of total RNA by the TruSeq Stranded mRNA Library Preparation Kit (Illumina). The Illumina NextSeq. 500 was used for sequencing. CLC Genomics Workbench 9 (Qiagen) was used to map the RNA-seq FASTQ reads to the human reference genome (hg19). Differential expression analysis was performed using the Deseq2 package in R [322] using a significance cutoff of *P*adj < 0.01, unless specified otherwise. Analysis of mock and *T. gondii*-infected HFF cells was based on datasets previously published and deposited into the Sequence Read Archive (SRA): SRR2644999, SRR2645000, SRR2645001, SRR2645002, SRR2645003, and SRR2645004. Hierarchical clustering of log2 transformed RPKM data was performed using MeViewer TM4 software. Data were either clustered as is or linearly mean centered using Euclidian distance. Color scales were adjusted for presentation purposes. RNAseq data have been deposited in the NIH short read archive (accession numbers pending).

For RT-qPCR analyses, RNA was isolated as described above and cDNA generated using the iScript cDNA synthesis kit (Bio-Rad), followed by qPCR using a StepOnePlus Real-Time PCR System (ThermoFisher). The ΔC_T method was used to determine gene expression and normalized to the human actin C_T of each sample. Primer sequences were as follows: Actin—ACTGGGACGACATGGAGAAAAA (Forward, 5'-3'); GCCACACGCAGCTC (Reverse, 5'-3'). CCL22—GTGGTGTTGCTAACCTTC (Forward, 5'-3'); GGCTCAGCTTATTGAGAATC (Reverse, 5'-3'). Pre-designed primers were ordered for CCL17 (Qiagen) and CCR7 (Sigma).

2.4.5 Microscopy

Cell monolayers and placenta explants were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 1x PBS. Primary antibodies were incubated for 1h at room temperature, followed by washing, then secondary antibodies conjugated to Alexa Fluor (Invitrogen) fluorophores for 30min at room temperature. Following washing, cells/explants were mounted with DAPI-Vectashield (Vector Laboratories) and imaging performed on an Olympus FV1000 laser scanning confocal microscope, a Zeiss LSM 710, or an Olympus IX83 inverted microscope. In some cases, imaged were adjusted for brightness and contrast using Photoshop or Fiji/Image J. Image J was used for image analyses. Transmission electron microscopy was performed as described previously [301].

Reagents and antibodies used for immunostaining studies include Alexa Fluor 594 or 633 conjugated phalloidin (Invitrogen), cytokeratin-19 (Abcam), LAMP2 (Santa Cruz), SAG-1 (mouse monoclonal D61S; ThermoFisher).

2.4.6 CCL22 ELISA

CCL22 ELISAs were performed with the human CCL22/MDC DuoSet ELISA (R&D Systems) as per the manufacturer's instructions.

2.4.7 Luminex

Conditioned media from cells was analyzed by multiplex luminex by the University of Pittsburgh Cancer Institute (UPCI) Cancer Biomarkers Facility: Luminex Core Laboratory that is supported in part by award P30CA047904.

2.4.8 Statistics

All statistics were calculated using GraphPad Prism. Experiments were performed with independent preparations of PHT cells and second trimester villous explants. The data are presented as the mean ±SD. The individual statistical analyses and associated *P* values are described in the individual figure legends.

3.0 A *Toxoplasma gondii* dense granule protein GRA28 induces CCL22 in primary trophoblasts and cell lines of monocyte lineage

Congenital toxoplasmosis is a serious concern during pregnancy, but little is known about interactions between *Toxoplasma gondii* and the placenta. Previously, we found placental trophoblasts induce the chemokine CCL22 specifically in response to *T. gondii* infection. In this current study, we identify GRA28 as the parasite effector necessary and sufficient for CCL22 induction and lay the foundation to begin to understand the mechanism behind this highly specific phenotype.

3.1 Introduction

Toxoplasma gondii infection during pregnancy can result in devasting consequences for the developing fetus: neurological disorders, vision loss, and even death. Early detection (and treatment) of *T. gondii* infection is associated with decreased disease severity [254, 255], however, current diagnostics rely upon detecting the anti-*Toxoplasma* antibody response—a response that may take as long as several weeks to reach detectable levels.

Previously, we not only found that primary placental trophoblasts restrict *T. gondii* infection, but that *T. gondii* infection also elicits a strong induction of immunomodulatory genes. Geneset Enrichment Analysis (GSEA) of differentially regulated genes during infection demonstrates a clear distinction in the responses of PHT cells contrasted with that of the JEG-3 trophoblast cell line: the PHT cells respond to *T. gondii* infection with up-regulation of genes

associated with immune response, immune system process, and defense response; while JEG-3 cells induce genes related to wound-healing and tissue/organ morphogenesis (**Appendix A, Figure 24**). To better understand the PHT cell's response to *T. gondii* infection, we examined the induction of the chemoattractant CCL22. Furthermore, this CCL22 response is specific to *T. gondii* and not induced in response to the related parasite *Neospora caninum*. In this current work, we identify *T. gondii* dense granule protein GRA28 as both necessary and sufficient for CCL22 induction. Moreover, we attempt to understand the mechanism by which GRA28 mediates CCL22 induction and establish a foundation for future *in vivo* studies to examine the impact of CCL22 secretion on transplacental transmission of *T. gondii* and pregnancy maintenance.

3.2 Results

3.2.1 *Toxoplasma gondii* induces CCL22 in monocytes as well as primary trophoblasts

T. gondii infection of PHT and monocyte/macrophage cells causes high induction of CCL22, a T_H2 and T_{Reg} chemoattractant (**Figures 13 & 16**). As shown in **Figure 16A** & **16B**, this induction in THP1 monocytes is shared among the three types of *Toxoplasma* common to Europe and North America (Types I, II, III). The degree of CCL22 induction increases both during the course of infection (**Figure 16A & 16B**) and in proportion with the multiplicity of infection (**Figure 16C**). As we previously saw with PHT cells, infection of THP1 monocytes with *Neospora* does not upregulate CCL22 production (**Figure 16A**).

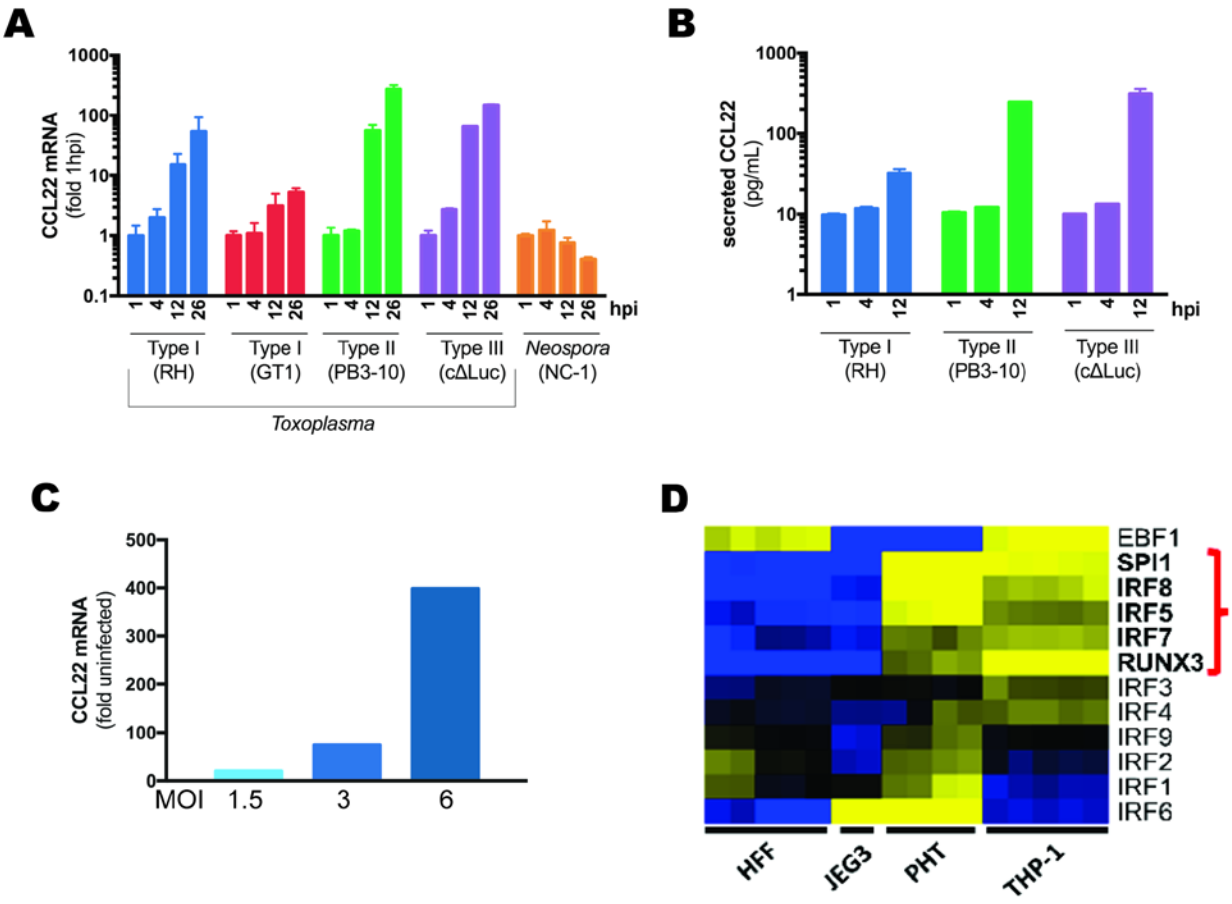


Figure 16 *T. gondii* Infection Induces CCL22 in THP1 Monocytes

(A, B) Timecourse of CCL22 induction and secretion during *T. gondii* infection of THP1 monocytes; MOI=1. (C) CCL22 induction by THP1 cells is dependent upon parasite burden, 26hpi. (D) Identification of host transcription factors uniquely expressed in PHT and THP1 cells but not HFF or JEG3 cells; analysis performed by Carolyn Coyne and Jon Boyle.

