



A local innate immune response against *Trypanosoma cruzi* in the human placenta: The epithelial turnover of the trophoblast



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ABSTRACT

Congenital Chagas disease, caused by *Trypanosoma cruzi*, is partially responsible for the progressive globalization of Chagas disease despite of its low transmission rate. The probability of congenital transmission depends on complex interactions between the parasite, the maternal and fetus/newborn immune responses and placental factors, being the latter the least studied one.

During transplacental transmission, the parasite must cross the placental barrier where the trophoblast, a continuous renewing epithelium, is the first tissue to have contact with the parasite. Importantly, the epithelial turnover is considered part of the innate immune system since pathogens, prior to cell invasion, must attach to the surface of cells. The trophoblast turnover involves cellular processes such as proliferation, differentiation and apoptotic cell death, all of them are induced by the parasite. In the present review, we analyze the current evidence about the trophoblast epithelial turnover as a local placental innate immune response.

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Contents

1. Chagas disease	123
2. Congenital Chagas disease	124
2.1. The parasite	124
2.2. Mother and fetus/newborn immune response	124
2.3. Placenta	125
3. The trophoblast: A key factor in <i>T. cruzi</i> congenital infection	125
3.1. Trophoblast turnover	125
3.2. Cell proliferation	126
3.3. Cell differentiation	126
3.4. Apoptotic cell death	126
4. Epithelial turnover as part of the innate immune system	127
5. Conclusion	128
Acknowledgements	128
References	128

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1. Chagas disease

American trypanosomiasis or Chagas disease is a neglected zoonotic disease caused by the protozoan parasite *Trypanosoma*

cruzi (*T. cruzi*). Chagas disease is endemic in Latin America where ten million people are estimated to be infected [46]. However, due to population mobility, the presence of Chagas disease has been increasingly detected in other non-endemic continents such as North America, Oceania and Europe [52]. The transmission of the parasite occurs through transfusion, organ transplantation or vertical routes. More than 10 000 deaths are estimated to occur annually from Chagas disease; its incapacitating effects and mortality are one of the biggest public-health problems in Latin America. The 10-year mortality rate may range from 9% to 85%, depending on the extent of the cardiac damage induced by the parasite [3,49].

Chagas disease manifests first with an acute phase, lasting for about 2 months, characterized by high parasitaemia. Most cases are asymptomatic or present nonspecific symptoms. Then, it turns into a chronic phase, where the parasites hides in target tissues, especially in the heart and digestive muscles [3]. In case of pregnancy, *T. cruzi* is also able to reach the placenta and consequently infect the developing fetus [9,35].

2. Congenital Chagas disease

WHO has considered recently, that 1,125,000 women in fertile age are infected with *Trypanosoma cruzi* in Latin America; being the incidence of congenital infection 8668 cases per year [56]. About 50% of the congenital cases were detected in Mexico, Argentina and Colombia. In endemic countries, an average congenital transmission rate of 5% has been reported in infected mothers [30]. However, in Bolivia congenital transmission rates about 11% has been observed [50]. Congenital infection can occur in both acute and chronic phases of maternal infection, be repeated at each pregnancy, and observed from one generation to another. Moreover, migration of pregnant woman contributes to the “globalization” of Chagas disease. For instance, cases of congenital Chagas disease have been currently reported in USA, Europe (mainly Spain), and Japan [8]. Congenital *T. cruzi* infection is associated with premature labor, low birth weight, and stillbirths [1,53]. Neonatal mortality can occur in untreated severe congenital Chagas disease. Older studies report high morbidity and mortality rates [5]. However, the recent studies in endemic as well as non-endemic countries, report non-lethal congenital cases [8] and a surprising the degree of normality in most congenitally infected children [4].

T. cruzi can be transmitted from mother to child across the placenta and through the birth canal [16]. During transplacental transmission, the parasite reaches the fetus by crossing the placental barrier [8,35]. The fact that only a percentage of the infected mothers transmit parasites to their fetuses raises the question of the ability of the placenta as well as the immunological status of mother and fetus/newborn to impair the parasite transmission. Therefore, the congenital transmission of the parasite is product of a complex interaction between the parasite, the maternal and fetus/newborn immune responses and placental factors [35,39], being the latter the least studied one. Here we analyze the current evidence about the epithelial turnover of the placental lining epithelia (the trophoblast) as a local innate immune response. Additionally, host genetics might play a role in the susceptibility to acquiring congenital infection. It has been demonstrated, that mutations in matrix metalloproteinase genes, particularly ADAM12 and MMP2, were associated with susceptibility to congenital infection [34].

