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Toxoplasmosis and abortion: pro- and anti-inflammatory cytokines gene expression of the host immune cells

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Abstract

Background: Toxoplasmosis during pregnancy is known for inducing variable serious outcomes. In many previous studies, pregnancy was evaluated as a single event while in reality; it has different distinct immunological stages depending on gestational time and possible external factors as infectious agents. A state of immunological balance as well as a state of maternal-fetal tolerance should be established; otherwise, atypical unwelcomed outcomes might occur as a result of such infection

Aim: This work aimed to study the gene expression profile of some pro- and anti-inflammatory cytokines in *Toxoplasma* seropositive cases

Results: All studied cytokines were significantly upregulated in group I, with a remarkable bias towards IFN-γ, denoting a predominant inflammatory state in group I. In group II; tested cytokines were approximately in a balanced state with a little shift towards anti-inflammatory cytokines (IL-10 and TGF-β). A significant positive correlation was detected between pro-inflammatory together ($p = 0.002$, $r = 0.96$) and between anti-inflammatory cytokines together ($p = 0.0008$, $r = 0.99$) in group II, indicating the harmonized and synchronized expression of both pro- and anti-inflammatory cytokines. In group III, IL-10 was relatively higher with negative correlation with IFN-γ ($p = 0.006$, $r = 0.93$), indicating the possible role of IL10 in saving some of the pregnancies' outcomes in group III.

Conclusion: The present study suggests a potential role of these cytokines in the upcoming prognostic or/and therapeutic concerns of toxoplasmosis among pregnant women.

Keywords: *Toxoplasma*, q-PCR, IFN-γ, TNF-α, IL10, TGF-β

Background

Toxoplasma gondii (*T. gondii*) is an intracellular parasite with very low host specificity as it probably infects almost all mammals and birds as well. It is endemic worldwide; however, according to the geographic location, 15 to 85% of the human populations are asymptomatically infected. Infection during pregnancy may lead to dangerous outcomes [1]. Abortions may occur in addition to stillbirths, intrauterine growth retardation, preterm deliveries, or fetal anomalies [2].

Although spontaneous abortion due to congenital toxoplasmosis is previously documented, yet it is controversial, to what extent toxoplasmosis is a risk factor and can cause repeated abortion [3, 4]. The previous studies relied on serological data to achieve their objectives, which was not convincing enough as an evidence to understand the relatively complex relation between the parasite and the host immune system [5]. The mechanisms by which *Toxoplasma* interacts with the host's immune system are still questionable and need further investigations to illuminate such important research area [6].

Host control against *T. gondii* infection requires the induction of strong parasite-specific immune response in the host [7]. Following *Toxoplasma* infection, neutrophils rapidly migrate to the site of infection, then

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activate dendritic cells (DC) for induction of T-helper 1 (Thl) inflammatory response [8–10]. The infected host generates a strong cytokine-dependent parasite-specific interferon-γ (IFN-γ) response [11, 12]. Elevated level of IFN-γ (Th1 cytokine) inhibits *Toxoplasma* replication and promotes its transformation from tachyzoite to bradyzoite stage [7, 13]. T-helper 2 (Th2) immune response provokes a significant increase in both *T. gondii* intracellular replication and invasion [14].

Pregnancy was described as a "Th2" or anti-inflammatory status, and any shift in the type of cytokines produced would lead to pregnancy complications [15, 16]. In previous studies, pregnancy was evaluated as a single event while in reality; it has different distinct immunological stages depending on gestational time and possible external factors as infectious agents [17, 18]. Therefore, a state of immunological balance as well as a state of maternal-fetal tolerance should be established; otherwise, atypical unwelcomed outcome might occur [19].

With the application of advanced analytical molecular technologies, more exploration of *Toxoplasma* growth, differentiation, and host-parasite interaction could be achieved [5]. A variety of methods have been used for gene expression quantification, including northern blotting, serial analysis of gene expression (SAGE), DNA microarrays, and quantitative reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The introduction of real-time quantitative PCR techniques is proven useful in various applications, including pathogen detection, gene expression and regulation, and allelic discrimination [20]. The aim of this work was to identify the modulated proinflammatory versus anti-inflammatory cytokine gene expression in the intended study group.

Subjects and methods
Type of study: a case-control study
The present study included a total of 61 cases, who were seropositive for toxoplasmosis. Cases were further subdivided according to variable pregnancy outcome into three groups: group I—19 cases complaining of repeated abortions (at least 3 times) with no history of successful pregnancies, group II—28 cases that were multiparous with no history of abortion, and group III—14 cases that had successful pregnancies' outcomes, but also suffered a history of some abortions. To exclude other causes of repeated abortion, further exclusion criteria were set in this study, as women with known thrombophilia, systemic lupus, antiphospholipid syndrome, or any other autoimmune diseases causing recurrent abortions, women with known uterine congenital anomalies, or multi-fetal pregnancies. Immunocompromised women, or those receiving immunosuppressive medications, and any maternal/fetal bacterial or viral infections that cause abortion were also excluded from the study.