As was noted in the previous chapter, CCL22 is induced in a cell-type specific manner and is not observed during infection of HFF or the JEG3 trophoblast cell line (**Figure 13**). We hypothesized this cell-type specificity could be related to expression of a unique subset of genes only found in primary trophoblasts and monocytes. Preliminary analysis of gene expression datasets publicly available on NCBI identified a set of host gene candidates that may help mediate CCL22 expression (**Figure 16D**), however transfection of the individual gene candidates into

JEG3 and HeLa CCL2 cells followed by infection (or later co-transfection with the parasite effector, GRA28) did not cause CCL22 induction (data not shown). However, it is possible that CCL22 induction requires the presence of multiple cell type-specific transcription factors or a host protein that was not identified in the initial screen of host gene candidates. To identify host cell binding partners of GRA28, I developed a construct to produce and secrete a mass quantity of recombinant GRA28 from *E. coli* (**Appendix A, Figure 25**). The rGRA28 has a c-terminal SUMO tag to permits purification via a nickel column. In future studies, fractions of cytosolic and nuclear proteins from RAW or THP1 cells will be incubated with pre-bound rGRA28 in order to pull-down and identify protein interactors by mass spectrometry. Alternatively, studies utilizing proximity-dependent biotin identification (BioID) could be used to identify these binding partners.

3.2.2 CCL22 induction during *Toxoplasma* infection is dependent upon dense granule protein GRA28

As was shown in the previous chapter, CCL22 induction during *T. gondii* infection is controlled by MYR1 expression (**Figure 14F**). MYR1 is a component of the *Toxoplasma* translocon for secretion of effector proteins beyond the parasitophorous vacuole into the host cytoplasm during intracellular infection [153, 154], and therefore unlikely to directly cause the induction of CCL22. In order to identify the true parasite effector, a list of candidate genes was generated by Elizabeth Rudzki and Jon Boyle based upon comparative genetics of *T. gondii, Hammondia hammondi,* and *Neospora caninum* (**Figure 17A**). Both *T. gondii* and *H. hammondi* are capable of inducing CCL22 in THP1 cells (S. Wong, E. Rudzki, & J.P. Boyle, personal communications), while *N. caninum* cannot. These three parasites are closely related and are highly syntenic: 99% of the *T. gondii* genome shares homology with *H. hammondi* [323], while

85% is shared in common with *N. caninum* [307]. Knock-out (KO) parasites were generated by Elizabeth Rudzki for each candidate parasite gene and tested for CCL22 induction in the THP-1 monocyte cell line. By this method, GRA28 was identified as the necessary parasite effector (Rudzki & Boyle, personal communications). I validated the necessity of GRA28 for CCL22 induction in primary trophoblasts by performing infections of second trimester placental villi with two independently generated GRA28-KO parasites (**Figure 17B, 17C**).

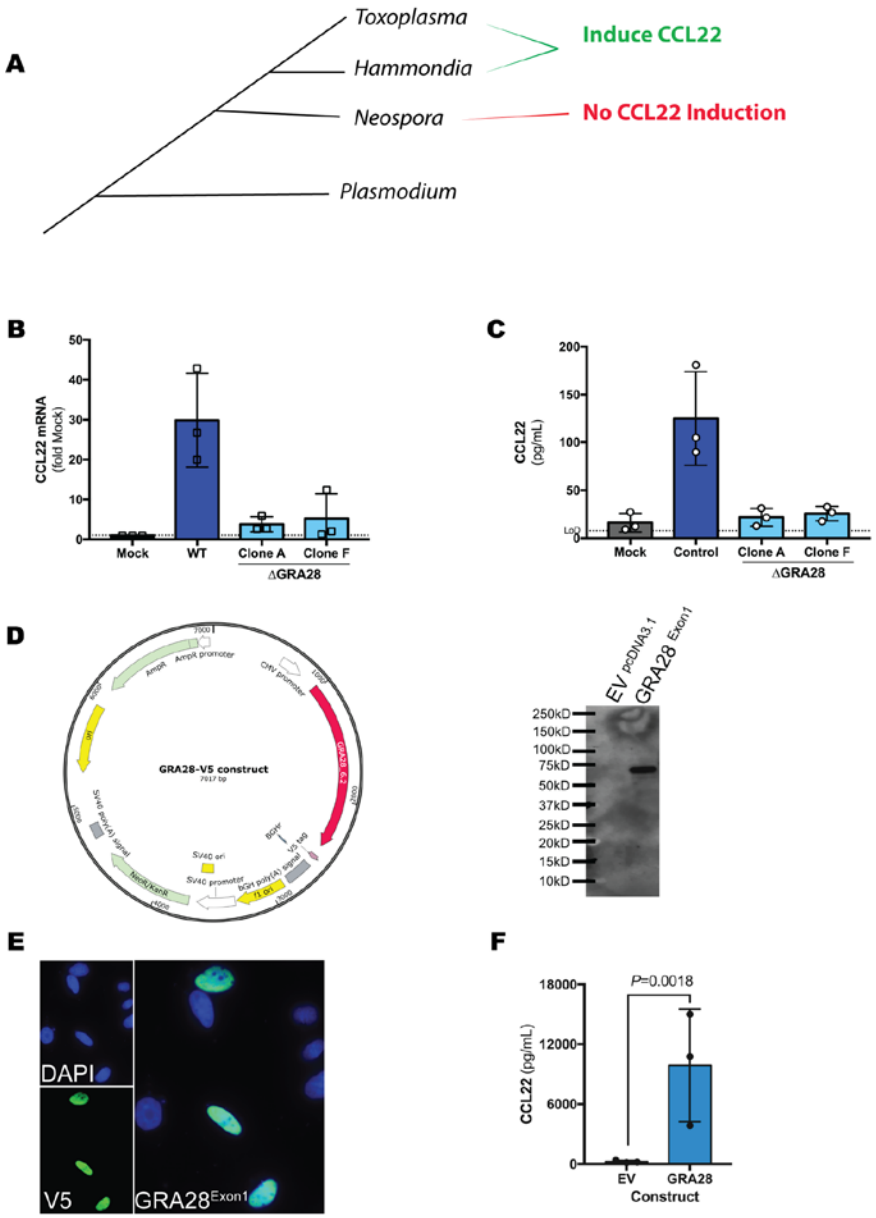


Figure 17 GRA28 Is Necessary & Sufficient for CCL22 Induction

(A) *Toxoplasma*, *Hammondia*, and *Neospora* are closely related parasites, but only *Toxoplasma* and *Hammondia* are capable of inducing CCL22 in PHT cells and THP1 monocytes. (B, C) Infection of second trimester chorionic villi with two independently generated GRA28-KO parasites fail to induce CCL22. (D, E) Ectopic expression of $GRA28_{Exon1}$ in HeLa cells. (F) Ectopic expression of GRA28 construct is sufficent for CCL22 induction by RAW macrophages.

