BRIEF COMMUNICATION

High level of soluble HLA-G in amniotic fluid is correlated with congenital transmission of *Toxoplasma gondii*

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Abstract The expression of human leukocyte antigen (HLA)-G on cytotrophoblast cells contributes to maternal–fetal tolerance. Soluble forms of HLA-G (sHLA-G) can be detected in amniotic fluid (AF) and a decrease of sHLA-G is known to be correlated to fetal loss. In this work we investigated the role of sHLA-G in the transplacental passage of the protozoan parasite *Toxoplasma gondii*, responsible for congenital toxoplasmosis in about 30% of fetuses when primary infection (PI) occurs during pregnancy. We determined the sHLA-G concentration in 61 AF from women with PI and 24 controls. Our results showed higher sHLA-G levels in AF from PI than in controls (p < 0.001). Moreover sHLA-G level from congenitally infected fetuses (n= 12) was higher than in fetus in whom congenital infection was ruled out (n=49, p < 0.05). These data suggest that sHLA-G could participate in immunomodulation necessary to avoid fetal loss due to *Toxoplasma* infection, but that over-expression could favor congenital transmission.

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1. Introduction

The expression of human leukocyte antigen (HLA)-G on cytotrophoblast cells contributes to maternal–fetal tolerance by inhibiting lysis by maternal natural killer (NK) cells [1,2] and by mediating suppression of allo-cytotoxic T (CTL) response against the fetus [3,4]. These non-classical major histocompatibility complex (MHC) class Ib genes display seven alternative splicing products, five of which encode truncated extracellular domains [4,5]. The resulting isoforms...
are either membrane-bound (4 isoforms) or expressed in soluble forms (sHLA-G) (3 isoforms) [6–9]. Recent studies reported elevated levels of sHLA-G in the serum during HIV infection [10] but also in other infectious diseases, which could contribute to immunotolerance [11,12].

Toxoplasmosis acquired during pregnancy can affect the fetus in around 30% of cases, depending on the gestational age, mostly resulting in brain or eye damage. The intracellular protozoan parasite Toxoplasma gondii can invade and multiply within trophoblast cells [13], but the mechanisms by which this otherwise effective barrier can fail to protect the fetus and let some pathogens go through, remain to be elucidated. Whereas an efficient immune response against Toxoplasma requires a T helper (Th)-1 cytokine pathway response involving IFN-γ [14–16], the placental microenvironment is rich in IL-10 and is designed to ensure maternal–fetal tolerance through a Th-2 driven immune response. Indeed, human trophoblast cells have been shown to express mRNA of TGF-β, IL-1α, IL-1β, IL-6, IL-10 and TNF-α, but not of IL-2 or IFN-γ [17], which are deleterious to pregnancy. Taken together, placental infection with Toxoplasma could give rise to a detrimental combination of cytokines, which could be even compensated through immunomodulatory mechanisms or lead to fetal loss when infection occurs in early pregnancy. In some instances, these compensatory immunomodulation could favor transplacental passage of parasites. We postulated that HLA-G could participate to this immunomodulation.

In this study we retrospectively determined the concentration values of sHLA-G isoforms using an enzyme-linked immunosorbent assay (ELISA) in the amniotic fluids of three groups of patients: i) control pregnant women without any history of Toxoplasma primo-infection during pregnancy, ii) women with Toxoplasma primo-infection during pregnancy leading to congenital infection, and iii) women with Toxoplasma primo-infection during pregnancy without fetal transmission. In addition, we quantified the expression of cytokines of interest, i.e., IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, in AF by flow immunocytometry.

