MAJOR ARTICLE

Infection of Placental Trophoblasts by *Toxoplasma gondii*

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How the intracellular parasite *Toxoplasma gondii* causes placental inflammation and infects the fetus is unknown. By use of a culture model of primary human trophoblasts, we examined the consequences of infection by a virulent strain of *T. gondii*. Infection fractions (parasitophorous vacuoles per trophoblast nuclei) ⩽0.9 were observed 1 day after challenge at an inoculum ratio of *T. gondii* to nuclei of 10. The culture content of infectious *T. gondii* increased 45-fold in 48 h. Two days after infection, almost 30% of trophoblast nuclei became apoptotic, and 30%–35% of nuclei were lost. Almost 90% of apoptotic nuclei were not adjacent to a parasitophorous vacuole, suggesting infection protected against apoptosis. However, there was no *T. gondii*–dependent accumulation of putative cytotoxic factors, such as tumor necrosis factor–α, that could mediate paracrine killing. Both mature and immature trophoblasts can be productively infected, and uninfected, but not infected, cells undergo apoptosis.

Toxoplasma gondii, a common protozoan parasite responsible for both severe congenital birth defects and fatal toxoplasmic encephalitis in immunocompromised people, exists in 3 forms: oocysts, tachyzoites, and tissue cysts filled with bradyzoites [1]. Tachyzoites (invasive forms) are polarized oval structures (4–8 μm long) that are capable of invading nucleated mammalian cells. Tissue cysts form within the host cell and may contain up to several thousands of bradyzoites (infective stages) that remain alive for the lifespan of the host. Congenital fetal toxoplasmosis may result in abortion, stillbirth, or severe mental retardation; infections in late pregnancy may be asymptomatic but present with retinal or neurologic damage later in life [2].

Worldwide, the risk of infection with *T. gondii* is 0.1%–1% of all pregnancies [3]. The risk of transmission after a primary maternal infection varies from 20% (in the first trimester) to 70% (in the third trimester) [4], but fetuses infected early in development are most severely affected [5]. Antimicrobial treatment after maternal seroconversion is ineffective, possibly because transmission may occur before detection of seroconversion or because of antibiotic catabolism [6–9]. Reactivation of latent disease, secondary to maternal immunosuppression (such as AIDS) or corticosteroid therapy, may also result in fetal infection [10]. Severity of the disease correlates with the extent of placental damage [11]. Clearly, the placenta plays a major role in prevention and expression of fetal disease, and an understanding of this role is crucial to prevention of congenital toxoplasmosis.

All fetal and maternal tissues are separated by a fetal epithelium (the trophoblast), the greatest area of which is in the villous placenta, the site of nutrient and gas exchange [11]. Within the villous placenta, a single multinucleated cell layer (the syncytiotrophoblast [ST]) contacts maternal blood within the intervillous space. Beneath the ST reside replicating progenitors (cytotrophoblasts [CT]) that are separated by a basal lamina from the fetal villous stroma containing vascular endothelium, fibroblasts, and macrophages. Damage to the villous placenta is almost always accompanied by

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inflammation, either in the intravillous space (intervillositis) [12] or within the fetal villi (villitis) [11] and, in severe cases, is accompanied by loss of the protective trophoblast. Extensive placental damage may lead to fetal loss or intrauterine growth retardation [11].

Although *T. gondii* infection is seen in the villous trophoblast from placentas associated with congenitally infected infants [13], the relationship between trophoblast infection and placental damage has not been studied. Furthermore, the only in vitro studies of placental trophoblast infection were carried out in rodent cells [14]. Lack of experimental evidence of *T. gondii* infection of placental trophoblasts is the result of the absence of experimental models that allow reproducible long-term studies of pure populations of primary trophoblasts of well-defined metabolic and differentiation states. We have developed such a model: highly purified (>99.99%) villous CT [15, 16] that can be maintained in culture for weeks as mononuclear cells (CT-like cultures) or differentiated into multinucleated syncytialized cells (ST-like cultures) by treatment with epidermal growth factor (EGF) [17, 18]. Such CT- and ST-like cultures have been used as infection models for human immunodeficiency virus [16] and cytomegalovirus (CMV) [19].

In the present study, we asked whether CT- and ST-like cultures of primary villous trophoblasts from different individuals can be productively infected by a virulent strain of *T. gondii*, and, if so, what the consequences are. We found that these cultures can be readily infected, that infection is followed initially by increased apoptosis (predominantly of uninfected cells), and that *T. gondii*–induced apoptosis is not mediated by tumor necrosis factor (TNF)-α or any other long-lived soluble intermediate.

MATERIALS AND METHODS

Isolation and purification of term villous trophoblasts. Placentas were obtained after normal, term delivery or cesarean section from uncomplicated pregnancy. Villous CT were isolated by both trypsin-DNase digestion of minced chorionic tissue and immunoabsorption onto immunoglobulin-coated glass-bead columns, as described elsewhere [16], with anti-CD9 (house preparation, clone 50H.19), anti-major histocompatibility complex (MHC) class I (W6/32; Harlan Sera-lab), and anti-MHC class II (house preparation, clone 7H3) antibodies, for immunoelimination. The purified cells were routinely cryopreserved and, after thawing, were washed in Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). The cells were seeded at 10^5/microwell/100 μL of 10% FBS, in 96-well tissue culture dishes (NUNC 167008; Gibco), and were incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere, the nonadherent cells and debris were removed by washing with prewarmed IMDM, and the cells were replenished with IMDM made in 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). Syncytialization of cultured CT was induced by treatment with 10 ng/mL of human EGF (Prepro-Tech) for 5 days [15, 16, 20] and was assessed by immunostaining fixed cells, with anti-desmoplakin monoclonal antibody (Sigma), to visualize desmosome-containing tight junctions [21], as described elsewhere [15]. Trophoblasts cultured 24 h without EGF are operationally termed "CT-like cultures," and those cultured 5 days with EGF are termed "ST-like cultures." All cultures contained <5 vimentin-positive cells (nontrophoblasts)/microwell.

Vero cell culture. The African green monkey–kidney cell line Vero [22] was propagated in 5% FBS-IMDM, in Corning T25 flasks, at 37°C in a 5% CO_2 incubator. The cells were routinely passaged and were plated when needed for maintenance and assay of *T. gondii*.