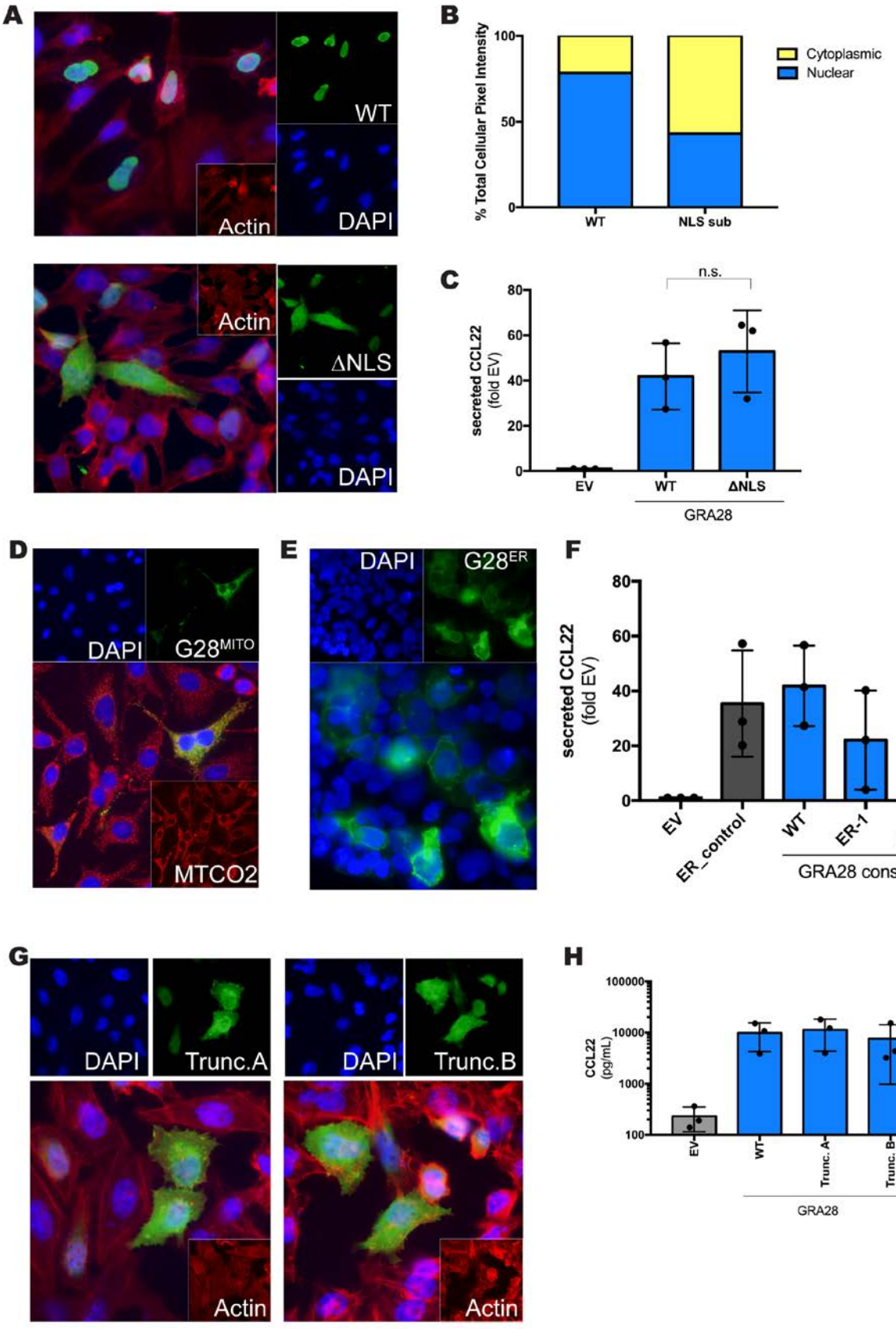
3.2.3 GRA28 is sufficient for CCL22 induction

After determining the necessity of GRA28 for CCL22 induction in human villous explants, I next examined whether ectopic expression of GRA28 alone is sufficient to induce CCL22. Based upon the GT1 (type I) genome on ToxoDB, I designed primers to create a plasmid for ectopic expression of GRA28 in mammalian cells. As various attempts to PCR-amplify the GRA28 mRNA transcript failed, I used genomic DNA as my template. Interestingly, while the computationally-derived annotation on ToxoDB describes GRA28 as a 3-exon gene (2-exons on the ME49, type II, genome), compiled transcriptomic data suggests the possibility of alternative splicing and the presence of an early stop codon 11 nucleotides after the annotated end of the first exon. This alternative transcript annotation, consisting of the first exon and 11 additional nucleotides at the 3' end, was used to synthesize the $GRA28_{Exon1}$ construct with a V5/His tag at the c-terminus. Transfection of this construct in the RAW macrophage cell line was sufficient for CCL22 induction (**Figure 17F**). In accordance with live infection, this phenotype is specific to macrophages, as transfection into HeLa cells and the JEG-3 trophoblast cell line did not induce CCL22 (**Appendix A, Figure 26**).

3.2.4 Nuclear localization may be important for GRA28 to mediate the induction of CCL22

Ectopically expressed $GRA28_{Exon1}$ localizes to the nucleus (**Figure 18A**), as has been previously shown with endogenously tagged GRA28 [151]. To investigate whether nuclear localization is required for CCL22 induction, I alanine-substituted the predicted nuclear localization signal (NLS) of GRA28 (**Figures 18A, 19B**). However, this did not completely

abrogate its nuclear localization (**Figure 18B**), and less stringent parameters for the NLS prediction algorithm revealed the possibility of several bipartite NLS. Thus, as an alternative method to prevent nuclear localization, I relocalized the GRA28 construct to other organelles in the host cell: the mitochondria and endoplasmic reticulum. $GRA28_{Exon1}$ tagged with a mitochondrial localization signal did not localize to the nucleus (**Figure 18D**). However, this relocalization appeared to be toxic, as the plasmid was poorly expressed and by 48h the few cells expressing the construct showed signs of apoptosis. Meanwhile, the $GRA28_{Exon1}$ construct tagged with an KDEL was successful in preventing nuclear localization (**Figure 18E**). However, the effect on CCL22 induction could not be assessed since both ER-$GRA28_{Exon1}$ constructs and the control ER-mCherry plasmid caused CCL22 induction upon transfection into RAW cells (**Figure 18F**). Swapping GFP for mCherry did not resolve this non-specific induction. To identify necessary regions of GRA28 for CCL22 induction, two truncation mutants were made (**Figures 18G, 19B**). However, these mutants were still capable of inducing CCL22 and also localized to the nucleus (**Figure 18G, 18H**). Therefore, it still remains unclear what areas of GRA28 are required to mediate CCL22 induction.


A
WT
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secreted CCL22
(fold EV)
80
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EV
WT
ΔNLS
GRA28
D
DAPI
G28MITO
MTCO2
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ER-1
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GRA28 constructs
G
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DAPI
Trunc.B
Actin
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CCL22
(pg/mL)
100000
10000
1000
100
EV
WT
Trunc. A
Trunc. B
GRA28

Figure 18 Mutations of GRA28 Construct & Impact on Localization & CCL22 Induction in RAW Macrophages

(A, B) Alanine-substitution of the predicted NLS increases the percentage of cytoplasmic GRA28-V5. (C) Mutation of the predicted NLS still permits CCL22 induction. (D, E, F) Relocalization mutants prevent nuclear accumulation of GRA28, but cannot be used to assess GRA28-mediated CCL22 induction. (G, H) Two truncation mutants, both still maintaining the N-terminus, are capable of inducing CCL22.

3.2.5 Computation analysis predicts the presence of SLiMs that may mediate the functions of GRA28

A recent study on *Toxoplasma*'s MYR translocon complex found that exportation of dense granule proteins is dependent upon their intrinsically disordered state, and the addition of a highly structured region prevents its translocation across the PV membrane [154]. As such, this likely explains the observation that most dense granule proteins are highly disordered and lack any predictable tertiary structure. Thus, instead of relying upon tertiary structure, it has been proposed that functions of dense granule proteins are driven by the presences of short linear motifs (SLiMs), 3 to 11 contiguous amino acids in length [156, 158]. SLiMs can have roles in signal transduction, regulatory function, and protein-protein interactions [158–160].

Computational analysis of GRA28 (type II, ME49 genome) for structure prediction found it to be highly intrinsically disordered with several putative mono- and bipartite nuclear localization signals (**Figure 19A, 19C**). Putative SLiMs were identified by analyzing the GRA28 construct with the Eukaryote Linear Motif (ELM) online motif database. ELM analysis found GRA28 to be a highly disordered protein possessing many potentially interesting SLiMs that could be involved with GRA28 function, including: binding sites for PP2A, USP7, and TRAF2 (**Figure 19C**; for full list of ELM hits see **Appendix A, Figure 27**). While the validity of these SLiMs

needs to be experimentally confirmed, especially as the small size of SLiM sequences consequently can result in a high number of false discoveries from computational prediction algorithms. Furthermore, it is also possible that some SLiM predictions may be true but be irrelevant for CCL22 induction; it is likely that GRA28 performs other functions in addition to stimulating CCL22 production.