Cases included in this study were selected from the outpatient's clinic of Obstetrics and Gynecology Department in Kasr Alainy Hospital, which is governmental, educational, and free-of-charge hospital, during the period from January 2014 to February 2015. All women included were of low educational and socioeconomic levels, without any significant demographic variations. Their age range was between 27 and 36 years old, and all were housewives.

In our study, the gene expression of 4 cytokines was investigated in selected study groups' women, who were seropositive for *T. gondii*, by measuring pro and anti-inflammatory cytokine mRNAs. These cytokines were interferon gamma (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin 10 (IL-10), and transforming growth factor–β (TGF-β). Blood samples were collected from all cases and tested serologically for anti-*Toxoplasma* IgG/IgM by OnSite Toxo rapid qualitative immune-chromatography test (ICT) (CTK, USA). Anti-*Toxoplasma* IgG antibodies were detected by ELISA, using Nova Tox IgG (Atlas link, Beijing). The cutoff (optical density (OD)) value was calculated against threshold 0.09 and samples giving higher OD were considered positive. The mononuclear layer was separated from each blood sample by Lymphoprep (Axis-Shield PoC, Norway) density gradient centrifugation, which is a simple and rapid method of purifying peripheral blood mononuclear cells (PBMC).

RNA extraction and reverse transcription PCR (RT-PCR)
According to the manufacturer's instructions, RNA extraction was done using Commercial kits (The SV Total RNA Isolation System) Promega Corpora   on 2800 Woods Hollow Road, Madison, WI 53711-5399 USA. Reverse transcription PCR was performed using HYBAID OmniGene 3 Block Thermal Cycler (Mid-Atlantic Lab.equip., UK). The recommended cDNA synthesis reaction mixture consisted of the following: M-MLV Reverse Transcriptase RNase H-200 U/μl (Solis BioDyne) (1 μl, 4 U/μl), 20mMdNTP mix (1.25 μl, 500 μM), 100 mM DTT (2.5 μl, 5 mM), and random hexamer μg of RNA (2.5 μl, 500 ng); RNase inhibitor (Solis BioDyne) (1 μl, 25 U), for 5× RT reaction buffer 1 (10 μl, 1×), with MgCl2 and DTT) 0.25 M Tris-HCl, 0.5 M KCl, 30 mM MgCl2, and 25 mM DTT; and RNA (5 μl, 2.5 μg) and DEPC treated H_2O to a 50-μl final reaction volume.

Microfluidic LabChip RT- real-time PCR
The procedure was applied on UltraFast LabChip Real-time PCR G2-3 System (NanoBioSys Inc., Korea). Real-time PCR reaction was prepared using SYBR Green I Real-Time PCR Master Mix Kit (NanoBioSys Inc., Korea) (Tables 1 and 2).

Table 1 Real-Time PCR Master Mix

Item	Volume
2× PCR Master Mix (polymerase, dNTPs)	8 μl
Sample (cDNA)	1.6 μl
Primers (forward/reverse) 1.0 μM (Table 2)	1.6 μl/1.6 μl
Distilled water	3.2 μl

For every patient, two PCR reaction mixtures were prepared, for the assay of cytokine mRNA and β-actin mRNA expression levels in RNA isolated from the samples collected from all cases. A 15-μL reaction mixture containing 10% (v/v) of synthesized cDNA and 1 μM of each primer were loaded into each channel of LabChip. LabChip filled with reaction mixture was placed onto the LabChip case and further injected into the ultra-fast LabChip real-time PCR G2–3 system. Real-time PCR was performed according to software protocol (pre denaturation at 95ÆC for 8 s then 30 cycles at 95ÆC for 8 s and 72ÆC for 14 s). As real-time PCR proceeded, real-time data were plotted on the screen and then sigmoidal curves for amplified DNAs appeared. After curve fitting, Ct (cycle threshold) values and reaction time of real-time PCR (15 min for 30 cycles) appeared on the screen. Each reaction included the no template control (NTC) to determine whether it was positive or negative. A sample with a Ct value less than 31 was considered positive, in cases where the Ct value of the NTC was not assigned; β-actin was treated as the internal control and results were expressed as cytokine/β-actin ratio. Target concentration was expressed in relation to the concentration of the housekeeping gene. For relative quantization, the values obtained were compared to those from the standard RNA dilutions which were amplified by the RT-PCR in parallel: relative cytokine expression = copy no of cytokines/copy no of β-actin = concentration of cytokines/concentration of β-actin [21].

The research work in this study was done in the Departments of Medical Parasitology and Clinical Pathology in the Faculty of Medicine, Cairo University, and Department of Molecular Biology, Faculty of Medicine, Ain Shams University.