***Maintenance and assay of* T. gondii.** The virulent Palo Alto Rh toxoplasma peritoneal fluid strain (obtained from J. Remington, Palo Alto Research Foundation, and maintained in Swiss Albino mice by the Provincial Laboratory for Public Health, Edmonton, Alberta) was propagated for routine use in Vero cells. High virulence was maintained by passage every 4 months in mice, by intraperitoneal injection and harvest of the peritoneal lavage between 3 and 7 days. Procedures were approved by the local animal welfare committee, according to the Canadian Council for Animal Welfare guidelines. After infectious challenge, Vero cells typically were lysed within 7 days. The supernatant was centrifuged at 80 *g* for 5 min, to sediment cells and other debris, and then was further centrifuged, at 525 *g* for 20 min, to pellet the parasite, as described elsewhere [23]. Because such *T. gondii* (enumerated under fluorescence by acridine orange-propidium iodide staining on a hemocytometer [24]) was >92% viable, numbers were routinely determined by phase-contrast microscopy, and *T. gondii* was suspended in IMDM, at a concentration of 10^7 tachyzoites/mL, for immediate use.

For routine assessment of the infectious capacity of a preparation, Vero cells were plated at 5×10^4 cells/100 μL, in 96-well dishes, were incubated overnight, and then were challenged in triplicate, as described for individual experiments. After 48 h, the wells were washed with PBS, were fixed in ice-cold methanol, and were stained either with Giemsa (or hematoxylin) or immunohistochemically, as described below for *T. gondii* P30 antigen. Infection was scored as the presence of an obvious parasitophorous vacuole (figure 1*A* and 1*B*) and was expressed as a fraction: number of parasitophorous vacuoles per number of host cell nuclei.

ST- and CT-like trophoblast cultures were challenged with *T. gondii* at levels indicated in individual figure legends. After a 2-h adsorption period, the nonadherent parasites were removed along with the supernatant, and the cells were washed

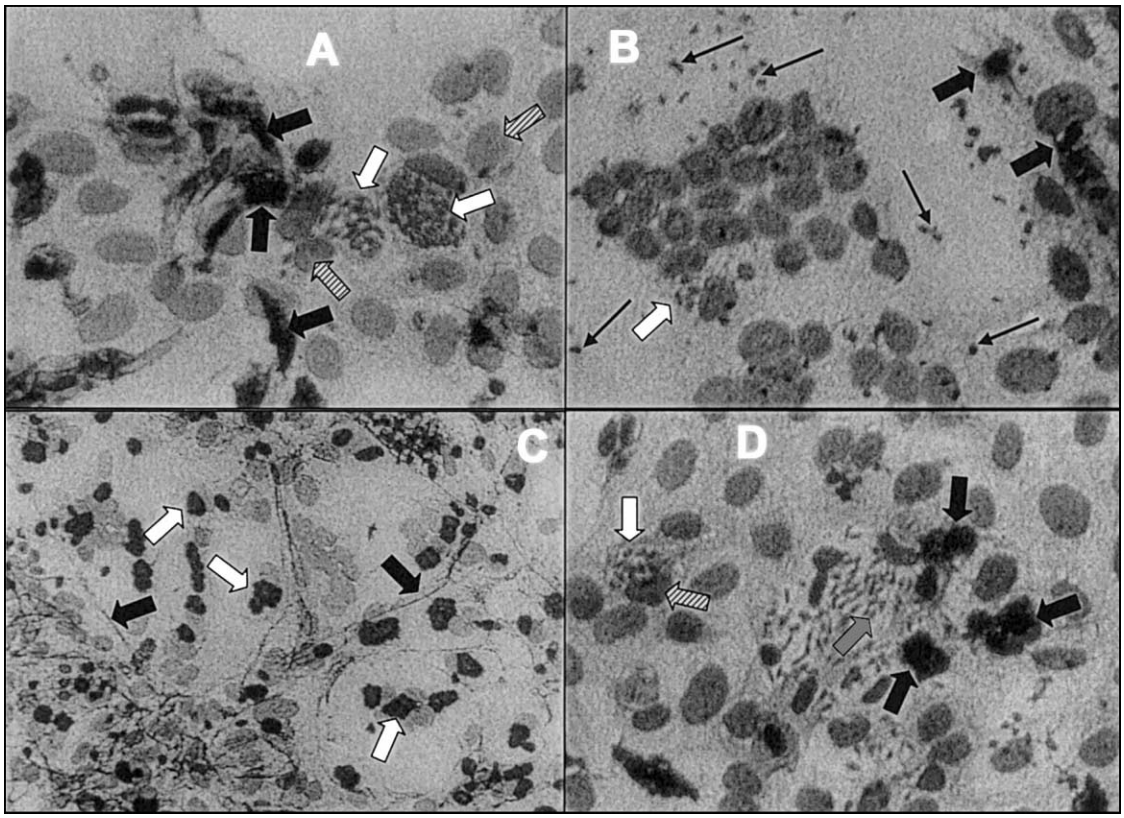

Figure 1. Photomicrographs of *Toxoplasma gondii* infection of primary trophoblasts in culture. Purified cytotrophoblasts were cultured as cytotrophoblast (CT)–like *(A* and *D)* and syncytiotrophoblast (ST)–like *(B* and *C)* cells, in 96-well dishes, were challenged with 2×10^5 *T. gondii* tachyzoites/microwell, were further cultured for 24 h, and then were fixed and stained, as described in Materials and Methods. *A,* CT-like cultures processed for terminal deoxynucleotidyl transferase–mediated dUTP-biotin DNA–nick-end labeling (TUNEL) analysis then counterstained with hematoxylin. Arrows point to TUNEL-positive trophoblast nuclei *(black arrows),* to parasitophorous vacuole *(white arrows),* and to TUNEL-negative nuclei *(striped arrows). B,* ST-like cultures processed as described in *A.* Narrower arrows point to single tachyzoites. *C,* ST-like culture stained for both trophoblast desmoplakin *(black arrows)* and *T. gondii* antigen P30 *(white arrows),* and then counterstained with hematoxylin. *D,* CT-like culture processed for TUNEL analysis, then counterstained with hematoxylin. Arrows indicate TUNEL-positive trophoblast nuclei *(black arrows),* a parasitophorous vacuole *(white arrow),* a TUNEL-negative nucleus *(striped arrow),* and a recently lysed vacuole *(gray arrow).* Bars, 25 μm.

twice with warm medium, were refed with medium, and were incubated for various times at 37°C, after infection.