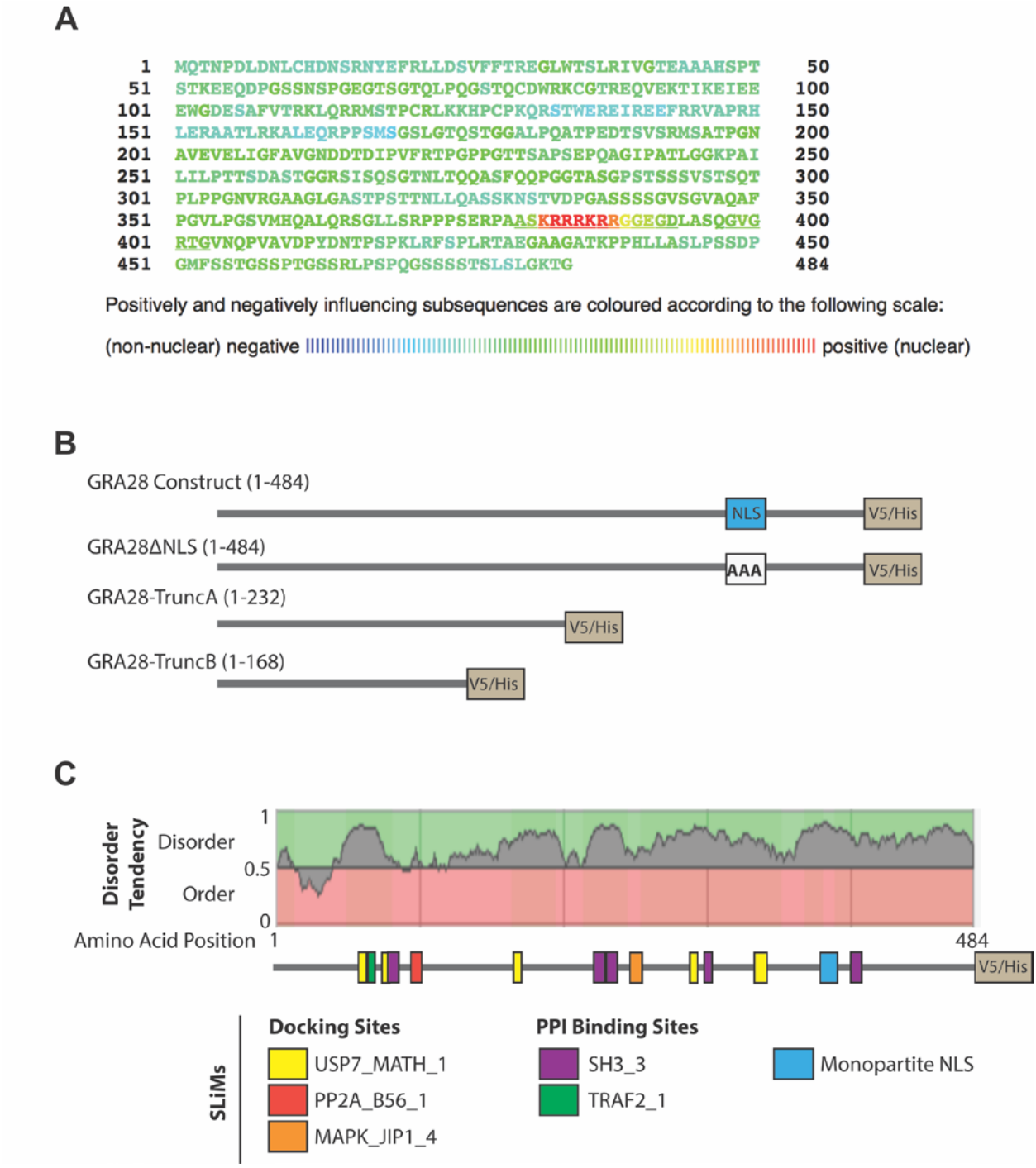


Figure 19 Computational Analysis of GRA28 Construct

(A) Prediction of NLS by NucPred [324]. (B) GRA28 mutant constructs. (C) Select hits from ELM analysis of GRA28 construct. Full analysis is available in Appendix A, Figure 25.

3.2.6 Establishing an *in vivo* model to examine the effect of CCL22 induction on *Toxoplasma* transmission and maintenance of pregnancy

In addition to defining GRA28's mechanism of CCL22 induction, it is important to understand the role of CCL22 in congenital toxoplasmosis and pregnancy maintenance. Towards this goal, we have begun to establish a mouse model of congenital toxoplasmosis and parasite-placental interactions. *Ex vivo* infection of murine placentas causes high secretion of CCL22 (E. Rudzki & J.P. Boyle, personal communications), and microscopic examination of infected tissue finds high levels of infection (**Figure 20**). *In vivo* infections of pregnant mice at embryonic day 8 (E8) of pregnancy (performed by E. Rudzki & J.P. Boyle) also results in high parasite burden in the placenta by day E12 (**Figure 21**). Interestingly, infection is primarily localized to the decidua and junctional zone, with few parasites observed to invade the chorionic labyrinth. Matching histology with hematoxylin and eosin is located in **Appendix A, Figure 28**. In conjunction with the GRA28-KO parasite, future studies will utilize this *in vivo* model to better interrogate the roles of GRA28 and CCL22 on congenital transmission and pregnancy outcome.

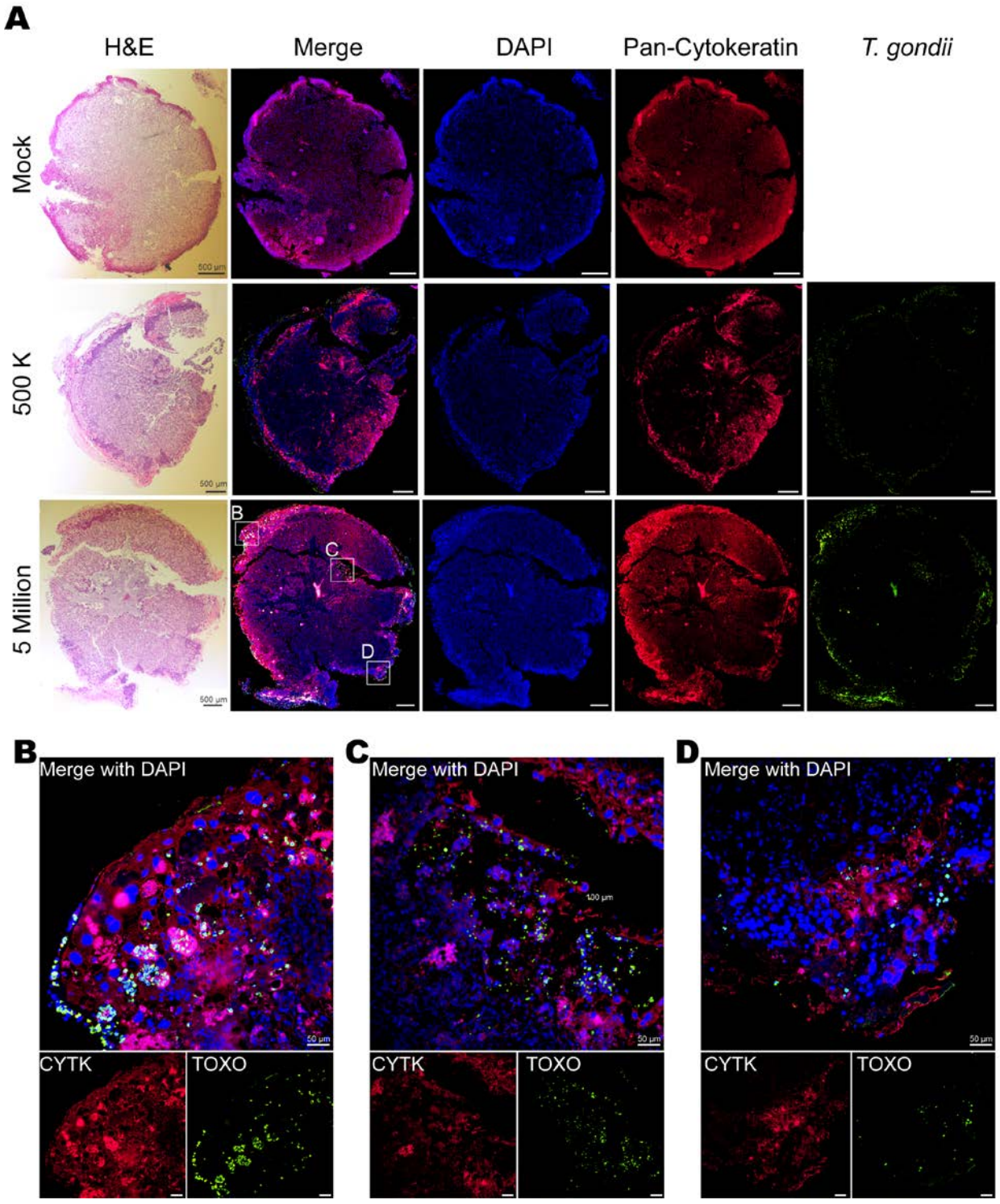


Figure 20 Immunofluorescence Staining of Placenta Tissue Sections Infected *Ex Vivo*

Sections of murine placentas infected *ex vivo* were stained for pan-cytokeratin and *T. gondii*. (A) Degree of fluorescence for *T. gondii* correlates with infectious dose: mock, 500,000 parasites, and 5 million parasites (type I,

RH). 4x images were stiched together using Photoshop to create an image of the full specimen. Scale bar represents 500 µm. (B, C, & D) Selected images taken at 20x of the 5 million-infected placenta; scale bar represents 50 µm.