2.1. The parasite

T. cruzi is a haemoflagelated protozoan of the *Kinetoplastida* Order and *Trypanosomatidae* family [57]. *T. cruzi* display great

biological, biochemical and genetic diversity, therefore different strains of the parasite have been identified and classified into six discrete typing units (DTU) [54,57]. *T. cruzi* strains corresponding to different DTUs might have relevant consequences on congenital transmission and fetal/neonatal pathology. Thus, strains from DTUs II/V/VI are the most predominant observed in congenitally infected children [45]. Particularly, in infected newborns of northwestern Argentina *T. cruzi* from DTU V has been described [15]. On the other hand, parasitaemia is associated with the risk of congenital transmission. Thus, a high parasitaemia, as in acute infection, correlates with a higher transmission rate (30–50%). In chronic infected patients, with very low parasitaemia, the transmission rate is between 1 and 21% [6,43,50]. Though appearing a low rate of infection, chronically infected pregnant women represent a high risk of maintenance of Chagas disease both in endemic and non-endemic areas.

2.2. Mother and fetus/newborn immune response

The immune system is fundamental to protect the mother against the environment and to prevent damage to the fetus. During pregnancy the maternal immune system is characterized by a reinforced network of cellular and molecular recognition, communication, trafficking and repair; it raises the alarm to maintain the well being of the mother and the fetus. On the other side, the fetus provides a developing active immune system that will modify the way the mother responds to the environment, providing a uniqueness of the immune system responses during pregnancy [42].

A crucial factor to stop, limit, or permit the development of fetal/neonatal infection relates to the capacity of the mother and fetus/newborn to mount innate and/or specific immune response(s) against pathogens. Clinical studies have shown a strong association between intrauterine infections and pregnancy disorders such as abortion, preterm labor and intrauterine growth retardation [37]. For instance, maternal infection or inflammation can lead to preterm birth. The exact mechanisms remain unclear, but inflammation may alter the appropriate maintenance of epigenetic profiles during pregnancy. Neonatal T cells, for instance, produce very little IFN- γ due to a number of factors, including low-level expression of the transcription factor T-box gene that encodes transcription factor 2 (Tbet2) and epigenetic factors, such as hypermethylation of the *Ifn- γ* promoter, preventing a potentially dangerous Th1 responses. The basal inhibition of IFN- γ could be altered by the maternal cytokine environment, promoting a deleterious inflammatory response [13]. On the other hand, it has been proposed that subclinical viral infections of the placenta could sensitize women to intrauterine bacterial infection or even to the placental microflora itself, changing the placental response to local microorganisms and, therefore, increases the risk of an inflammatory response resulting in preterm birth [48]. We have recently reported, that *T. cruzi* modulates the ERK1/2 MAPK pathway, which is related to cytokine gene expression, in human placental chorionic villi explants (HPCVE) [12].

As described above, congenital *T. cruzi* infection is associated premature labor, low birth weight, and stillbirths [1,35,53].

Production of pro-inflammatory cytokines can be observed in uninfected babies born to infected mothers [8]. Contrarily, the levels of inflammation markers and activation of NK cells are rather low in congenitally infected newborns [29]. These data highly suggest a protective role of such innate defenses in an uninfected newborn from infected mothers. On the other side, maternal *T. cruzi*-specific IgG antibodies play protective roles in mothers and in fetuses when antibodies are transferred through the placenta and also may contribute to a reduction in parasitaemia [8].

2.3. Placenta

The placenta is the principal site for the exchange of nutrients and gases between the mother and fetus. This organ plays an important role in hormone, peptide, and steroid synthesis necessary for a successful pregnancy [2,35]. The human placenta is classified as a hemochorial villous placenta in which the free chorionic villi, formed by the trophoblast and the villous stroma, are the functional units. The trophoblast contacts maternal blood in the intervillous space and is separated by a basal lamina from the villous stroma, which is connective tissue containing the vascular endothelium, fibroblasts and macrophages [2,20,35,41]. Trophoblast, basal laminae and villous stroma with the endothelium of fetal capillaries form the placental barrier that must be crossed by different pathogens, including *T. cruzi*, to infect the fetus during vertical transmission [7,20,35](Fig. 1). During acute infection, due to the high number of circulating parasites, intracellular parasites (amastigote nests) can be observed in the placenta. Moreover, rupture of these nests releases the parasites and cellular contents in the interstitium where they provoke an inflammatory reaction and necrosis of the placental villi [1,5].