Immunohistochemical staining. At different times after infection, infected and uninfected cultures were washed with PBS, were fixed in ice-cold methanol for 10 min, and were washed again with PBS. Endogenous peroxidase activity was neutralized by incubation for 30 min at room temperature with 3% H_2O_2, followed by incubation for 1 h at room temperature with 10% nonimmune goat serum (Zymed/Intermedico), to block nonspecific sites. Blocking solution was removed, 30 μL (end concentration, 5 μg/mL) of mouse monoclonal anti–*T. gondii* P30 antibody (Advanced Immuno Chemical) or its isotype control, IgG2a (Zymed/Intermedico), was added, the plates were incubated overnight at 4°C, the wells were washed with PBS, and secondary antibody (biotinylated goat anti-mouse IgG, Streptavidin Biotin System) (Histostain-SP Kit, Zymed) was added for 10 min. The wells were again washed with PBS, streptavidin-peroxidase was added at room temperature for 10 min, and wells were washed again and were incubated for 1 min with aminoethylcarbazole as a substrate, yielding a red precipitate. After counterstaining with hematoxylin, photographs were taken immediately. Cultures were stained for the tight-junction protein desmoplakin, which marks the cell boundaries of cultured trophoblasts [21], as described elsewhere [15].

Dextran sulfate inhibition of* T. gondii *infection. Dextran sulfate (500,000 Da; Pharmacia) or the same concentration of dextran (T500; Pharmacia) was added at 10, 1, 0.1, and 0 mg/mL to 2.5×10^5 *T. gondii* in 100 μL of medium and was incubated for 5 min, and the mixture was then transferred to a well containing a CT-like culture and was cultured for 24 h. The infection frequency was determined as described above.

Detection of apoptotic cells. The terminal deoxynucleotidyl transferase–mediated dUTP-biotin DNA–nick-end labeling (TUNEL) assay detects DNA fragmentation and is based on a method [25] modified for trophoblasts, as described else-

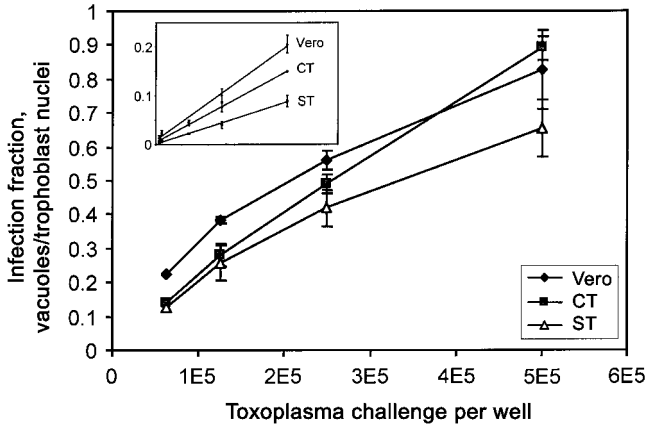

Figure 2. Dose-response relationship between challenge with *Toxoplasma gondii* and formation of parasitophorous vacuoles, in Vero cells *(diamonds),* cytotrophoblast (CT)–like cells *(squares),* and syncytiotrophoblast (ST)–like cells *(triangles),* 24 h after challenge. *Inset,* Infection fraction of 0–100,000 at challenge with *T. gondii* (separate experiment). Data are average ± SD of triplicate samples in 1 of 2 experiments with the same trends.

where [26]. The percentage of TUNEL-positive nuclei was determined from the average evaluation in 5 randomly selected 0.25-mm^2 fields in each microwell. The average total number of nuclei/0.25-mm^2 field was also determined, and the total nuclei per culture was calculated by multiplying by the ratio of well surface area/0.25 mm^2 (=128).

TNF bioassay and neutralization. Supernatants from infected and uninfected cells were frozen at −20°C, were thawed, and were assayed for TNF activity, with human TNF-α (a gift from Hofmann La Roche) standards, in the L929-8 bioassay, as described elsewhere [27]. The lowest level of detection is 1 pg/mL. To neutralize biologically active TNF-α released in trophoblast cultures, 20 μg/mL of polyclonal anti-human TNF-α antibody (ICN) was added to the culture at the time of challenge.

RESULTS

***Primary trophoblast infection by* T. gondii.** Immature CT-like and mature ST-like trophoblasts were challenged with 2×10^5 *T. gondii*/microculture (~4 *T. gondii*/trophoblasts nuclei) and were cultured for 24 h. When stained with Giemsa or hematoxylin, both CT-like cultures (figure 1*A*) and ST-like cultures (figure 1*B*) showed characteristic parasitophorous vacuoles containing 2–>20 parasites . These structures stained positive for the parasite-coat protein P30 [28], further identifying them as being of *T. gondii* origin (figure 1*C*; a ST-like culture stained with desmoplakin to visualize junctional boundaries between cells).

We next compared the susceptibility of trophoblasts and the highly susceptible African green monkey–kidney cell line Vero to *T. gondii* infection. After 24 h of challenge, the number of parasitophorous vacuoles relative to cell nuclei number (infection fraction [IF]) increased with increasing challenge with *T. gondii,* for all cell types. At IF <0.2, there was a linear relationship between IF and *T. gondii* dose, in which susceptibility followed the order Vero > CT-like cultures > ST-like cultures (figure 2, *inset*). In both CT- and ST-like cultures, the IF was maximal at 24 h and decreased thereafter (figure 3 shows the time course of infections of cells from 2 different placentas).

To formally determine whether the infection was productive, CT-like cultures were challenged with 2×10^5 tachyzoites, were cultured for 2 h, and were washed to remove unattached parasites. The washed cultures were either lysed and immediately