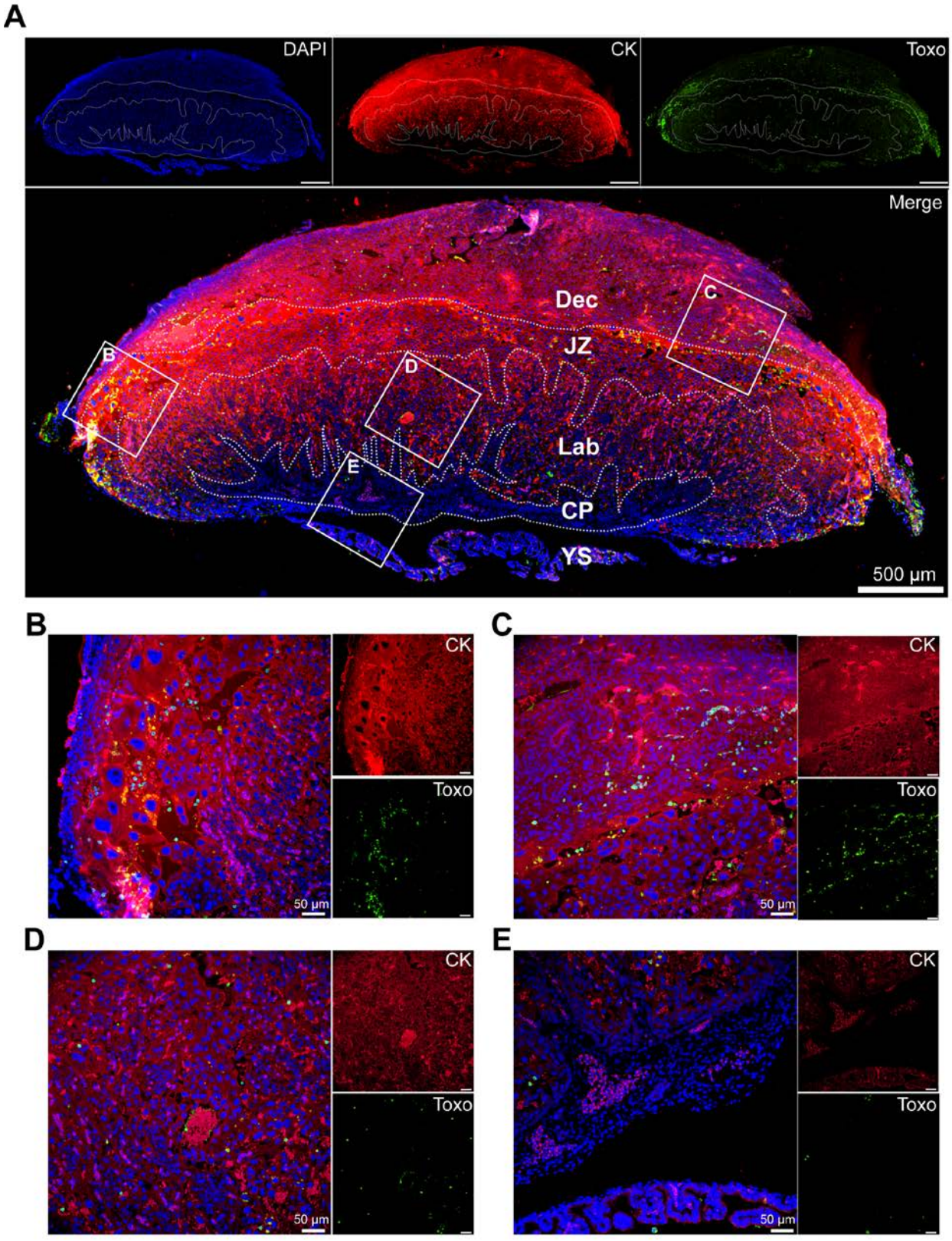


Figure 21 Immunofluorescence Staining of Placenta Tissue Sections from *In Vivo* Infection

Pregnant dams were infected at E8 of pregnancy with 1 million tachyzoites (ME49, type II) via a single intraperitoneal injection. Dams were euthanized on E12 and fetal tissues harvested. Sections of murine placentas infected *in vivo* were stained for pan-cytokeratin (CK, red) and *T. gondii* (Toxo, green). (A) A series of 10x images were stiched together using Photoshop to create a complete image of the full specimen; scale bar represents 500 µm. Tissue layers were identified and outlined by dotted white line: Dec, decidua; JZ, junctional zone; Lab, labrynth; CP, chorionic plate; YS, yolk sac villi. (B-E) Selected images were taken at 20x magnification; scale bar represents 50 µm.

3.3 Discussion and Concluding Remarks

Our present data illustrates the multifunctional role of placental trophoblasts: possessing a capacity to function like an immune cell in addition to their barrier function and epithelial lineage/origins. GSEA of differentially regulated genes in PHT cells following *T. gondii* infection (**Appendix A, Figure 24**) highlights the immunological barrier functionality of primary trophoblasts. Previously, we found *T. gondii* infection induces several immunomodulatory genes by primary trophoblasts, including the leukocyte chemoattractant CCL22. In the present study, we identify GRA28 as the parasite effector specifically driving CCL22 induction and begin to examine the mechanism by which it induces this phenotype. We found GRA28 to be both necessary and sufficient for CCL22 induction.

Interestingly, GRA28 presents a cell type-specific ability to induce CCL22: whereas only primary trophoblasts and monocyte/MØ are capable of producing CCL22 in response to parasite infection or ectopic GRA28 expression. Furthermore, this phenotype is not recapitulated in any of the tested trophoblast cell lines. This phenomenon may be explained by several hypotheses. First, the chromatin accessibility of the CCL22 promoter is more relaxed or open in these specific cell types. In this scenario, GRA28 may act directly as a transcription factor and bind to the CCL22

promoter or it may indirectly promote CCL22 transcription by interaction with endogenous transcription factors. Second, GRA28 may interact with a host factor that is uniquely expressed in primary trophoblasts and monocytes/MØ. Our initial analysis of host transcription factors for cell-specific CCL22 induction identified several candidates, yet transfection of these candidates into HeLa or JEG3 cells did not produce CCL22 induction. It is possible that (1) several of these transcription factors may be required together, (2) GRA28 interacts with a specific isoform not expressed by our plasmid constructs, or (3) GRA28 interacts with some other host factor all together. Interestingly, CCL22 was also induced when either the GRA28 construct or the control plasmid was relocalized to the endoplasmic reticulum. This correlation of CCL22 induction and increased protein localization at the endoplasmic reticulum suggests the CCL22 response may also be triggered by a cellular stress response pathway.

In order to better understand the mechanism of GRA28's function, I generated a construct for ectopic expression in mammalian cells. This construct localizes to the nucleus and is sufficient for CCL22 induction in RAW cells. Attempts to alter nuclear localization by mutating the predicted monopartite NLS or truncation found the c-terminus region (amino acids 169 to 484) to be dispensable for $GRA28_{Exon1}$ trafficking to the nucleus. Repeated computational analysis for NLS prediction sites with reduced stringency revealed the presence of several putative bipartite NLS sites along the length of the protein. A similar phenomenon has been observed with another *T. gondii* dense granule protein, GRA16, where no single predicted NLS was found to be required for nuclear localization [147]. Thus, we can currently only hypothesize whether the nuclear localization of GRA28 is required for CCL22 induction. Future experiments could attempt to disrupt all predicted NLS to examine whether nuclear localization and CCL22 induction are abrogated.

I also computationally analyzed GRA28 for the presence of any SLiMs that could be mediating CCL22 induction. As many dense granule proteins are predicted to possess high intrinsic disorder, functional domains of GRA proteins may consist of very short linear motifs rather than classical globular domains [158]. The flexibility of disordered regions promotes translocation by the MYR complex across the parasitophorous vacuole [154–156], and the short size is also evolutionarily advantageous as new SLiMs may arise (or disappear) with a single point mutation [161–163]. My analysis predicted the presence of several interesting SLiMs that may mediate CCL22 induction or other, novel functions of GRA28. However, as with all computational analyses, the validity of these predictions will need to be determined. It is not uncommon for SLiM interactions to be transient and possess weak binding affinities, both of which can cause difficulties in traditional pull-down assays for the identification of protein-protein interactions. As such, one alternative strategy for validating these SLiMs may be to tag GRA28 constructs with a biotin ligase in order to achieve a more complete network of GRA28 protein-protein interactions.

In conclusion, our studies have identified the *T. gondii* dense granule protein, GRA28, as both necessary and sufficient to induce CCL22 in primary trophoblasts and monocytes/MØ. Due to the asymptomatic nature of *T. gondii* infection in the healthy adult, it is difficult to identify maternal infection in a timely manner to prevent vertical transmission. Current diagnostic methods rely upon detecting maternal seroconversion, however, the timing of maternal antibody response may not be detectable until weeks after infection. In our future studies, we hope to utilize this robust CCL22 phenotype as a biomarker for congenital infection and improved diagnostic for congenital toxoplasmosis. Towards that end, we have obtained clinical samples from women who seroconverted during pregnancy and are examining whether there are any correlations with

congenital toxoplasmosis diagnoses. Our future studies will also examine the impact of CCL22 on transplacental transmission of *T. gondii* and pregnancy maintenance.

3.4 Materials and Methods

3.4.1 Cell Culture

All cell and tissue cultures were incubated at 37°C and 5% CO_2 and all media were supplemented with 10% FBS. THP1 cells were grown in RPMI-1640 media (HyClone); HFF and RAW cells were grown in Dulbecco's Minimum Essential Medium (DMEM; Lonza); HeLa CCL2 cells were grown in Eagle's Minimum Essential Medium (EMEM; Lonza) supplemented with 1x nonessential amino acids (HyClone) and 1mM sodium pyruvate (HyClone); and JEG-3 cells in Eagle's Minimum Essential Medium (EMEM; Lonza).

3.4.2 Mid-gestation placental explants

Human placental tissue from less than 24 weeks gestation was obtained from the University of Pittsburgh Health Sciences Tissue Bank through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. Chorionic villi were dissected and cultured in DMEM/F12 (1:1) supplemented with 10% FBS, penicillin/streptomycin, and amphotericin B. For *T. gondii* infections, isolated tissue was infected immediately following isolation with $1x10^6$ parasites for ~24hrs.

3.4.3 Parasites

Type I (RH) *T. gondii* tachyzoites were used for this study. GRA28-KO parasites were generated by E. Rudzki. All parasites were maintained by continual passage in human foreskin fibroblast (HFF) cultures incubated at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 50 μg/mL penicillin/streptomycin, and 2mM glutamine. For infections, infected monolayers were scraped and syringe-lysed to release the tachyzoites. These parasites were then pelleted at 800 x *g* for 10 minutes, resuspended in fresh media, filtered through a 5 μm filter, and counted to determine the appropriate dilution for infection. Mock inoculum was produced by filtering out the tachyzoites with a 0.2 μm filter.