Table 2 Sequence of primers used for quantitative real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GTGGGGCGCCCCAGGCACCAG	TCCTTAATGTCACGCACGATTTC
TNF-α	ACCATGAGCACTGAAAGCAT	AGATGAGGTACAGGCCCTCT
IFN-γ	TTGGGTTCTCTTGGCTGTTA	AAATATTGCAGGCAGGACAA
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
IL-10	GGTTGCCAAGCCTTGTCTGA	AGGGAGTTCACATGCGCCT

Ethical considerations

Each patient individually signed an informed written consent, before being included in the present study. The current study was carried out in accordance with The Code of Ethics of The World Medical Association of Helsinki for experiments in Humans.

Statistical analysis

The data collected in this study were evaluated using the PRISM® 5.01(GraphPad Software Inc., San Diego, CA, USA.) Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparisons between two groups were made using the Student's t test. For the analysis of numerical data when samples failed to show a normal distribution, the Mann-Whitney U test was used for comparison between two groups, while the Kruskal-Wallis test was used for comparing 3 groups together. The Pearson chi-square test was used for categorical data. The relation between variables was examined with correlation analysis using the Spearman rank correlation equation. p values of < 0.05 were considered as statistically significant.

Results

The present work comprised a total of 61 women, whose sera were serologically positive either for IgG alone or for both IgG and IgM *Toxoplasma* antibodies at least by 2 testing techniques. However, none of these seropositively reactive women had apparent constitutional symptoms of toxoplasmosis. The detection of combined seropositivity IgG + IgM against *Toxoplasma* was significantly higher in group I ($p = 0.001$) (74% (14/19)) compared to group II and group III (21% (6/28) and 43% (6/14), respectively) as shown in Table 3 and Fig. 1.

Concerning pro- and anti-inflammatory cytokine gene expression, seven representative samples were selected from each of the three study groups, taking into consideration to include the IgM-positive samples; this comprised a total of 21 samples, with 2 samples of them (first in group I, second in group II) being only IgG anti-*Toxoplasma* serologically positive, but these both samples were positive to *Toxoplasma* B1 gene by qPCR (unpublished data). The gene expression values of different cytokines in the three groups were analyzed individually in relation to other cytokines tested and in relation to different groups as demonstrated in Table 4. The detected IFN-γ expression in groups I, II, and III showed the highest level (mean = 0.294 ± 0.108) in group I. The detected differences in IFN-γ expression levels in the three study groups collectively were statistically significant ($p = 0.0001$) as shown in Fig. 2a and Table 4.

Table 3 Results of serological assays for detection of anti-*Toxoplasma* antibodies among cases in the study groups

Serum anti-*Toxoplasma* antibodies	Group I (abortion)	Group II (no abortion)	Group III (successful outcome and abortion)
IgG + IgM (%)	14 (74%)	6 (21%)	6 (43%)
IgG (%)	5 (26%)	22 (79%)	8 (57%)
Total (100%)	19 (100%)	28 (100%)	14 (100%)
Chi-square test (*p* value)	0.001	0.001	0.00594

Concerning TNF-α, it was the highest expressed (mean = 0.217 ± 0.053) in group I when compared to groups II and III (mean = 0.060 ± 0.020 and mean = 0.094 ± 0.018, respectively). Significant differences were obtained between TNF-α expression levels in group I and group II (*p* = 0.0006) and between group II and group III (*p* = 0.007). The difference detected among the three study groups was remarkably statistically significant (*p* = 0.0003) as shown in Fig. 2b and Table 4.

Regarding the anti-inflammatory cytokine gene expression analysis, IL-10 expression tended to be balanced in the three groups. Differences in IL-10 expression between each two groups or between the three groups were statistically non-significant (*p* = 0.16) (Table 4 and Fig. 2c). Similarly, TGF-β gene expression was higher in group I (mean = 0.124 ± 0.35), when compared to groups II and III (mean = 0.075 ± 0.034 and mean = 0.092 ± 0.031, respectively) (Table 4 and Fig. 2d). The difference in TGF-β gene expression was statistically significant only between group I and group II (*p* = 0.03) (Fig. 2d)

but was non-significant among the three study groups (*p* = 0.089) as shown in Table 4 and Fig. 2d.

IFN-γ showed the highest level of expression together with TNF-α level, that both (IFN-γ and TNF-α) were found upregulated than the other two anti-inflammatory cytokines (IL-10 and TGF-β), denoting a predominant inflammatory state in the group I of women, who suffered from repeated abortion without any successful gestational outcome as shown in Fig. 3a. In group1, the differences between both pro-inflammatory IFN-γ and TNF-α and both anti-inflammatory IL-10 and TGF-β cytokine gene expression levels were significant with detailed *p* values shown in Fig. 3a.