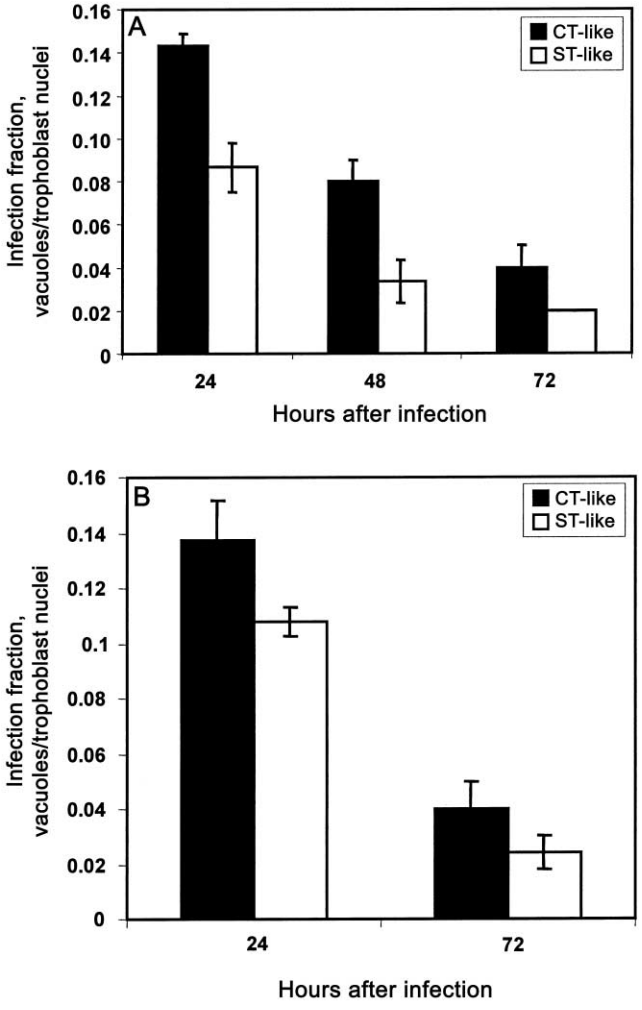

Figure 3. Relationship between time after challenge with *Toxoplasma gondii* and the infection fraction (IF) (ratio of parasitophorous vacuoles to trophoblast nuclei) in target cells. *A and B,* IF as a function of time after challenge (10^5 parasites/well) in 2 independent experiments with 2 different preparations of primary trophoblasts. Where error bars are shown, data are average ± SD of triplicate samples. CT, cytotrophoblast; ST, syncytiotrophoblast.

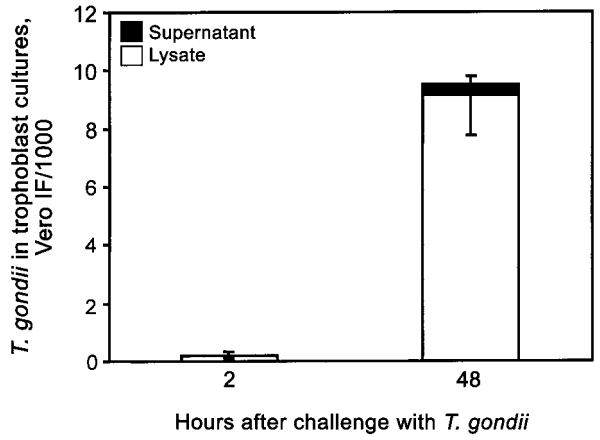

Figure 4. Increase in infectious *Toxoplasma gondii* content in cytotrophoblast (CT)–like cultures 2–48 h after infection. CT-like cultures were washed with medium 2 h after addition of *T. gondii* (10^5/well), then either were lysed for assessment of the infection fraction (IF) (ratio of parasitophorous vacuoles to trophoblast nuclei) in Vero cells (2-h histogram) or fresh medium added, the culture continued for 48 h, and both medium and cell lysate were assessed for IF (48-h histogram). Data are average ± SD pooled from 8 cultures in 2 independent experiments, each with a different placental preparation.

assayed for IF, by the Vero assay described above (figure 4), or fresh medium was added, and the culture continued for 48 h, at which time both supernatant and lysates were assayed for IF (figure 4). The results show very few cell-associated infectious parasites after the 2-h challenge and a 45-fold increase in total culture infectious-parasite content 48 h later. The observation of productive infection is in accordance with the presence of both parasitophorous vacuoles and, after a wave of vacuole lysis 24 h after challenge, numerous single tachyzoietes attached to almost every cell in the culture (figure 1*B*).

Interactions between *T. gondii* and host cell sulfated proteoglycan (inhibitable with dextran sulfate) have been shown to be essential for its attachment to a variety of cell types, including epithelial cells [29]. We found that infectious challenge with 2×10^5 *T. gondii*/microculture of both CT-like trophoblasts and Vero cells was inhibited approximately to the same degree (77% ± 7.4% for Vero and 66% ± 7.1% for CT-like cultures, data not shown) by 10 mg/mL dextran sulfate (relative to the same concentration of nonsulfated dextran).

T. gondii *–induced trophoblast loss.* The above data show that both CT-like and ST-like trophoblast cultures support a productive infection of *T. gondii* and thus that these cultures are suitable models for examination of *T. gondii*–induced villous-trophoblast damage. We therefore next asked whether *T. gondii* infection led to cell loss. Because of the multinucleated nature of trophoblasts, especially ST-like cells [18], changes in cell numbers were estimated from nuclei numbers. We found that infection resulted in minimal (~15%) loss of nuclei in both CT-like (figure 5*A*) and ST-like (figure 5*B*) cultures 24 h after challenge, but, by 72 h, infected cultures had lost 40%–60% of nuclei, relative to uninfected controls.

We next asked whether this loss of cells was accompanied by apoptosis, as determined by DNA nicking detected by the TUNEL assay. We found that *T. gondii* infection strongly stimulated trophoblast apoptosis, however, with different kinetics for CT- and ST-like cultures (figure 6). The apoptosis frequency for *T. gondii*–infected CT-like cultures remained at ~27% from 24 to 48 h after challenge. In contrast, the frequency for ST-like cultures was very low (~2%) at 24 h but increased (to ~17%) by 48 h.