2. Patients and methods

2.1. Patients and samples

The group of patients with Toxoplasma infection during pregnancy consisted of 58 women followed within the framework of the French prevention program for congenital toxoplasmosis, which relies on hygienic recommendations to avoid contamination and monthly serological screening of Toxoplasma-seronegative pregnant women. French guidelines recommend rapid specific treatment in case of serologic conversion and prenatal diagnosis. Indeed it can be drawn from several studies that the longer the delay to treat, the higher materno-fetal transmission rates and the poorer vision outcome in infants [18–20]. For prenatal diagnosis, amniotic fluids (AF) were collected after 16 weeks of gestation and/or 4 weeks after maternal infection and analyzed for T. gondii detection by PCR and mouse inoculation, as usually [21,22]. The mothers received specific therapy consisting of spiramycin (9 MIU/day); this was replaced by a combination of pyrimethamine and a sulfonamide (sulfadoxine or sulfadiazine) when prenatal diagnosis was positive. After birth, placentas were analyzed for T. gondii detection [23] and infants underwent a one-year clinical and serological follow-up, so that the final diagnosis of congenital toxoplasmosis could be made definitively. Congenital infection was stated on the basis of the following criteria: (i) persistence of specific IgG at the age of at least 9 months; and/or (ii) positive prenatal diagnosis and/or presence of specific IgM in neonatal serum and/or neosynthesison of specific antibodies assessed by comparative immunoblotting against the mother’s serum. Absence of congenital infection was established by the disappearance of maternal IgG in two consecutive blood samples in the absence of any treatment. Congenital infection was diagnosed in 12 neonates and ruled out in 46 cases.

The control group consisted of 24 women without Toxoplasma infection during the course of pregnancy, who were proposed AF puncture for karyotyping indication. In 23/24 cases, karyotype result was normal. All women provided written informed consent before AF collection, as required by French legislation and by the Ethical Committee of the University Hospital of Rennes. AF sample was divided into several aliquots and stored at −80 °C before analysis. Patients’ characteristics are described in Table 1.

2.2. Determination of specific soluble HLA-G by ELISA

sHLA-G concentration was evaluated by a specific sandwich ELISA using MEM-G/9 and HRP-anti2-microglobulin as

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Trimester of Toxoplasma maternal infection (n/N)</th>
<th>Term of pregnancy at AF sampling (WA) (mean ± SEM)</th>
<th>Echographic signs due to Toxoplasma infection (n/N)</th>
<th>In utero treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected fetuses (n=12)</td>
<td>2/12</td>
<td>30.1 ± 1.6</td>
<td>ICC 2/12</td>
<td>Pyrimethamine + sulfonamide</td>
</tr>
<tr>
<td>Non infected fetuses (n=49)</td>
<td>37/49</td>
<td>21.5 ± 0.6</td>
<td>No</td>
<td>Spiramycin</td>
</tr>
<tr>
<td>Control group (n=24)</td>
<td>na</td>
<td>18.04 ± 2.11</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

AF; amniotic fluid; WA; week of amenorrhea; ICC; intracranial calcifications; na; not applicable.

Table 1 Characteristics of the patients’ groups.
capture and detection antibodies respectively, as previously described [24]. Briefly, microtitration plates (Corning costar, Issy-les-Moulineaux, France) were coated overnight at 4 °C with MEM-G/9 (10 μg/mL). Plates were saturated with phosphate-buffered saline (PBS), 2% bovine serum albumin for 30 min. All AF samples were tested in triplicate and incubated for 1 h. After a 3-fold repeated washing step, plates were incubated for 1 h more with detection antibody (HRP-anti β2-microglobulin), then for 30 min in the dark with the substrate (ortho-phenylenediaminedihydrochloride; DAKO). Between each step, plates were washed 3 times with PBS containing 0.05% Tween 20. All incubation steps were performed at room temperature. After addition of H2SO4 (1 N), optical densities were measured at 490 nm. This ELISA detected both HLA-G5 molecules and sHLA-G1 molecules (HLA-G1 shedding form). Total sHLA-G levels were determined from a five-point standard curve using dilutions of calibrated HLA-G5 as standard reagent, and results were expressed as ng/mL. This specific ELISA was validated by the Wet Workshop for Quantification of sHLA-G [25].