In Fig. 3b, the four cytokines were approximately in a balanced state in group II, with a little shift towards the 2 anti-inflammatory cytokines (IL-10 and TGF-β). This group of multiparous women with no history of abortions in spite of anti-*Toxoplasma* seropositivity did not show an observable elevation of any particular cytokine gene expression, as shown in Fig. 3b.
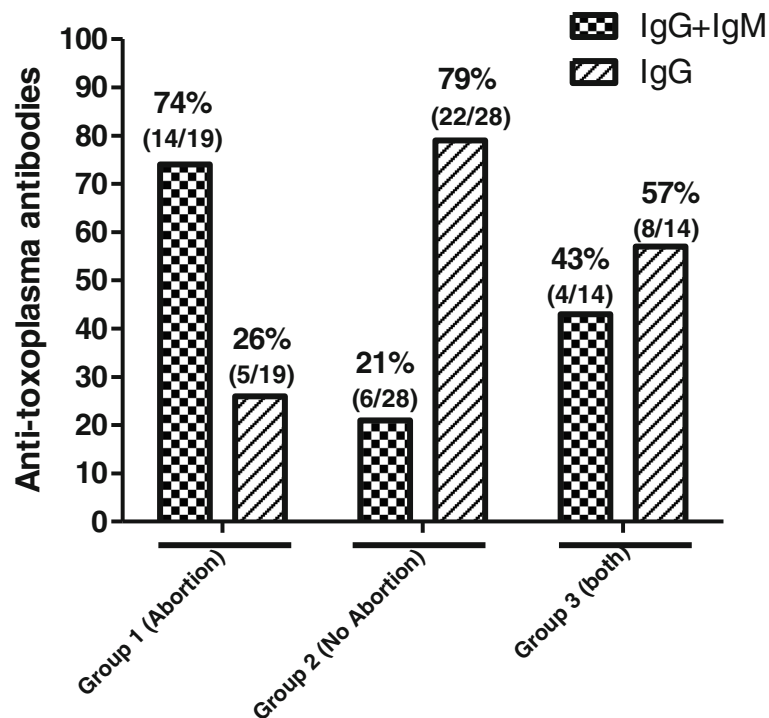