Mechanism of* T. gondii*–induced trophoblast apoptosis

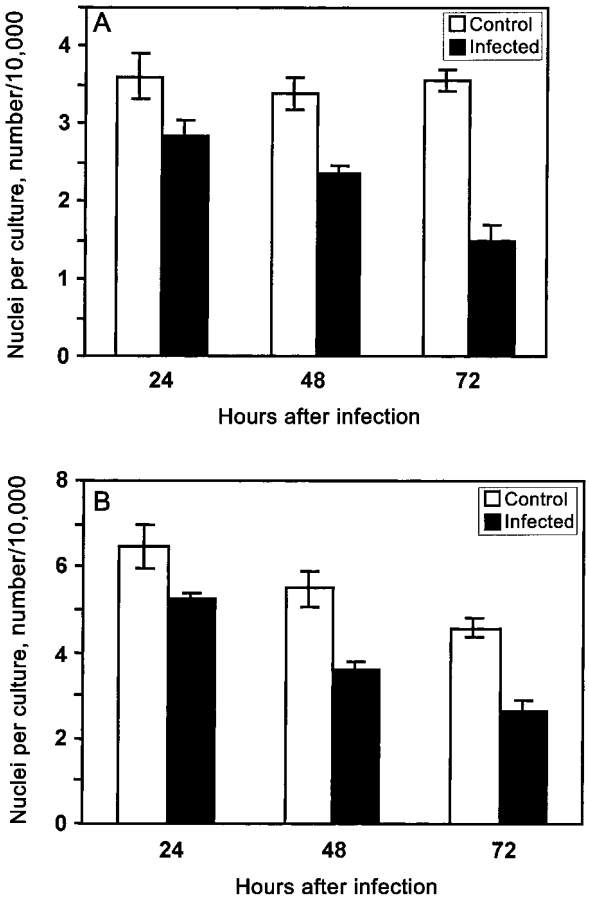

Figure 5. Loss of nuclei from cultures of primary trophoblasts infected with *Toxoplasma gondii.* Cytotrophoblast (CT)–like cultures *(A)* and syncytiotrophoblast (ST)–like cultures *(B)* were prepared and were challenged with *T. gondii,* such that the infection frequency after 24 h was 0.2–0.3, all as described in Materials and Methods. At the indicated times, the cultures were stained with hematoxylin, and the number of nuclei per well were evaluated as described in Materials and Methods. Data are average ± SD of triplicate wells from 1 of 3 experiments performed with cells from different placentas, all of which showed the same trend in percentage of cell loss but have different numbers of cells per well (trophoblast adherence varies between batches [17]).

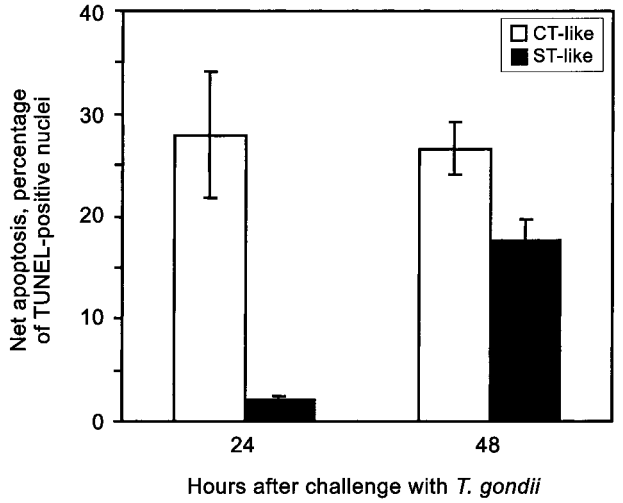

Figure 6. Apoptosis in trophoblast cultures infected with *Toxoplasma gondii.* Cytotrophoblast (CT)—like cultures *(white bars)* and syncytiotrophoblast (ST)—like cultures *(black bars)* were prepared, were challenged with 2×10^5 *T. gondii*/microwell, were cultured for the indicated periods of time, and then were assessed for apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP-biotin DNA–nick-end labeling (TUNEL) analysis, all as described in Materials and Methods. Data are average ± range of triplicate wells from 2 independent experiments performed with cells from different placentas.

and loss. *T. gondii*–infected lymphohematopoietic cells are resistant to apoptosis induced by multiple stimuli but are not resistant to cytotoxic T lymphocyte–induced cytolysis [30]. To determine whether the trophoblasts undergoing apoptosis were infected, we examined infected cultures 24 and 48 h after challenge, for proximity of obvious vacuoles to TUNEL-positive nuclei. *T. gondii* parasitophorous vacuoles are predominantly juxtanuclear [31, 32] and, in trophoblasts, frequently distort (indent) nuclear shape (figure 1*A*). Thus, close apposition of a TUNEL-positive nuclei to a vacuole indicates apoptosis in an infected cell. We found that 89.8% ± 0.7% of nuclei directly adjacent to a parasitophorous vacuole were TUNEL negative (figure 1*C* and 1*D*). The remaining 10% of nuclei adjacent to vacuoles were TUNEL positive, and most (6.30% ± 0.73%) of these were near vacuoles showing signs of recent disintegration (figure 1*D*). We conclude that productive *T. gondii* infection is associated, at least initially, with resistance to *T. gondii*–stimulated apoptosis in the cultures.

These data showing that, predominantly, uninfected cells are undergoing *T. gondii*–stimulated apoptosis also indicate paracrine induction. We have shown that CMV infects both CT- and ST-like cultures [19] and induces loss of uninfected cells in a manner inhibited by neutralizing antibody to TNF-α [33]. We therefore asked whether *T. gondii*–induced apoptosis and loss of uninfected cells was also caused by paracrine release of TNF-α. However, we found that *T. gondii* infection did not stimulate TNF production from these cultures ($\leq$1 pg/mL, data not shown). Furthermore, neutralizing antibody to TNF-α at levels that inhibit >80% of apoptosis stimulated by 10 ng/mL TNF-α did not inhibit *T. gondii*–induced apoptosis at either 24 or 48 h after *T. gondii* infection (figure 7*A*). We conclude that, unlike CMV, *T. gondii* does not induce trophoblast apoptosis, by stimulating release of TNF-α.