2.3. Cytokine dosages

A panel of cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ) was quantified in AF from infected and non-infected fetuses by flow immunocytometry using Cytometric Beads Array (BD Biosciences, Mountain View, CA) on a FC500 cytometer (Beckman Coulter, France). Results were expressed in pg/mL.

2.4. Statistical analysis

Data are expressed as mean±SEM for each group of patient. Differences between groups were analyzed using non-parametric tests (Mann-Whitney test). Correlations between variables were evaluated using the Spearman rank correlation test. Statistical analysis was performed using GraphPad Prism 5.02 software. Differences were considered significant when the p-value was <0.05, and graduated as * (p<0.05), ** (p<0.01), and *** (p<0.001).

3. Results

sHLA-G concentration in AF was markedly different in the 3 groups of patients (Fig. 1A). In AF from Toxoplasma-infected fetuses, sHLA-G level was significantly higher (93.9±12.8 ng/mL) than in AF from non-infected fetuses (63.3±4.3 ng/mL, p<0.05) and AF from the control group (43.6±2.4 ng/mL, p<0.001). In addition, sHLA-G was also detected at higher concentrations in AF from non-infected fetuses compared to the control group (p<0.01). No differences were observed between male and female fetuses, whether infected or not. As sHLA-G concentration is known to decrease along gestation, we determined the term of pregnancy when AF sampling was performed. It appeared that the mean time period for AF sampling was 30.1±1.6 and 21.5±0.6 weeks of amenorrhea in the groups of infected and non-infected fetuses, respectively, ruling out the hypothesis that earlier sampling during gestation would be responsible for the higher sHLA-G levels observed in infected fetuses. The sHLA-G concentrations were also analyzed according to the trimester of maternal seroconversion (Fig. 1B). A significant difference was observed for infections that occurred during the second trimester of pregnancy, showing lower levels in the group of non-infected fetuses (p<0.01). No statistical analysis could be drawn for the first and third trimesters, because of too small sample data.

Cytokine dosage could be performed in 40 AF samples. IL-6 was detected in 34/40 amniotic fluids (mean 410.2±4.4 pg/mL), whereas IFN-γ was rarely detected (12/40 samples) and at very low levels (mean 18.6±10.9 pg/mL), regardless the fetus was infected or not (Fig. 2). IL-6 was detected in 8 of 8 (100%) infected fetuses and in 21 of 32 (66%) non infected fetuses tested. IL-2, IL-4 and IL-10 were undetectable and TNF-α was detected in only 3/40 AF. There was no difference in any cytokine response regarding the sex of the fetus, despite a trend in favor of higher levels of IL-6 in...
male compared to female fetuses (532±150 versus 288±57 pg/mL, p=0.26). However, when taking into account a mean concentration rate of IL-6>2.6 ng/mL defined by others as a significant threshold detection to diagnose intra-amniotic inflammation [26], it can be considered that only one patient had an IL-6 titer above this threshold (3.16 ng/mL), corresponding to a non-infected fetus.