However, the typical amastigote nests can not be observed in placentas from mothers with chronic Chagas disease [22] nor in human placental chorionic villi explants (HPCVE) infected with the parasite [20]. In the latter, only a few parasite antigen and DNA can be identified [20,40]. Interestingly, isolated trophoblast cells are less susceptible to *T. cruzi* infection as compared to other mammalian cells, such as fibroblast [18]. Moreover, infections of the placenta are not commonly observed. Taking together, these evidences suggest that anti-parasite mechanisms exist in the placenta of women suffering chronic Chagas disease. Considering that trophoblast as a lining epithelia forms an anatomical barrier and constitutes therefore part of the innate immune system, we have proposed that it plays a fundamental role in protecting the placenta and fetus against pathogens.

3. The trophoblast: A key factor in *T. cruzi* congenital infection

The major constituent of the human placenta is the trophoblast, the first cell lineage that develops before any embryonic tissue

arises. Between morula and blastocyst stages, the trophoblast lineage forms a cover around the early embryo (the embryoblast) [31]. The human trophoblast differentiates into two major subtypes [19,24]: a) Cells that invade maternal uterine tissue and differentiate into the *extravillous trophoblast*, and b) Cells that remain within the placenta and differentiate into the *villous trophoblast* forming the epithelial cover of the placental villi and constituting part of the placental barrier [24,35] (Fig. 1).

The *villous trophoblast* is composed of two cellular layers: the syncytiotrophoblast (ST) and the cytotrophoblast (CT). The CT displays high proliferative properties, whereas the differentiated ST loses its generative capacity and is no longer able to proliferate. The ST is a multinucleated layer that forms the outer surface of placental villi and comes into direct contact with maternal blood [31,32,41]. This cell layer is a typical syncytium with plasma membranes only on the apical and basal sides. The ST is continuous and normally uninterrupted, covering all villous trees of the human placenta. It is generated and maintained through syncytial fusion by incorporation of CT cells [32,41]. As described above, the trophoblast forms a physical barrier, that need to be maintained intact, in order to protect the organism from the attack of pathogens. The normal epithelial turnover is one mechanism that assures the integrity of this anatomical barrier. Moreover, the epithelial turnover has been considered part of the innate immune system due to the fact that pathogens, prior to cell invasion, must attach to the surface of cells. As these cells are continuously eliminated, the attached pathogens are removed with them [39]. Therefore, it is reasonable to postulate that the trophoblast turnover could be considered as a defense mechanism against pathogens.

3.1. Trophoblast turnover

Trophoblast turnover implies precise orchestration of different cellular processes that include *cell proliferation* of the CT, *cell differentiation* (meaning the syncytial fusion by incorporating CT cells into a non replicative ST and differentiation of CT cells previous fusion with the ST) and *cell death* [32,41]. Cell death in the trophoblast manifests by formation of apoptotic ST knots, which are released into the maternal blood. The apoptotic knots

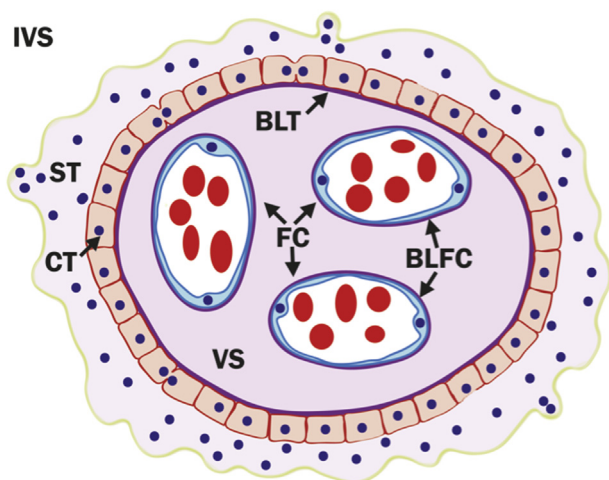


Fig. 1. Placental barrier: The placental barrier is composed of syncytiotrophoblast (ST), cytotrophoblast (CT), fetal connective tissue of the villous stroma (VS), fetal capillary (FC) and basal lamina between villous stroma and trophoblast (BLT) and around fetal endothelium (BLFC). Maternal blood circulates in the intervillous space (IVS).