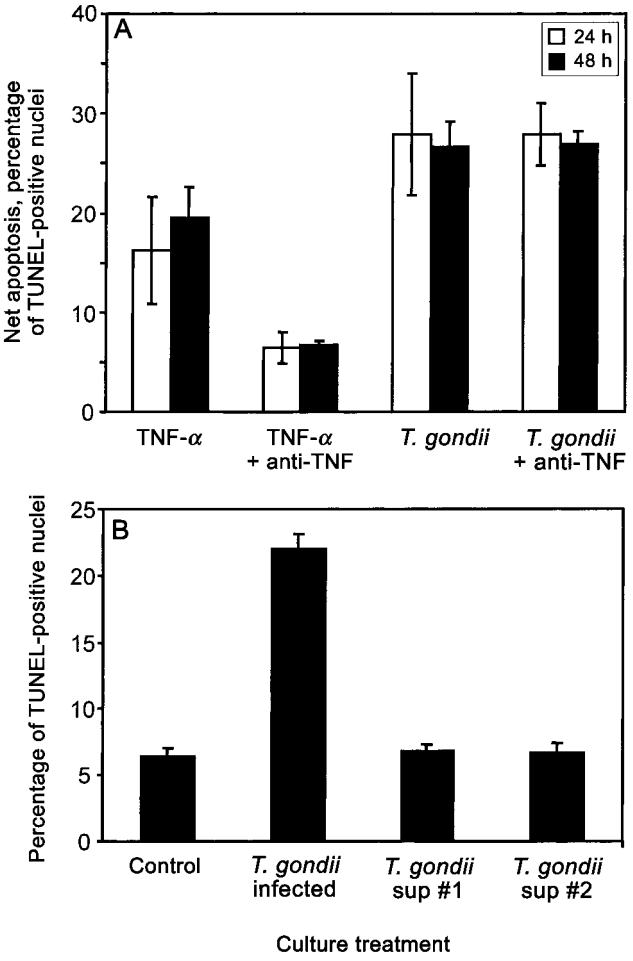

Figure 7. *Toxoplasma gondii*–induced apoptosis not mediated by supernatant (sup) tumor necrosis factor (TNF)–α or by measurable accumulation of a soluble death factor, in culture supernatants. *A,* Cytotrophoblast (CT)–like cultures challenged with either 2×10^5 *T. gondii*/microwell or TNF-α (10 ng/mL) or combination of either 1 of those with antibody to TNF-α, as depicted, and terminal deoxynucleotidyl transferase–mediated dUTP-biotin DNA–nick-end labeling (TUNEL) analysis performed after a 24-h culture. Depicted are the mean ± range of 2 independent experiments performed with cells from different placentas. Net apoptosis indicates that, in absence of treatment, CT apoptosis has been subtracted from values. *B,* CT-like cultures challenged with 2×10^5 *T. gondii* and cultured for 24 h. Supernatants from the 24-h *T. gondii*–infected cultures were filtered through a 0.22-μm filter and were immediately transferred (at 50% v/v) to fresh CT-like cultures, and TUNEL analysis was performed after 24 h. *T. gondii* sups 1 and 2 indicate supernatants from *T. gondii*–infected cultures of cells from 2 different placentas. Data are mean ± SD of triplicate samples from 1 experiment.

To determine whether apoptosis was mediated by supernatant accumulation of any death-inducing activity, we asked whether supernatant from a *T. gondii*–infected culture filtered through a 0.22-μm filter would induce apoptosis. The results show that such supernatants induced no apoptosis or cell loss (figure 7*B*). Thus, if a soluble factor is mediating apoptosis of uninfected cells, it must be acting locally within an unstirred layer above the cultured cells, be very labile, or be removable by the 0.22-μm filter.

DISCUSSION

Because *T. gondii* tachyzoites bind and enter almost all nucleated mammalian cells [34], including mouse trophoblasts [14], human trophoblast infection was highly predicted. Nonetheless, this is the first report that primary human villous trophoblasts can be productively infected by *T. gondii.* We have found that *T. gondii* infects immature CT-like cultures somewhat better than it does mature ST-like cultures and that both are marginally less susceptible to infection than is the African green monkey–kidney cell line Vero. We have also found that dextran sulfate inhibits trophoblast (and Vero cell) infection by *T. gondii*, suggesting initial binding to a sulfated proteoglycan. Such binding sites are essential for *T. gondii* attachment to a variety of cell types, including epithelial cells [29]. These data suggest that, in humans, the first step of in utero transmission of *T. gondii* is very likely productive infection of the villous ST. The data also provide the basis for a model to study both the pathogenesis of *T. gondii* infection in the placenta and the mechanisms of innate resistance in the trophoblast.

As early as 24 h after challenge with *T. gondii,* parasitophorous-vacuole maturation disperses progeny tachyzoietes throughout the culture and associates parasites to almost every cell in the culture (figure 1*B*). After an initial-infection IF of 0.2–0.3, this dispersal of infectious progeny and general attachment to uninfected cells would be predicted to increase the IF (ratio of parasitophorous vacuoles to trophoblast nuclei), because the IF can be >0.8 in naive (not previously exposed) cultures. However, rather than increasing, the IF decreases in the next 48 h of culture. This decrease in IF suggests induced resistance in the uninfected trophoblasts in the culture. Possible innate resistance mechanisms available to villous trophoblasts are induction of reactive oxygen [35, 36] and nitrogen intermediates [37–39], the enzyme indoleamine 2,3-dioxygenase (IDO) that converts tryptophan to kynurenine [23, 40], or other, as-yet-undefined mechanisms [41]. The nature of *T. gondii*–inducible trophoblast resistance to *T. gondii* infection is presently under investigation.

Uncontrolled *T. gondii* induction of type 1 cytokines, such as IFN-γ and TNF-α, lead to bystander immunopathologies in mice [42]. The *T. gondii*–induced damage observed in trophoblast cultures cannot be caused by an acquired immune response but may be mediated by endogenous production of the same cytokines. Indeed, the only cytokine demonstrated to induce villous-trophoblast apoptosis and cell loss is TNF-α, the effects of which are enhanced by IFN-γ [20, 43] and which is expressed by human trophoblasts [33, 44]. Human CMV infection of villous trophoblasts causes loss of up to half of cultured cells in the first 24 h of exposure [33]. The CMV-induced cell loss is caused by apoptosis and is mediated by TNF-α, which is, in turn, induced by CMV immediate-early gene products. However, *T. gondii* infection does not induce measurable (1 pg/mL) TNF-α production nor is neutralizing antibody to TNF-α able to inhibit *T. gondii*–induced cell loss and apoptosis. We conclude that, although placental infections by CMV and *T. gondii* both result in trophoblast loss in vivo and in culture, the mechanisms of trophoblast loss are very different.

We found that ~90% of *T. gondii*–induced trophoblast apoptosis manifested in nuclei at a distance from parasitophorous vacuoles. Because parasitophorous vacuoles are juxtanuclear [31], this observation argues that apoptosis is not occurring in cells containing intact parasitophorous vacuoles. The resistance of *T. gondii*–infected cells to apoptosis has been observed elsewhere [30]. However, the observation that ~7% of apoptosis manifests in nuclei near disintegrating vacuoles suggests that infected trophoblasts may undergo apoptosis at the time of parasite egress. The absence of *T. gondii* vacuoles near most apoptotic trophoblast nuclei may be a consequence of timing—that is, nicked nuclear DNA detectable by TUNEL lasts longer in culture than do indications of disintegrating *T. gondii* vacuoles. An alternative explanation is bystander killing, in which infected cells release or express agents cytotoxic to neighboring cells. Bystander apoptosis occurs in trophoblast cultures infected with CMV [33], but apoptosis in uninfected cells in the vicinity of *T. gondii*–infected cells has not been explored [31]. Bystander killing implies release of cytotoxic factors by the infected cells. However, as discussed above, we find that filtered supernatants from *T. gondii*–infected trophoblast cultures undergoing apoptosis do not stimulate apoptosis when transferred to uninfected trophoblasts. Thus, if cytotoxic factors are being released by infected cells, they are either sensitive to the culture context (act very locally, are very labile, and/or do not survive supernatant transfer or are *T. gondii*–dependent depletions of essential medium components) or are large enough to be removed by a 0.22-μm filter. Examples of the former category are rapidly decaying reactive oxygen and nitrogen intermediates, *T. gondii*–dependent IDO expression, and subsequent depletion of medium tryptophan (see above). Examples of cytotoxic substances that could be removed by filtration are plasma membrane-tethered members of the TNF superfamily (reviewed in [45]), such as Fas ligand, the expression of which is increased by *T. gondii* infection in ocular cells [46]. Possibilities in both categories are being investigated.