4. Discussion

Toxoplasmosis is a worldwide infection, though its prevalence may differ among countries. In France, where the prevalence was historically high, a national prevention program was instituted in the late 1970s to inform, prevent and screen infection in pregnant women. Prenatal diagnosis is routinely performed since 1992 and pregnancies and offspring are monitored carefully. It has been drawn from retrospective studies and meta-analyses in the literature that early maternal anti-parasitic treatment can reduce the fetal transmission rate, but the micro-environmental factors determining the actual transplacental passage of parasites are largely unknown. Since *T. gondii* tachyzoites are able to infect placental trophoblastic cells [13], the parasites are located directly at the interface between maternal and fetal compartments. Therefore, cytotrophoblasts play a key role in the maternal–fetal transmission of the parasites, depending on how their microenvironment promotes immunotolerance or anti-inflammatory inflammatory response. In the case of primary-acquired toxoplasmosis, it has been extensively demonstrated that a Th1-type immune response mostly based on CD8+ T cells and IFN-γ production is determinant in the control of *Toxoplasma* infection [16,27]. However, excessive IFN-γ synthesis and inflammatory response may result in fetal death and abortion, a well-known manifestation of *Toxoplasma* infection in early pregnancy, which was reported to be dependent on IFN-γ in mice [28]. The regulation of the balance between immunologic anti-parasitic defense and maternal–fetal tolerance is therefore critical for the outcome of pregnancy itself. Such an undesired effect of inflammatory anti-infectious response is also described in other models of infection, such as listeriosis [17]. Along with T cells, the role of NK cells in the control of maternal–fetal transmission of *T. gondii* was clearly shown in a model of Rag-2⁻/⁻ mice [29]. But in this case also, there is a need for a sharp regulation between NK-mediated lysis of infected cells and protection of fetal cells to NK lysis.

Protection of the fetus against the maternal immune system relies on HLA-G expression by cytotrophoblast cells, warranting a pregnancy-protective Th2-type immune response [3], but alternatively could favor fetal infection. It has been stated that sHLA-G molecules present in the amniotic cavity could be secreted by cytotrophoblast cells [30], as well as by amnion epithelial cells [31]. In this work, we analyzed the level of sHLA-G released in amniotic fluid and showed that sHLA-G levels were significantly higher in AF from pregnancies exposed to *Toxoplasma* primo-infection than in control AF (p<0.001), and are in agreement with the hypothesis that HLA-G may contribute to downregulate the inflammatory response due to infection in order to maintain gestation. Other authors showed that there was a down-regulation of HLA-G expression by cytotrophoblast cells induced by human cytomegalovirus gene products, a mechanism that could account for spontaneous pregnancy loss following HCMV infection [32]. In our series, all fetuses were born alive, consistent with an adequate down-modulation of inflammatory response. However, sHLA-G was detected at higher concentration in case of congenital infection, than when fetuses were not infected (p<0.05), suggesting that excessive immunotolerance induced by HLA-G could be associated with transplacental transmission. Indeed, it has been shown in a mouse model of congenital toxoplasmosis, that a Th-2 response, in particular through IL-4 synthesis, is associated with an increase of the transplacental passage of *T. gondii* [33].

Contrasting with the data of Emmer et al. [34] but in agreement with Kusanovic et al. [35], we did not detect any relation between the level of sHLA-G and the sex of the offspring.

Our investigation of associated immunological markers through cytokine dosages in AF did not allow us to further explain the progression of parasites into the fetal compartment, since we did not observe significant differences in terms of cytokine pattern response between infected and non-infected newborns. Most cytokines were barely detected. IL-6 was the most frequently detected one and was present in 8/8 AF from infected fetuses, but the significance of this observation remains unclear. It is well-known that pro-inflammatory cytokines are deleterious for the fetus [36], being often associated with preeclampsia and decrease of HLA-G levels [37,38]. They are therefore usually absent or detected at very low levels in placenta [17] and amniotic fluid [39], though to our knowledge, little data report their detection threshold.

In conclusion, despite a small sample size, we provide here original data showing elevated levels of sHLA-G in amniotic fluid from women with acquired toxoplasmosis during pregnancy, with maximal concentrations when the fetus was congenitally infected. We postulate that HLA-G could play a role in the immunomodulation necessary to avoid fetal loss, but potentially leading to maternal–fetal transmission of *T. gondii* in case of over-expression, a hypothesis that could provide new insights in the pathophysiology of congenital toxoplasmosis. Of course, these results must be considered as a hypothesis generating study and

![Figure 2](image-url)
need to be confirmed on a larger series before drawing definitive conclusions.

Acknowledgment
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References


are related to the sex of the offspring, Eur J Immunogenet 30 (2003) 163–164.