In vivo infection of pregnant Swiss-Webber mouse was performed on day E8 of pregnancy, where day E0 was defined as the day a vaginal plug was observed after co-housing with a male. Infection consisted of a single intraperitoneal injection of 1 million tachyzoites (type II, ME49). The dam was euthanized on day E12, and fetal tissues were collected for histology.

3.4.4 Plasmid construction

The first predicted exon of GRA28 (excluding the 18 nucleotides upstream of the predicted signal peptide) was amplified from genomic DNA and TOPO cloned into a pcDNA3.1 vector with a c-terminus V5/His tag (Invitrogen). Primer sequences were as follows: GCCACCATGCAGACAAATCCTGATCTCGATAATCTATG (forward, 5'-3'); GTCCTCCCAATTGTCCGGTCTTACTCCGGTCTTACCC (reverse, 5'-3'). To alanine-substitute the predicted NLS, the following primers were used: CGCAAGTAAGGCAGCTGCAGCAGCTGCAGGTGGCGAAGGTG (forward, 5'-3');

CTTCGCCACCTGCAGCTGCTGCAGCTGCCTTACTTGCGGC (reverse, 5'-3'). To create truncations at the c-terminus, the following reverse primers (5'-3') were used: AGCTGATGTTGTACCCGGAGGCCCCG (truncation A) and CATACTTGGAGGTCGCTGCTCAAGAGCTTTTC (truncation B).

3.4.5 Transfections

Transfection of JEG-3 and RAW cells was performed using Lipofectamine 3000 (ratio of 2µg DNA to 2µL of P3000 reagent and 4µL of Lipofectamine 3000 reagent. Transfection of all other cells was performed with XtremeGene HP (ratio of 0.5µg of DNA to 1.5µL of transfection reagent).

3.4.6 CCL22 ELISA

CCL22 ELISAs were performed with the human or mouse CCL22/MDC DuoSet ELISA (R&D Systems) as per the manufacturer's instructions.

3.4.7 Microscopy

Cell monolayers were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 1x PBS. Primary antibodies were incubated for 1h at room temperature, followed by washing, then secondary antibodies conjugated to Alexa Fluor (Invitrogen) fluorophores for 30min at room temperature. Following washing, cells/explants were mounted with DAPI-Vectashield (Vector Laboratories) and imaging performed on an Olympus IX83 inverted microscope. In some

cases, imaged were adjusted for brightness and contrast using Photoshop or Fiji/Image J. Image J was used for image analyses. Reagents and antibodies used for immunostaining studies include Alexa Fluor 594 or 633 conjugated phalloidin (Invitrogen), anti-V5 (Invitrogen), MTCO (Abcam).

3.4.8 Computational Analysis

Prediction of protein structure and short linear motifs was performed using the Eukaryotic Linear Motif resource (ELM), located at http://elm.eu.org. Nuclear localization signal prediction was performed with NucPred, available at https://nucpred.bioinfo.se/cgi-bin/single.cgi. N-terminal signal peptide cleavage site of GRA28 was identified using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

3.4.9 Western Blotting

Cells were lysed in cold RIPA buffer (Millipore) supplemented with 1x proteinase inhibitor cocktail (Thermo Scientific), and lysate was cleared by centrifugation at 13000 x*g* for 15min at 4°C. Protein concentrations were determined by BCA assay (Thermo Scientific), and 30µg samples were separated on a 4-20% Tris-HCL gel (Bio-Rad). Proteins were transferred to PVDF membranes and blocked with 10% nonfat dried milk in PBS before antibody probing. Blots were developed for detection with chemiluminescence.

3.4.10 RT-qPCR

RNA was isolated from cultures using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and the associated DNase digestion set (Sigma). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad), followed by qPCR using a StepOnePlus Real-Time PCR System (ThermoFisher). The ΔC_T method was used to determine gene expression and normalized to the human actin C_T of each sample. Primer sequences were as follows: Human Actin—ACTGGGACGACATGGAGAAAAA (Forward, 5'-3'); GCCACACGCAGCTC (Reverse, 5'-3'). Human CCL22—GTGGTGTTGCTAACCTTC (Forward, 5'-3'); GGCTCAGCTTATTGAGAATC (Reverse, 5'-3').

3.4.11 Immunohistochemistry

Tissue sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol (100%, 95%, 80%), and washed with ddH_20. Antigen retrieval was performed with slides submerged in 10mM citrate buffer (pH 6.0) and heated in a standard microwave for 3 5-min intervals. Slides were cooled to room temperature and incubated with rodent block for 30mins (from the Mouse on Mouse Polymer IHC Kit from Abcam). Sections were incubated with anti-Toxoplasma (LS Bio, 1:25) and anti-pan cytokeratin (Invitrogen, 1:50), diluted in 1% BSA in PBS, and incubated overnight in a humidified chamber at 4^oC. The next day, slides were rinsed thrice in TBS-T (Tris-buffered saline, 0.1% Tween-20) and incubated with secondary antibodies conjugated to Alexa Fluor (Invitrogen) fluorophores for 30min at room temperature. Following washing, sections were mounted with DAPI-Vectashield (Vector Laboratories) and imaging performed on an Olympus IX83 inverted microscope using a UC90 color CCD camera

(Olympus). In some cases, imaged were adjusted for brightness and contrast using Photoshop or Fiji/Image J. H&E staining was performed by the Rangos Histology Core.

Appendix A Supplementary Figures

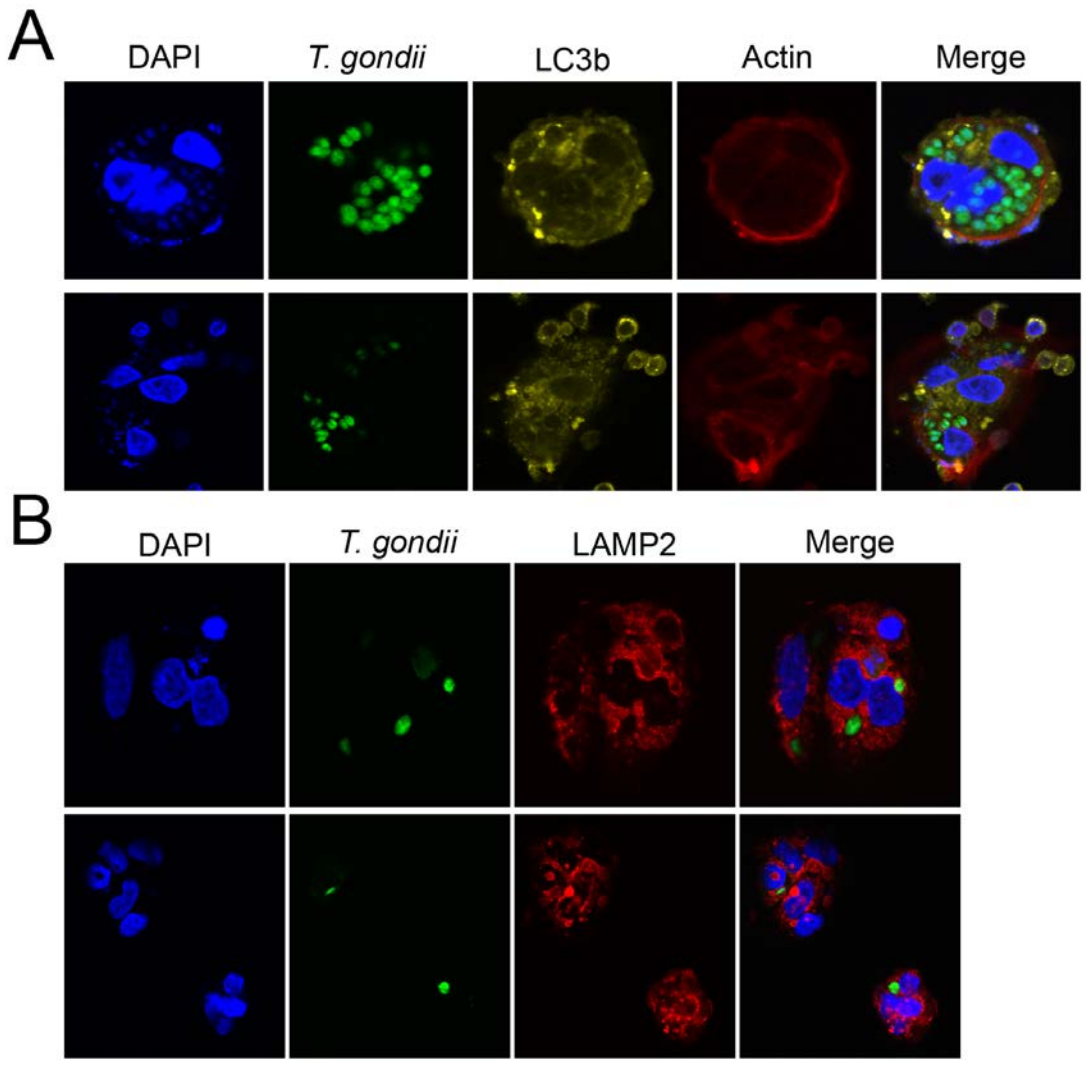


Figure 22 Markers of Autophagy & Lysosomal Degradation Do Not Associate with *T. gondii*

Immunofluorescence microscopy of PHT cells infected with *T. gondii* (YFP-RH, multiplicity of infection of 4) (green). (A) LC3B staining is shown in yellow, actin is in red, and DAPI-stained nuclei are shown in blue at 8 h postinfection. (B) Lysosome-associated membrane protein 2 (LAMP2) is shown in red and DAPI is shown in blue at 24 h postinfection.