The major contribution of this study is the characterization of a primary trophoblast culture model for nonimmunocyte-mediated tissue damage in the placenta. We propose that *T. gondii*–induced trophoblast damage is central to the process of in utero transmission to the fetus, the mechanism of which remains unknown [34]. A hematogenous infection would target the ST, which faces maternal blood; thus, any *T. gondii*–induced apoptosis could be either direct in the ST or indirect in underlying CT. The villous trophoblast, like all epithelia, constantly undergoes renewal, with a turnover of ~3.5 weeks at term [47]. Increased ST apoptosis accompanies pregnancy-associated disorders, such as preeclampsia [48] and intrauterine growth retardation [49], both of which manifest in increased placental pathology [50–52]. The link between increased villous-trophoblast apoptosis and placental pathologies, although apparently obvious, is not completely understood. Common to most forms of villitis is loss of the ST layer, the loss of which could be caused either by increased ST apoptosis and shedding or by decreased CT-renewal capacity. Our results suggest that *T. gondii* infection may target either or both of these components of trophoblast turnover: either direct ST apoptosis late in the infection cycle or depletion of underlying CT by bystander killing. Because loss of the ST barrier would allow access to the underlying fetal mesenchyme, by progeny parasites or infected maternal leukocytes, understanding the mechanism of loss is crucial to understanding the mechanism of *T. gondii* passage across the villous placenta. Both of the suggested mechanisms of increasing trophoblast turnover—direct *T. gondii*–induced ST apoptosis or bystander CT apoptosis—can be studied in the experimental model presented in this study.

We used a type 1 *T. gondii* strain (virulent in mice) for these studies, but type 2 *T. gondii* strains (with low virulence in mice) more commonly vertically transmit in human pregnancy [7, 53]. However, type 2 strains are also virulent in human fetal tissue [54], and the mouse may not be an accurate model for vertical transmission of *T. gondii* [55]. Nonetheless, whether low-virulence *T. gondii* strains induce human trophoblast apoptosis remains to be determined. Also remaining to be determined is whether the increased transmission rate in the third trimester of pregnancy is related to increased sensitivity of near-term trophoblasts to the pathogenic effects reported in this article.

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References

1. Remington JS, McLeod R, Thulliez P, Desmonts G. Toxoplasmosis. In: Remington JS, Klein JO, eds. Infectious diseases of the fetus and newborn infant. 5th ed. Toronto: WB Saunders, **2001**:205–346.
2. Wong SY, Remington JS. Toxoplasmosis in pregnancy. Clin Infect Dis **1994**; 18:853–61.
3. Stray-Pedersen B. Toxoplasmosis in pregnancy. Baillieres Clin Obstet Gynaecol **1993**; 7:107–37.
4. Sever JL, Ellenberg JH, Ley AC, et al. Toxoplasmosis: maternal and pediatric findings in 23,000 pregnancies. Pediatrics **1988**; 82:181–92.
5. Desmonts G, Couvreur J. Congenital toxoplasmosis: a prospective study of 378 pregnancies. N Engl J Med **1974**; 290:1110–6.
6. Gilbert RE, Gras L, Wallon M, Peyron F, Ades AE, Dunn DT. Effect of prenatal treatment on mother to child transmission of *Toxoplasma gondii*: retrospective cohort study of 554 mother-child pairs in Lyon, France. Int J Epidemiol **2001**; 30:1303–8.
7. Ajzenberg D, Cogne N, Paris L, et al. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J Infect Dis **2002**; 186:684–9.
8. Foulon W, Villena I, Stray-Pedersen B, et al. Treatment of toxoplasmosis during pregnancy: a multicenter study of impact on fetal transmission and children's sequelae at age 1 year. Am J Obstet Gynecol **1999**; 180: 410–5.
9. Gratzl R, Sodeck G, Platzer P, et al. Treatment of toxoplasmosis in pregnancy: concentrations of spiramycin and neospiramycin in maternal serum and amniotic fluid. Eur J Clin Microbiol Infect Dis **2002**; 21:12–6.
10. Biedermann K, Flepp M, Fierz W, Joller-Jemelka H, Kleihues P. Pregnancy, immunosuppression and reactivation of latent toxoplasmosis. J Perinat Med **1995**; 23:191–203.
11. Benirschke K, Kaufmann P. Pathology of the human placenta. 4th ed. New York: Springer-Verlag, **2000**.
12. Joshi VV. Handbook of placental pathology. New York: Igaku-Shoin, **1994**.
13. Fox H. The placenta and infection. In: Redman CW, Sargent I, Starkey PM, eds. The human placenta. Oxford: Blackwell Scientific, **1993**: 313–33.
14. Ferro EA, Bevilacqua E, Favoreto-Junior S, Silva DA, Mortara RA, Mineo JR. *Calomys callosus* (Rodentia: Cricetidae) trophoblast cells as host cells to *Toxoplasma gondii* in early pregnancy. Parasitol Res **1999**; 85:647–54.
15. Yui J, Garcia-Lloret MI, Brown AJ, et al. Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. Placenta **1994**; 15:231–46.
16. Kilani R, Chang L-J, Hemmings D, Guilbert LJ. Placental trophoblasts resist infection by multiple HIV-1 variants even with CMV co-infection but support HIV replication after provirus transfection. J Virol **1997**;71: 6359–72.
17. Guilbert LJ, Winkler-Lowen B, Sherburne R, Rote NS, Li H, Morrish DW. Preparation and functional characterization of villous cytotrophoblasts free of syncytial fragments. Placenta **2002**; 23:175–83.
18. Morrish DW, Dakour J, Li H, et al. In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium. Placenta **1997**; 18:577–85.
19. Hemmings DG, Kilani R, Nykiforuk C, Preiksaitis JK, Guilbert LJ. Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts. J Virol **1998**; 72:4970–9.
20. Garcia-Lloret M, Yui J, Winkler-Lowen B, Guilbert LJ. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. J Cell Physiol **1996**; 167:324–32.