Fig. 1 Anti-*Toxoplasma* antibodies in different study groups

Table 4 Mean values for gene expression for different cytokines in different groups. Calculated p value comparing the 3 study groups using the Kruskal-Wallis test, with a p value <0.05 considered significant
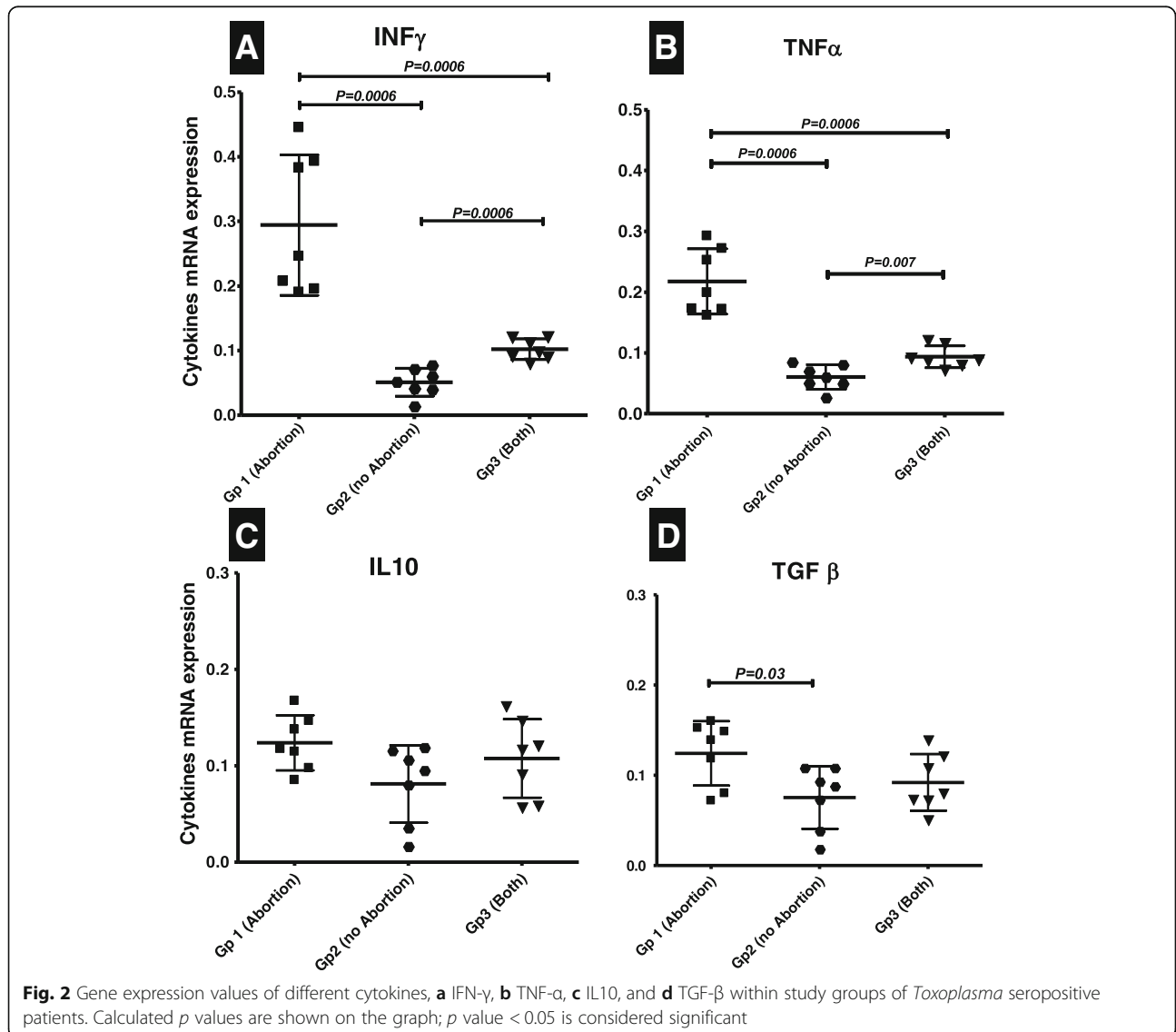
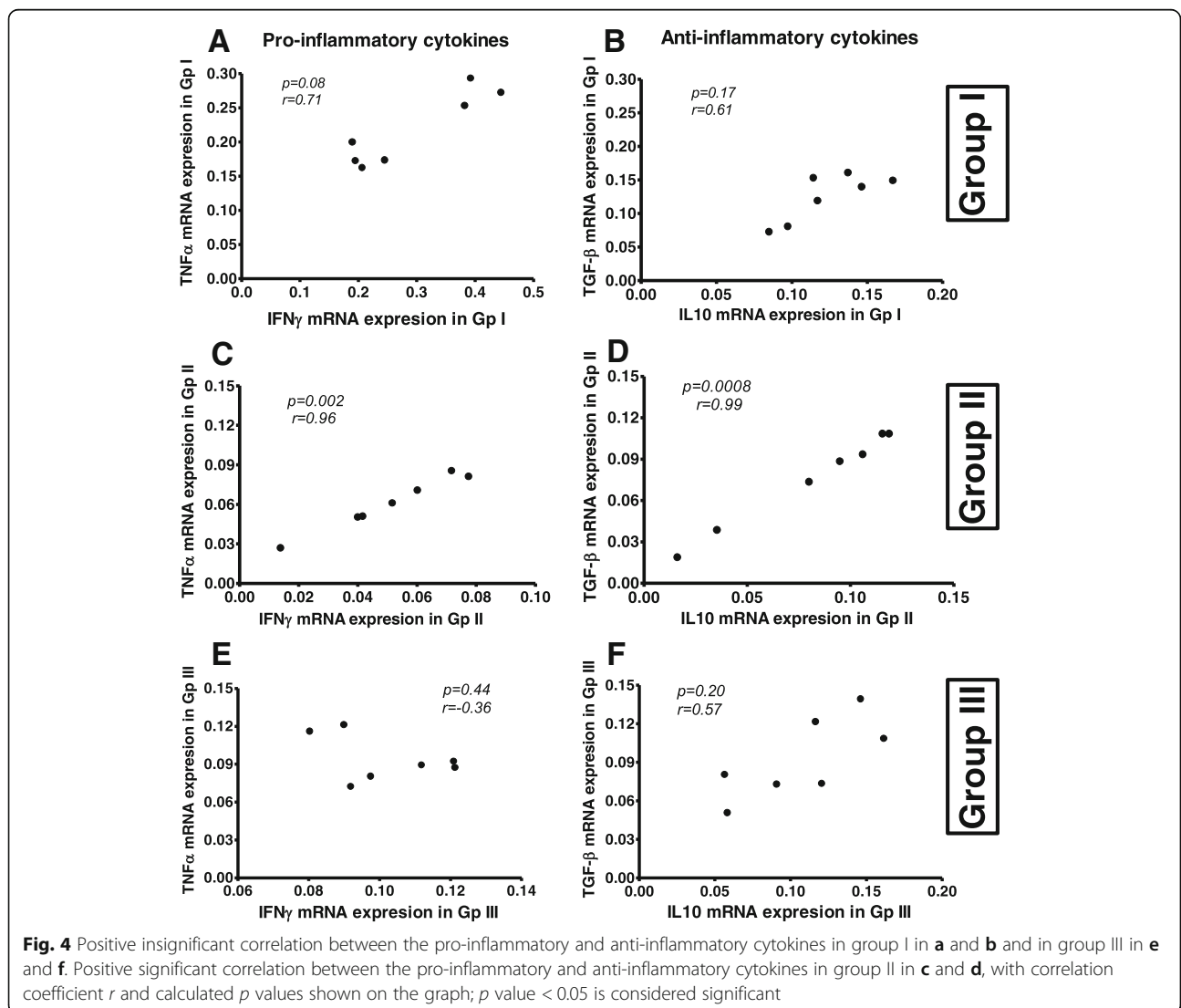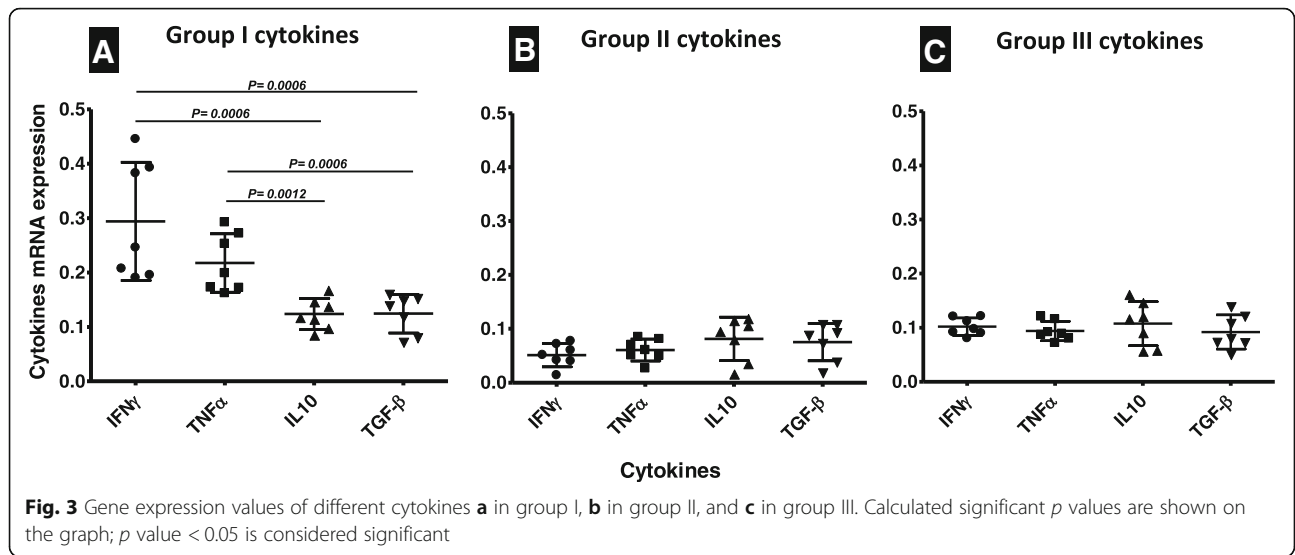|	Group I (abortion)	Group II (no abortion)	Group III (successful outcome and abortion)	Kruskal-Wallis test (p value)
IFNγ (Mean ± St.Dev.)	0.294 ± 0.108	0.051 ± 0.021	0.102 ± 0.016	0.0001
TNFα (Mean ± St.Dev.)	0.217 ± 0.053	0.060 ± 0.020	0.094 ± 0.018	0.0003
IL10 (Mean ± St.Dev.)	0.124 ± 0.028	0.081 ± 0.040	0.107 ± 0.040	0.1632
TGF-β (Mean ± St.Dev.)	0.124 ± 0.035	0.075 ± 0.034	0.092 ± 0.031	0.0894