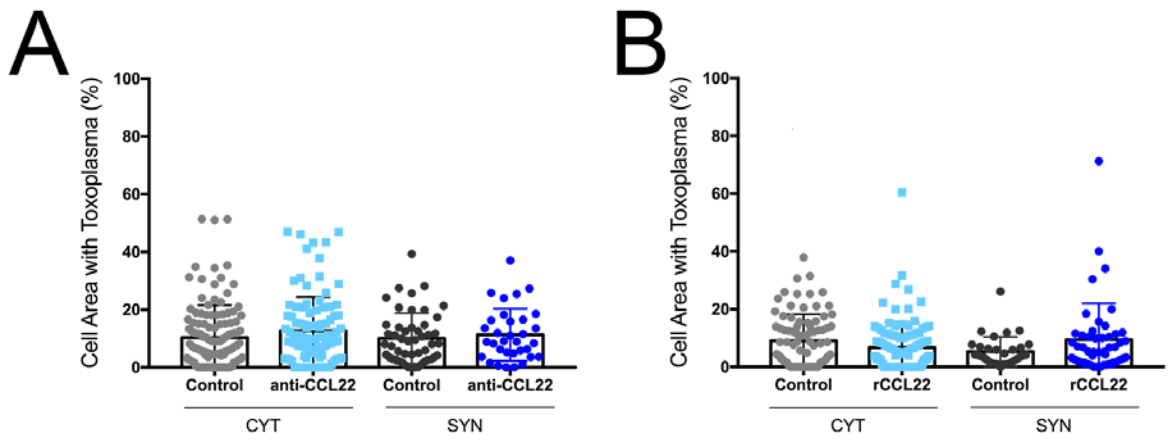
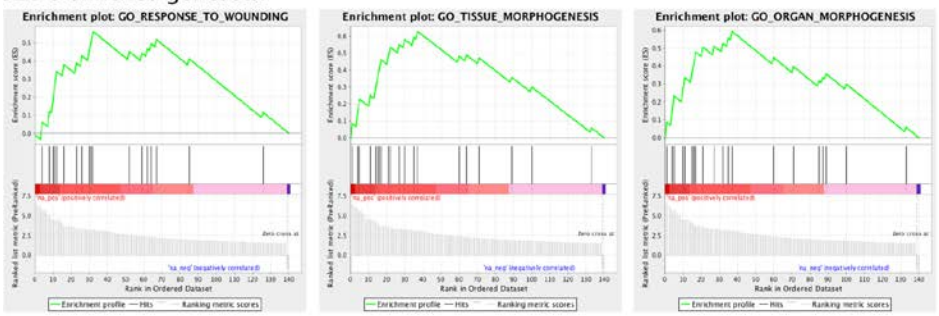


Figure 23 CCL22 Is Not Anti-Parasitic

PHT cells were superinfected with 2.16×10^5 YFP-RH parasites for 24 h and stained with cytokeratin 19, phalloidin, and DAPI in order to distinguish cell type and boundary. Degree of infection was determined by parasite area as percentage of host cell, from images taken of two technical replicates from one PHT preparation. (A) PHT cultures were treated with a neutralizing antibody to CCL22 at the time of infection. (B) Cultures were pretreated with 1 ng/ml of rCCL22 for 24 h prior to infection.

JEG-3 enriched genesets:



PHT-enriched genesets:

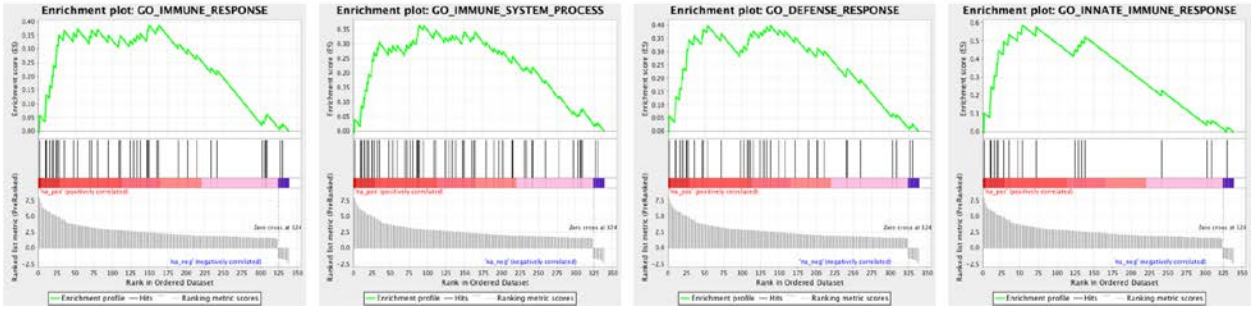


Figure 24 Geneset Enrichment Analysis (GSEA) of Differentially Regulated Genes Following Infection of JEG-3 or PHT cells

GSEA was performed on pre-ranked lists of differentially regulated genes (fold-change +/- 1.5 log_2; p-adj value <0.01) in either JEG-3 cells or PHT cells. Genesets used for analysis include "Hallmark," "Immunological Signatures," and "Gene Ontology." However, only comparison to the "Gene Ontology" geneset resulted in the identification of genesets significantly enriched (FDR<25%; nominal pvalue <1%).

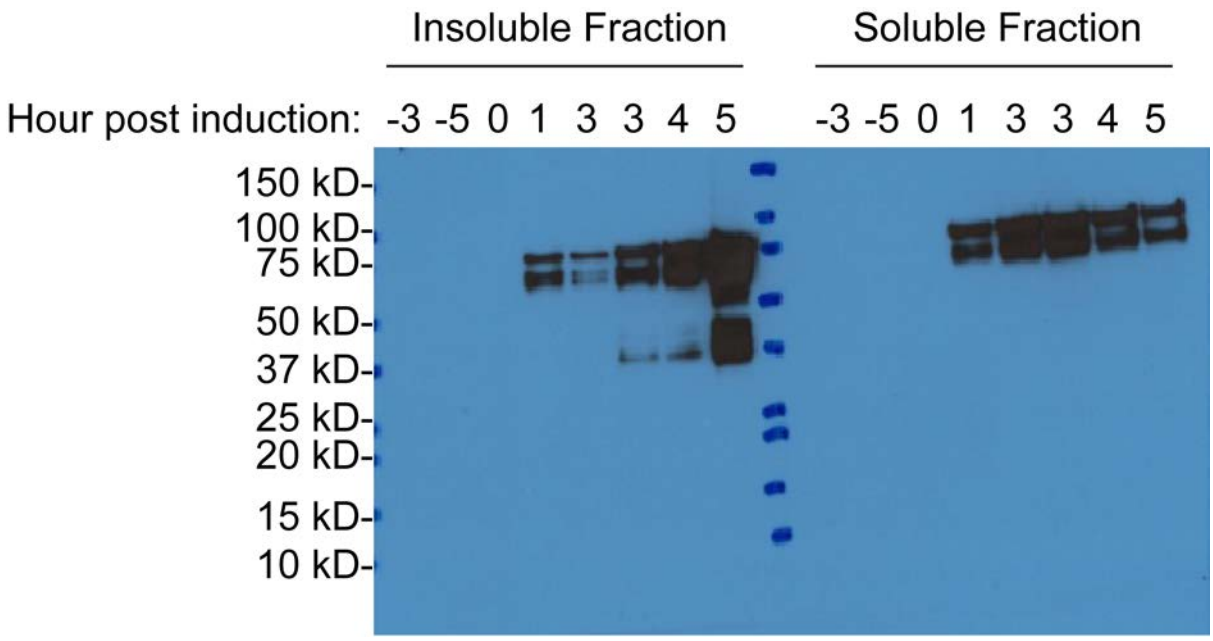


Figure 25 *E. coli*-Expressed Recombinant GRA28

Inducible induction of GRA28 construct by *E. coli* by the addition of 1mM Isopropylthio-β-galactoside (IPTG) to bacterial broth. This recombinant GRA28 is c-terminally tagged with SUMO instead of V5/His.

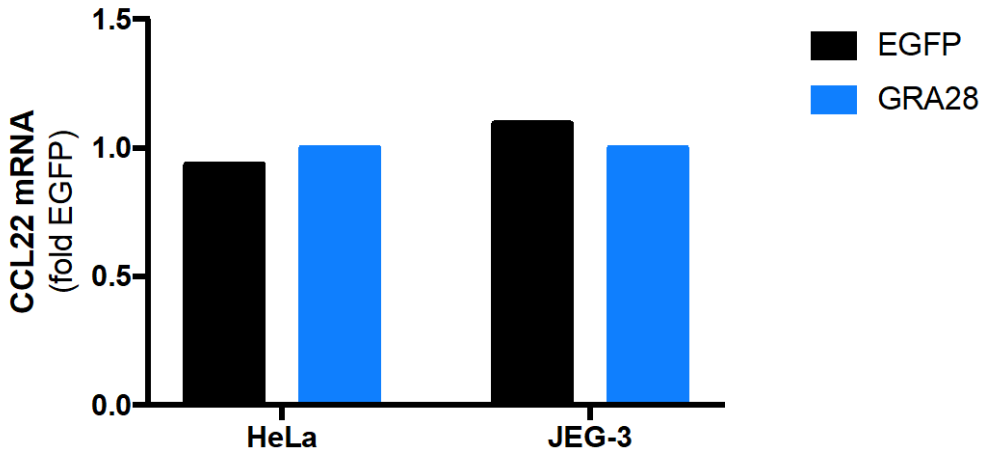


Figure 26 Ectopic Expression of GRA28 Construct Does Not Induce CCL22 in HeLa or JEG-3 Cells

EGFP or GRA28 construct was transfected in HeLa and JEG-3. CCL22 transcripts were measured using qPCR and normalized to EGFP samples.