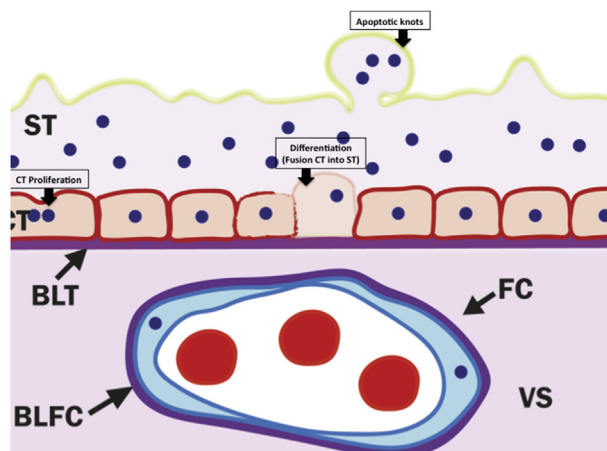


Fig. 2. Trophoblast turnover: The trophoblast, a continuously renewing epithelia, is formed by two cellular layers: The basal cytotrophoblast (CT) and the superficial syncytiotrophoblast (ST). Cells of the CT are the only ones with proliferating capacity (1); CT cells fusion into the ST (2) and the continuous incorporation of the CT are counterbalanced by the formation of apoptotic knots (3) that are eliminated into the maternal blood present in the intervillous space (IVS). Underlining the trophoblast, villous stroma (VS) containing fetal capillary (FC) and respective basal laminae (BLT and BLFC) can be observed.

counterbalance the continuous incorporation of CT cell into the ST [21,24,41] (Fig. 2).

3.2. Cell proliferation

As previously described above, cells of the CT are the only ones showing capacity for cell proliferation [41]. We have shown, that *T. cruzi* induces cellular proliferation in the trophoblast. In BeWo cells, a cell line widely used in trophoblast studies [19], *T. cruzi* induces significant DNA synthesis, increase of the percentage of cells in the S and G₂/M cell cycle phases and the expression of different proliferation markers, particularly AgNORs, PCNA and Ki67 (Droguett et al. submitted). In HPCVE, *T. cruzi* also increases DNA synthesis as well as the PCNA proliferation marker (Fig. 3). AgNORs, PCNA and Ki67 are all widely used as proliferation markers. However, PCNA acts also as a molecular coordinator in multiple different cellular functions, including DNA damage repair and avoidance, cell cycle control, cell survival, chromatin assembly, gene transcription, epigenomic maintenance and sister-chromatid cohesion [55]. Likely, the observed increase of PCNA expression could also be a response to *T. cruzi*-induced cell damage. Indeed, we have previously demonstrated disorganization of human placental chorionic villi explants (HPCVE) [20] as well as apoptosis [10,21] after *ex vivo* incubation of the explants with this parasite. Importantly, Ki67 considered as a more reliable proliferation marker that is present during all active phases of the cycle [41], was highly significant the above mentioned study.

3.3. Cell differentiation

Growth, expansion and maintenance of the ST throughout pregnancy depend mainly on the continuous incorporation of CT cells into the ST, implying that CT cell differentiate and fuses into the ST [32,41]. We have previously shown, that particularly low concentrations of the parasite induce cell differentiation in the trophoblast. *T. cruzi* induces protein expression of β -human

chorionic gonadotropin (β -hCG) and syncytin [39], both considered as major biochemical markers of trophoblast differentiation [2]. Additionally, in the trophoblastic cell line BeWo, we demonstrate parasite-induced cell fusion by the two-color fusion assay as well as desmoplakin re-distribution (Fig. 4) [39]. Interestingly, the intracellular replicative form of the parasite can be observed in fused cells (Fig. 4 C, F). On the other hand, the induction of trophoblast differentiation has been related to MAPK signal transduction pathway activation [23]. In this context, we have shown previously, that ERK1/2 MAPK is also activated by a low parasite challenge [12].