In group III, the gene expression level of IL-10 was the highest (mean 0.107 ± 0.040) compared to other cytokines in the same group, however, with no statistical significance ($p = 0.16$) as shown in Table 4 and Fig. 3c.
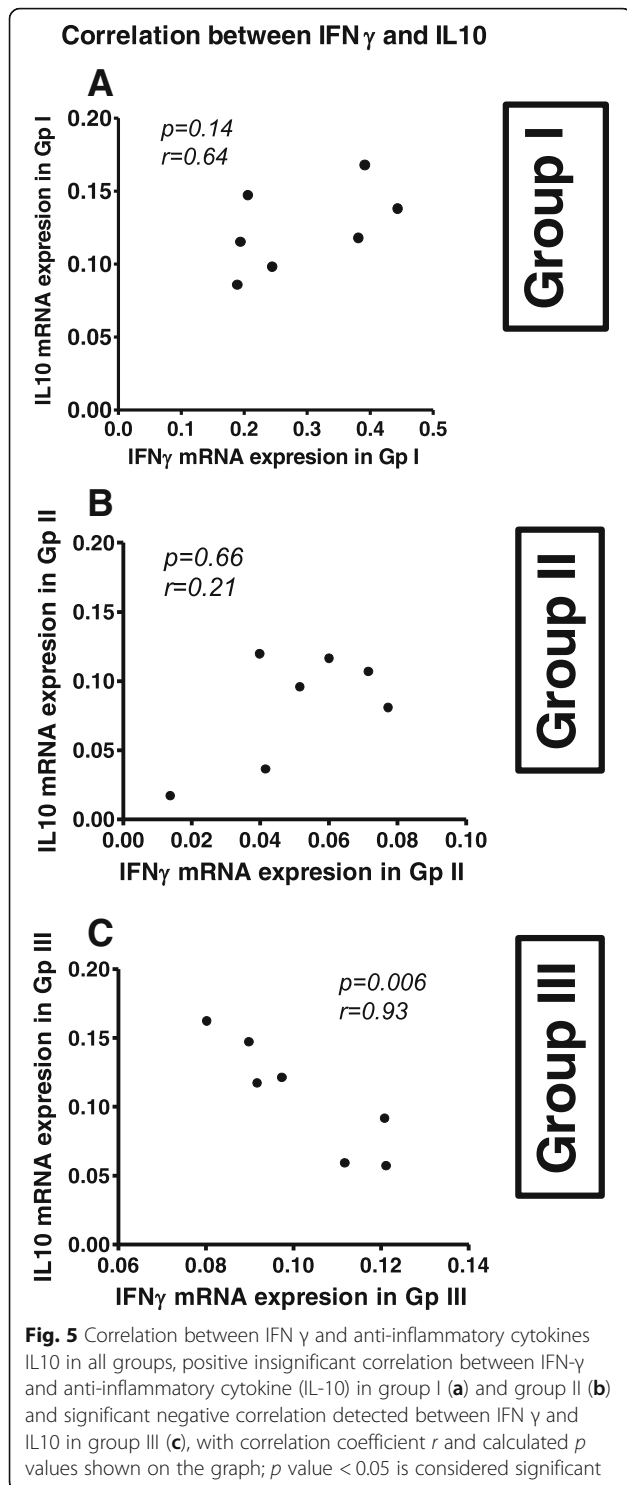
The analysis of associations between different cytokines in the three groups was illustrated in Figs. 4 and 5. In groups I and III, there was a positive correlation tendency yet statistically insignificant