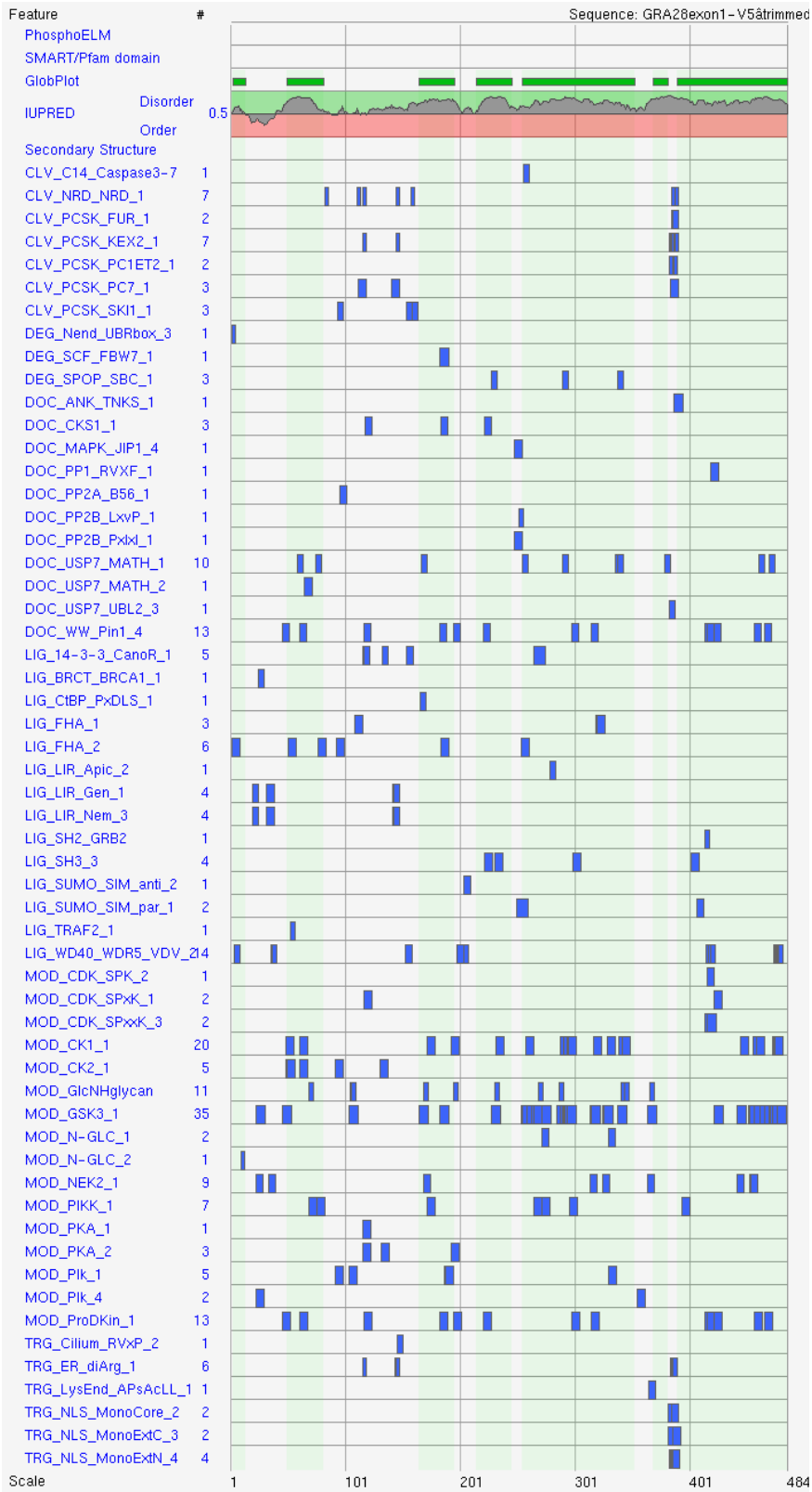


Figure 27 Full ELM Analysis of GRA28 Construct

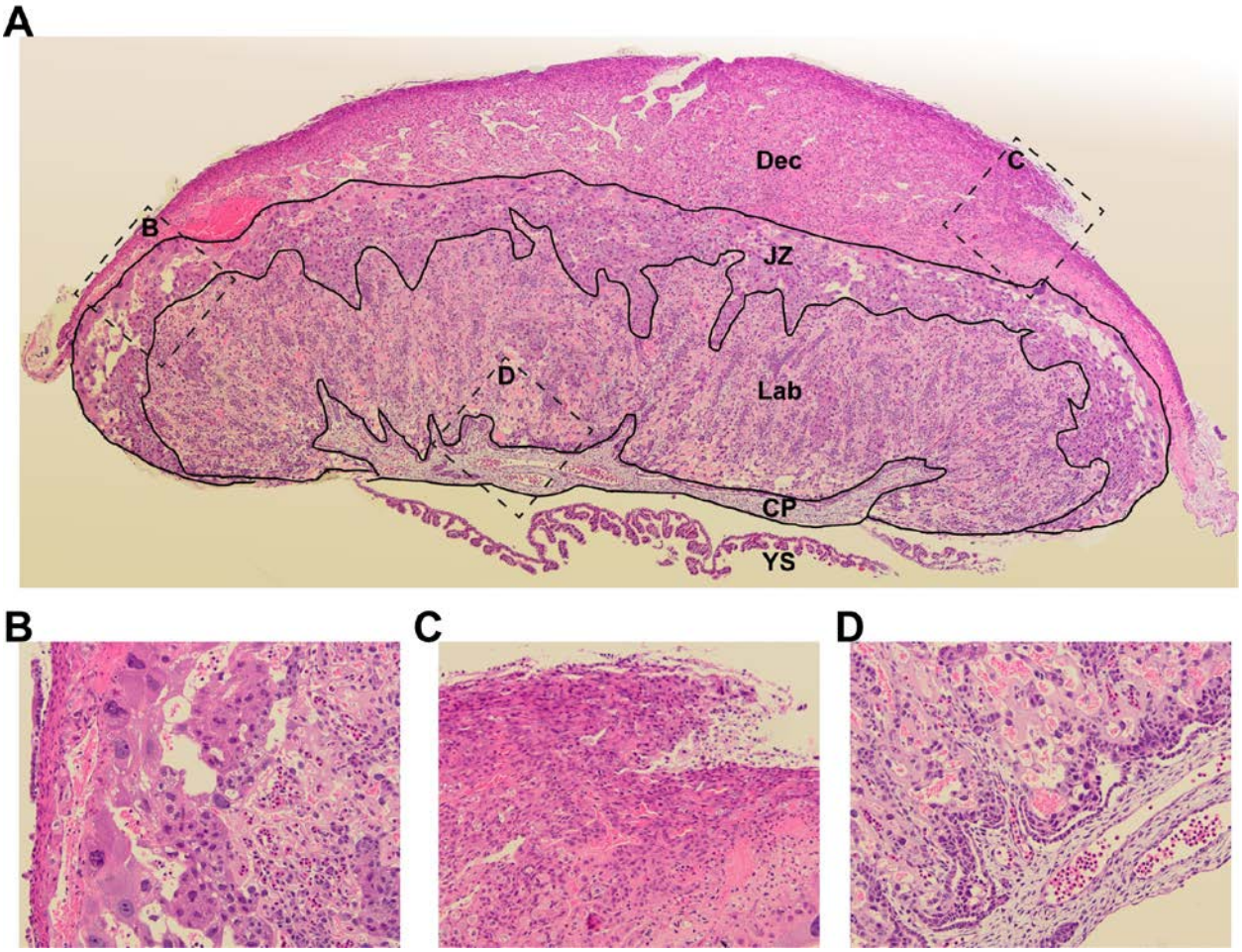


Figure 28 H&E Staining of Placenta Tissue Sections Infected *In Vivo*

A series of 10x images were stiched together using Photoshop to create a complete image of the full specimen. Tissue layers were identified and outlined by solid black line: Dec, decidua; JZ, junctional zone; Lab, labrynth; CP, chorionic plate; YS, yolk sac villi. (B-D) Selected images were taken at 20x magnification and roughly correspond to similar areas on the section that was stained for immunoflourescence.

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Ander SE, Rudzki EN, Arora N, Sadovsky Y, Coyne CB, Boyle JP (2018) Human Placental Syncytiotrophoblasts Restrict Toxoplasma gondii Attachment and Replication and Respond to Infection by Producing Immunomodulatory Chemokines. *MBio* **9**, e01678-17.

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