3.4. Apoptotic cell death

The continuous generation of apoptotic knots is part of the normal trophoblast turnover since it counterbalance the incorporation of CT cells into the ST [41]

On the other hand, host cell integrity has been considered as a valuable prerequisite for the survival of pathogens. The activation or prevention of cell death seems to be a critical factor in the outcome of an infection since it can facilitate or difficult the pathogen control and spreading. Apoptosis in the hosts can be managed during the infection with microorganisms including protozoa in order to limit the infection or facilitate the proliferation of intracellular pathogens [17,27]. In case of the trophoblast, *T. cruzi* induces apoptotic cell death. We have demonstrated previously, that the parasite induces the presence of picnotic nuclei, caspase 3 like activity, caspase cleaved cytokeratin 18 and induction of DNA fragmentation in HPCVE [21].

Interestingly, cell differentiation and apoptotic cell death are closely related in the trophoblast. Thus, CT cell differentiation and fusion is regulated, between other, by apoptotic cell death involved caspases [25,26]. In particular caspase 8, an apoptosis initiator caspase, has been proposed to regulate trophoblast differentiation and fusion. For instance, caspase 8 is activated in highly differentiated CT cells just prior to fusion and escorts the fusing cell content including the nucleus into the ST and it has not been found in

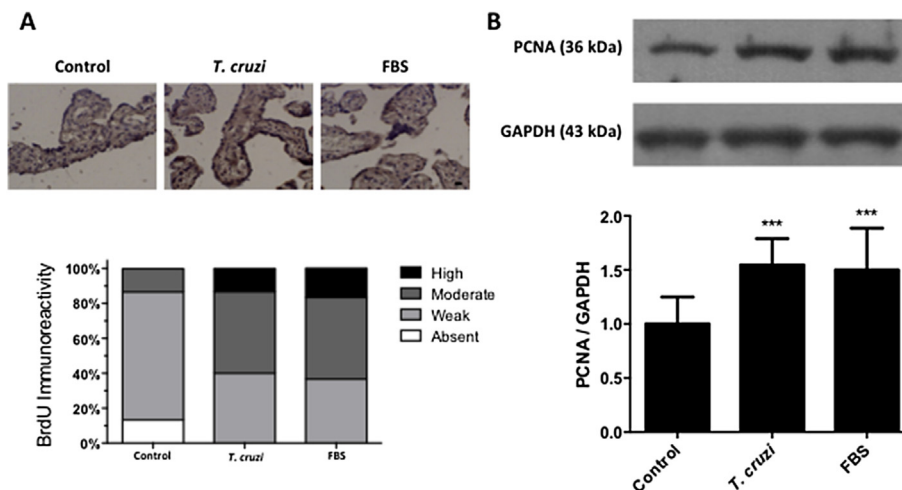


Fig. 3. *T. cruzi* induces DNA synthesis and PCNA proliferation marker in HPCVE: HPCVE were obtained as described previously [20] and incubated in FBS free medium in presence and absence of 10^5 /ml of trypomastigotes de *T. cruzi* (Ypsilon strain) during 24 h. Incubation with FBS (10%) was used as positive control. **A:** BrdU incorporation into DNA was measured by using a commercial kit according manufacturer's instructions (BrdU Immunohistochemistry Kit (Abcam® ab125306)). Bar scale: 10 μ m. For analysis of staining, ten fields were randomly selected, the localization and intensity of the immunoreactivity was analyzed by two independent observers using light microscopy and the signal intensity was scored as follows: -, absent; +, weak; ++, moderate; +++, high. Data were analyzed by Fisher's exact test ($p < 0.0001$) [11]. **B:** Protein expression of PCNA proliferation marker was determined by Western blotting. A representative western blots for PCNA (sc-7907, Santa Cruz Technologies®; 1:500) and GAPDH (sc-51905 Santa Cruz Technologies®; 1:500 v/v) is shown. Immunoreactive proteins were detected using enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham Biosciences® UK, Ltd.). The films were scanned, and NIH-Image v1.6 (NIH, Bethesda, MD) was used for the densitometric analysis of the bands. The bar graph represents the ratios of PCNA to GAPDH normalized with respect to the values obtained in control not infected HPCVE. All values are given as the means \pm S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. Data were expressed as the mean \pm standard deviation and analyzed using analysis of variance followed by Tukey's post-test (**** $p < 0.001$).