21. Douglas GC, King BF. Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. J Cell Sci **1990**; 96:131–41.
22. Yasumura Y, Kawakita M. The search for SV40 by means of tissue culture methods. Nippon Rinsho **1963**; 21:1201–19.
23. Nagineni CN, Pardhasaradhi K, Martins MC, Detrick B, Hooks JJ. Mechanisms of interferon-induced inhibition of *Toxoplasma gondii* replication in human retinal pigment epithelial cells. Infect Immun **1996**; 64:4188–96.
24. Borel E, Mayencon M, Kaiser K, Picot S, Peyron F. Fluorogenic detection of viable *Toxoplasma gondii*. Parasite **1998**; 5:371–3.
25. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol **1992**; 119:493–501.
26. Payne SG, Brindley DN, Guilbert LJ. Epidermal growth factor inhibits ceramide-induced apoptosis and lowers ceramide levels in primary placental trophoblasts. J Cell Physiol **1999**; 180:263–70.
27. Branch DR, Shah A, Guilbert LJ. A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. J Immunol Methods **1991**; 143:251–61.
28. Mineo JR, McLeod R, Mack D, et al. Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection. J Immunol **1993**; 150:3951–64.
29. Carruthers VB, Hakansson S, Giddings OK, Sibley LD. *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. Infect Immun **2000**; 68:4005–11.
30. Nash PB, Purner MB, Leon RP, Clarke P, Duke RC, Curiel TJ. *Toxoplasma gondii*-infected cells are resistant to multiple inducers of apoptosis. J Immunol **1998**; 160:1824–30.
31. Carruthers VB. Host cell invasion by the opportunistic pathogen *Toxoplasma gondii*. Acta Trop **2002**; 81:111–22.
32. Lindsay DS, Speer CA, Toivio-Kinnucan MA, Dubey JP, Blagburn BL. Use of infected cultured cells to compare ultrastructural features of *Neospora caninum* from dogs and *Toxoplasma gondii*. Am J Vet Res **1993**; 54:103–6.
33. Chan G, Hemmings DG, Yurochko AD, Guilbert LJ. Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha. Am J Pathol **2002**; 161:1371–81.
34. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. Int J Parasitol **2000**; 30:1217–58.
35. Arsenijevic D, Onuma H, Pecqueur C, et al. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet **2000**; 26:435–9.
36. Murray HW, Cohn ZA. Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. J Exp Med **1980**; 152:1596–609.
37. Adams LB, Hibbs JB Jr, Taintor RR, Krahenbuhl JL. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*: role for synthesis of inorganic nitrogen oxides from L-arginine. J Immunol **1990**; 144:2725–9.
38. Alexander J, Scharton-Kersten TM, Yap G, Roberts CW, Liew FY, Sher A. Mechanisms of innate resistance to *Toxoplasma gondii* infection. Philos Trans R Soc Lond B Biol Sci **1997**; 352:1355–9.
39. Smith SC, Guilbert LJ, Yui J, Baker PN, Davidge ST. The role of reactive nitrogen/oxygen intermediates in cytokine-induced trophoblast apoptosis. Placenta **1999**; 20:309–15.
40. Kudo Y, Boyd CA. The role of L-tryptophan transport in L-tryptophan degradation by indoleamine 2,3-dioxygenase in human placental explants. J Physiol **2001**; 531:417–23.
41. Dimier IH, Bout DT. Inhibition of *Toxoplasma gondii* replication in IFN-gamma—activated human intestinal epithelial cells. Immunol Cell Biol **1997**; 75:511–4.
42. Gazzinelli RT, Wysocka M, Hieny S, et al. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol **1996**; 157:798–805.
43. Yui J, Garcia-Lloret M, Wegmann TG, Guilbert LJ. Cytotoxicity of tumour necrosis factor-alpha and gamma- interferon against primary human placental trophoblasts. Placenta **1994**; 15:819–35.
44. Chen HL, Yang YP, Hu XL, Yelavarthi KK, Fishback JL, Hunt JS. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. Am J Pathol **1991**; 139:327–35.
45. Gruss HJ. Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. Int J Clin Lab Res **1996**; 26: 143–59.
46. Hu MS, Schwartzman JD, Yeaman GR, et al. Fas-FasL interaction involved in pathogenesis of ocular toxoplasmosis in mice. Infect Immun **1999**; 67:928–35.
47. Huppertz B, Frank HG, Reister F, Kingdom J, Korr H, Kaufmann P. Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments in vitro. Lab Invest **1999**; 79:1687–702.
48. Leung DN, Smith SC, To KF, Sahota DS, Baker PN. Increased placental apoptosis in pregnancies complicated by preeclampsia. Am J Obstet Gynecol **2001**; 184:1249–50.
49. Smith SC, Symonds EM, Baker PN. Increased placental apoptosis in intrauterine growth restriction. Am J Obstet Gynecol **1997**; 177:1395–401.
50. Salafia CM, Vintzileos AM, Silberman L, Bantham KF, Vogel CA. Placental pathology of idiopathic intrauterine growth retardation at term. Am J Perinatol **1992**; 9:179–84.
51. Salafia CM, Minior VK, Pezzullo JC, Popek EJ, Rosenkrantz TS, Vintzileos AM. Intrauterine growth restriction in infants of less than thirty-two weeks' gestation: associated placental pathologic features. Am J Obstet Gynecol **1995**; 173:1049–57.
52. Salafia CM, Pezzullo JC, Ghidini A, Lopez-Zeno JA, Whittington SS. Clinical correlations of patterns of placental pathology in preterm preeclampsia. Placenta **1998**; 19:67–72.
53. Fuentes I, Rubio JM, Ramirez C, Alvar J. Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. J Clin Microbiol **2001**; 39: 1566–70.
54. Costa JM, Darde ML, Assouline B, Vidaud M, Bretagne S. Microsatellite in the beta-tubulin gene of *Toxoplasma gondii* as a new genetic marker for use in direct screening of amniotic fluids. J Clin Microbiol **1997**; 35:2542–5.
55. Innes EA. Toxoplasmosis: comparative species susceptibility and host immune response. Comp Immunol Microbiol Infect Dis **1997**; 20: 131–8.