between both pro-inflammatory cytokines IFN-γ and TNF-α (Fig. 3a, e), as well as between both anti-inflammatory cytokines IL-10 and TGF-β (Fig. 3b, f). Such correlations were significantly positive in group II ($p = 0.002$ and $p = 0.0008$, respectively) as shown in Fig. 3c and d.

Furthermore, a positive insignificant correlation between pro-inflammatory cytokine IFN-γ and anti-inflammatory

Fig. 2 Gene expression values of different cytokines, **a** IFN-γ, **b** TNF-α, **c** IL10, and **d** TGF-β within study groups of *Toxoplasma* seropositive patients. Calculated p values are shown on the graph; p value < 0.05 is considered significant

Fig. 3 Gene expression values of different cytokines **a** in group I, **b** in group II, and **c** in group III. Calculated significant *p* values are shown on the graph; *p* value < 0.05 is considered significant

Fig. 4 Positive insignificant correlation between the pro-inflammatory and anti-inflammatory cytokines in group I in **a** and **b** and in group III in **e** and **f**. Positive significant correlation between the pro-inflammatory and anti-inflammatory cytokines in group II in **c** and **d**, with correlation coefficient *r* and calculated *p* values shown on the graph; *p* value < 0.05 is considered significant

Fig. 5 Correlation between IFN γ and anti-inflammatory cytokines IL10 in all groups, positive insignificant correlation between IFN-γ and anti-inflammatory cytokine (IL-10) in group I (**a**) and group II (**b**) and significant negative correlation detected between IFN γ and IL10 in group III (**c**), with correlation coefficient *r* and calculated *p* values shown on the graph; *p* value < 0.05 is considered significant

cytokine IL-10 in groups I and II was detected as shown in Fig. 5a–d. However, in group III, a strongly significant negative correlation was detected ($p = 0.006$, $r = -0.93$) between IFN γ and IL10 as shown in Fig. 5c. On the other hand, no association between IFN-γ and TGF-β was detected in all groups.

Discussion

Since the pathogenicity of the abortive consequence of pregnancy is explained through the net result of immunological state, which is mediated through cytokine release, our study was concerned with cytokine expression profile in *Toxoplasma*-seropositive cases with variant pregnancy outcomes.

Anti- *Toxoplasma* IgM appears 14 days after infection and disappears several months later, with the possibility of its early disappearance, or persistence over time, even for as long 3–4 years [22]. However, still, it is considered that the presence of a *Toxoplasma*-specific IgM antibody combined with high IgG titer indicates acute or reactivation of infection within the previous 3 months [23, 24].

Th1 cells are involved in cell-mediated inflammation and produce pro-inflammatory cytokines, which inhibit trophoblast growth and differentiation and produce cytokines as IFN-γ resulting in recurrent abortion [25, 26]. Additionally, Th1-type cytokines induce apoptosis that could jeopardize the trophoblast barriers separating the semi-allogenic fetus from the mother's immune system, leading to fetal rejection or abortion. These cytokines initiate the development of natural killer cells, lymphokine-activated killer cells, and CTL cells that cause fetal death, as they are capable of killing trophoblasts [27].

The immunological paradox of pregnancy is still not fully comprehended. It was proposed that a healthy pregnancy is accompanied by a decreased Th1/Th2 balance; nevertheless, other studies argued against this concept [28]. Pregnancy as a physiological condition is characterized by fluctuation between being pro-inflammatory and anti-inflammatory response depending upon the stage of gestation [29, 30]. Any dysfunction of this well-tailored immunological status leads to the production of pro-inflammatory cytokines such as IFN-γ and TNF-α and may be extremely harmful to pregnancy [31–33].