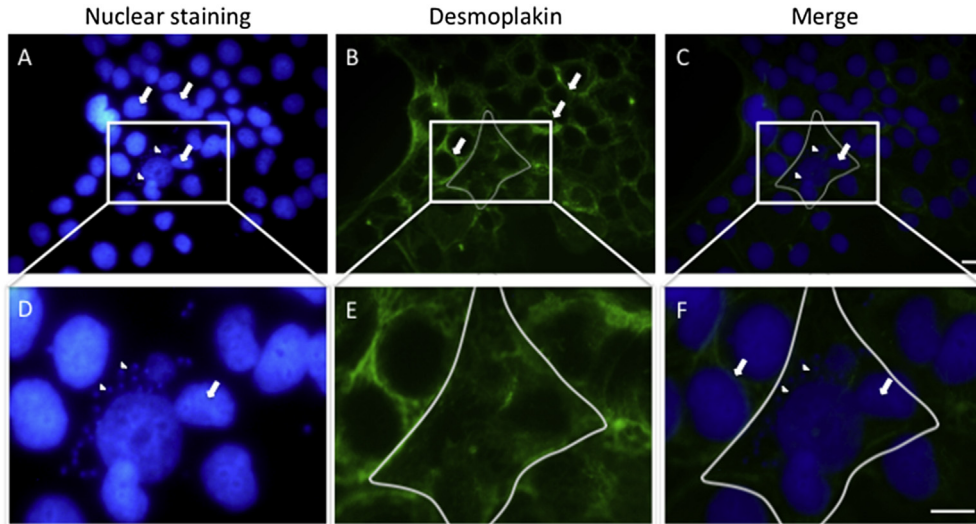


Fig. 4. Intracellular amastigotes can be observed in fused cells: BeWo cells were incubated for 48 h with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 as described previously [39]. **A and D:** infected BeWo cells stained with DAPI, with the white arrows indicating BeWo cell nuclei and the arrowheads indicating intracellular parasites, amastigotes were identified as described previously [38]. **B and E:** desmoplakin immunoreactivity (CBL173 Millipore®; 1:10 v/v) (cell limits: white arrows; fused cells: white demarcation) was determined by routine immunofluorescence methods as described previously [39]. **C and F,** merged images from **A, D** and **B, E** in which intracellular parasites are present in the cytoplasm of fused cells (parasite nuclei: white arrows; fused cells: white demarcation). Bar scales: 10 µm.

proliferating CT cells [31,47]. Moreover, fusion of the trophoblast can be visualized by localizing caspase 8 [26]. Here we show, that *T. cruzi* induces in HPCVE the expression of the cleaved (active fragment) of caspase 8 (Fig. 5A). Moreover the inhibition of caspase 8 increases the amount of parasite DNA in the HPCVE, indicating that the inhibition of cell differentiation and the start of apoptosis increase the infection in the placental tissue (Fig. 5B).

4. Epithelial turnover as part of the innate immune system

There are three types of defense mechanisms in innate immunity: a) the above-mentioned anatomical barrier; b) cellular innate immune responses; and c) humoral innate immune responses. Once the invading pathogens breach the anatomical barrier, innate immune cells are activated and secrete cytokines and chemokines to control pathogen replication [28,33].

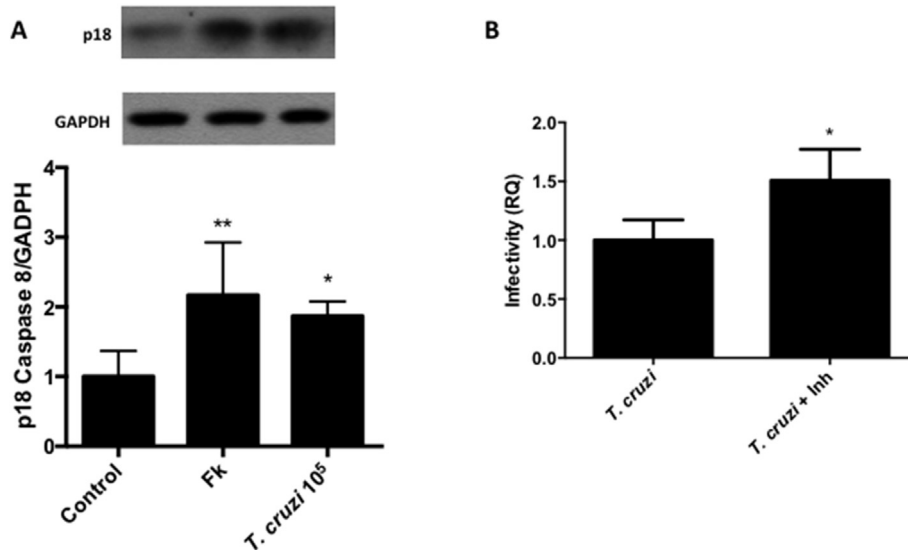


Fig. 5. *T. cruzi* induces the expression of the active fragment of caspase 8 in HPCVE, inhibition of the enzyme increases the infection of the parasite: HPCVE were obtained as described previously [20] and incubated in presence and absence of 10⁵/ml of trypomastigotes de *T. cruzi* (Ypsilon strain) during 48 h. Incubation with Forskolin (Fk) (100 µM) was used as positive control. **A:** Protein expression of cleaved fragment of caspase 8 (p18) was determined by Western blotting. A representative western blots for p18 (#9496, Cell Signalling®; 1:1000 v/v) and GAPDH (sc-51905 Santa Cruz Technologies®; 1:500 v/v) is shown. The bar graph represents the ratios of p18 to GAPDH normalized with respect to the values obtained in control not infected HPCVE. Immunoreactive proteins were detected using enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham Biosciences® UK, Ltd.). The films were scanned, and NIH-Image v1.6 (NIH, Bethesda, MD) was used for the densitometric analysis of the bands. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. Data were expressed as the mean ± standard deviation and analyzed using analysis of variance followed by Tukey's post-test (**p* < 0.05**; *p* < 0.01). **B:** Parasite DNA in HPCVE was quantified by Real-time quantification by qPCR using the ΔΔCt method as described previously [11]. Data are a comparison of parasite DNA in 1 ng of total DNA isolated from infected HPCVE. Data represents means ± SD and were analyzed by Student *t*-test. **p* < 0.05.

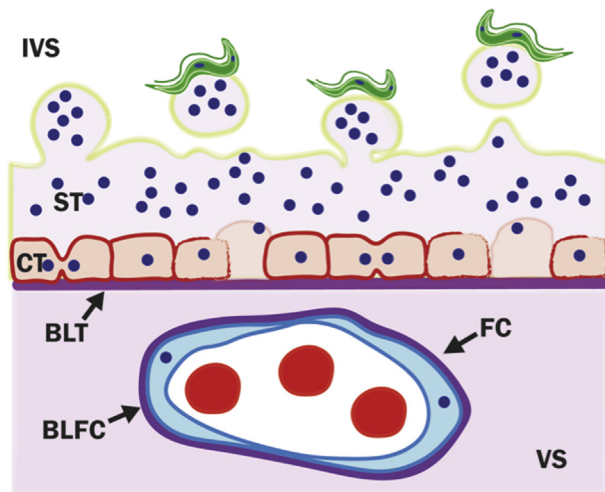


Fig. 6. The trophoblast turnover as a local placental defense mechanism against *T. cruzi*. During congenital transmission, trypomastigotes (in green) circulating in the intervillous space (IVS) must cross the placental barrier. The placental barrier, formed by the two layers of the trophoblast (cytotrophoblast (CT) and syncytiotrophoblast (ST)), the villous stroma (VS), the fetal capillaries (FC) and basal laminae between VS and trophoblast (BLT) as well as around the fetal endothelium (BLFC). In presence of the parasites, CT cell proliferation its fusion into the ST as well as the release of the apoptotic knots into the IVS is increased. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The epithelial turnover is considered to be a protective mechanism against pathogens in other organs, such as the skin and the gastrointestinal and urogenital tracts [14,36]. There is a basal level of epithelial renewal, which can be accelerated or decreased in response to various stimuli. Infection usually accelerates epithelial turnover [36]. For instance, in the intestinal epithelium, the turnover is twice as fast in conventional mice as in germ-free mice [51]. Other parasites such as nematodes have been shown to accelerate intestinal epithelial turnover [14]. In the urothelium, the basal level of tissue renewal is low (approximately 40 weeks in mice); however, during uropathogenic *Escherichia coli* infection, the turnover of these epithelia is rapidly accelerated and completed in 7 days [44].

It seems highly probable that differences in the probability of transmission of different pathogens might be related, at least partially, to the capacity to maintain the epithelial barrier.

5. Conclusion

Considering that *T. cruzi* induces cellular proliferation (Droguett et al. submitted), differentiation [39] and apoptotic cell death [21] in the trophoblast of HPCVE as well as in the trophoblastic cell line BeWo, together with the low transmission rate, we conclude that *T. cruzi* might accelerate the trophoblast turnover (Fig. 6), which could be considered as a local placental defense mechanism.

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