Increased production of some inflammatory cytokines during pregnancy could play an essential role in the development of severe forms of the *Toxoplasma* infection including abortion, which may be attributed to different *Toxoplasma* strains [34]. The ability of different forms of *T. gondii* to induce a Th1 response may be sufficient to induce abortion during the first trimester, when pregnancy-induced Th2 bias is low. In contrast, in the third trimester; pregnancy has induced a strong Th2 polarization that may counteract the *T. gondii*-induced Th1 response and prevent stillbirth or preterm labor. As a consequence, parasite survival may be favored in the third trimester and account for the increased rates of transmission [35].

In our study, cases in group I reported a high level of IgM and IFN-γ, in agreement with Abo Bacar et al. [36] who stated that IFN-γ is increased during acute *Toxoplasma* infection. Blader and Saeij [6] reported that IFN-γ

is produced in response to parasite stimulation of IL-12 expression, which is the critical cytokine for resistance to both acute and chronic *Toxoplasma* infections.

Our study revealed that women of group I showed a statistically significant increase in the expression of TNF-α, compared to groups II and III. The role of TNF-α in causing abortion could be due to its action as a pro-inflammatory cytokine that causes uterine contraction and necrosis of implanted embryo by thrombosing its blood supply leading to fetal expulsion [37, 38]. IFN-γ and TNF-α cause pregnancy failure by two independent but superimposed mechanisms: systemic inhibition of ovarian function and local induction of inflammatory process within implantation sites [39, 40].

Inversely; cases in group II did not suffer any abortions, in spite of being *Toxoplasma* seropositive; this could be explained by a well-adjusted status between two opposing immunological fronts, which was demonstrated in the current study, through convincing association between pro-inflammatory cytokines IFN-γ and TNF-α combined expression. On the other frontage, both anti-inflammatory IL-10 and TGF-β cytokine expression were strongly associated. This balanced and harmonized state seemed to be essential to achieve successful pregnancy outcome in group II, rather than higher levels of these cytokine expressions. Initiation of the Th2 pathway and production of anti-inflammatory cytokines promote embryonic and placental development and deficiency of these anti-inflammatory cytokines leads to poor placental development, subnormal growth, and even fetal death. Moreover; these anti-inflammatory cytokines antagonize the harmful action of Th1-dependent cytokines [16, 37, 41]. Cases included in group II showed no abortions; however, they did not record the highest expression levels of anti-inflammatory cytokines among our study groups.

In agreement with our conclusion, other studies reported that the balance between IFN-γ and other pro-and anti-inflammatory cytokines and the stage of gestation at which IFN-γ is produced are fundamental in determining whether IFN-γ plays a physiological or pathological role [42, 43].

In accordance with our results, Taher and Abdullah [40] reported an increase of IL-10 mean percentage in the placenta of aborted women infected with *T. gondii* compared to that produced by the placenta of aborted women uninfected with *T. gondii* and induced aborted women. The relatively high level of the anti-inflammatory cytokine (IL-10) accompanied by significant downregulation of pro-inflammatory cytokine (IFN-γ) expression, shown in group III, indicated the possible regulatory role of cytokine (IL-10) in saving some pregnancies' outcomes in this group. In this context, Gaddi and Yap [44] reported that modification of the immune response by TGF-β and IL-10 is crucial to the outcome of *Toxoplasma gondii* infection;

however; it looked like that the significantly high level of TGF-β expression in group I compared to group II was not sufficient to modulate or to control the significantly higher levels of the synergistic action of both IFN-γ and TNF-α.

Concerning the impact of IFN-γ on anti-inflammatory cytokines, there was no statistically significant correlation between IFN-γ and both anti-inflammatory cytokines IL-10 and TGF-β in groups I and II, which may imply a weak influence of IFN-γ on anti-inflammatory cytokines in these groups with two extreme limits of clinical forms of toxoplasmosis.

Our findings valued gene expression profiling in *Toxoplasma gondii* infections, in relation to the severity of clinical picture. This was in accord with Alizadeh et al. [45], who demonstrated that gene expression profiling was able to predict poor prognosis that was not predicted by clinical criteria alone. Identification of these high-risk patients by gene expression profiling may, in the future, influence treatment decisions as well as patient selection in clinical trials of new therapeutics [46].

Conclusion

This study demonstrated variable cytokine expression profiles regarding different pregnancy outcome in *Toxoplasma* seropositive women and emphasized that cytokine gene expression is a valuable parameter for estimating patho-immunological effect of *Toxoplasma* infection (latent or active), whether towards upregulating the pro-inflammatory cytokine axis resulting in an imbalanced immunological state in the first trimester resulting in lethal gestational consequences or maintaining a harmonized immunological balance, in spite of still harboring the infection as in multiparous women. This verifies the complex relation between pro-inflammatory and anti-inflammatory cytokines in relation to diverse pregnancy outcomes in *Toxoplasma* infection.

study and performed the statistical analysis. GMN participated in the design of the study and helped in statistical analysis. MS participated in the design of the study and helped in statistical analysis. MSB participated in the design of the study and carried out the serological and molecular assays. All authors read and approved the final manuscript.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research was approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University (N-51-2013). Written informed consents were obtained from participants in this study. All authors accepted the publication. The data used or analyzed during the study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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