# Tissue Transglutaminase on Trophoblast Cells as a Possible Target of Autoantibodies Contributing to Pregnancy Complications in Celiac Patients

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## Problem
Women with celiac disease (CD) are often affected by atypical presentations of the disease associated with reproductive disorders as a main extra-digestive complaint. Here, we analyzed if autoantibodies against tissue transglutaminase (tTG) in sera from CD patients with reproductive disorders could display direct effects through their interaction with tTG expressed on trophoblast cells and phagocytes inducing tissue damage and interfering in the clearance of trophoblast apoptotic bodies.

## Method of study
Sera from CD women with reproductive disorders were obtained, and their ability to induce apoptosis of Swan-71 (cytotrophoblast cell line) and to modulate the wound-healing and phagocytes process was tested.

## Results
Swan-71 cells expressed tTG and CD sera displayed a significant decrease in trophoblast cell migration and a delay in injury healing on trophoblast cells, compared with those observed with control sera. Moreover, CD sera significantly reduced trophoblast cell proliferation and increased apoptosis levels in comparison with those observed in the control sera. Finally, autoantibodies against tTG interfere in the clearance of trophoblast apoptotic bodies through a mechanism involving MFG-E8 (milk fat globulin-EGF factor 8)–tTG binding.

## Conclusion
The anti-tTG antibodies might contribute to trophoblast damage and disrupt the phagocytosis process of apoptotic bodies that could promote a pro-inflammatory microenvironment.

## Introduction

The constitution of the maternal–placental interface during decidualization and trophoblast invasion involves a pro-inflammatory response accompanied by a deeper tissue remodelation.[1–3] The embryo has to break through the epithelial lining of the uterus to implant, damaging the endometrial tissue to invade and replace the endothelium and vascular smooth muscle of the maternal blood vessels.[4–6] In this sense, the inflammatory response that characterizes the peri-implantation period will be physiologically

limited by regulatory and tolerogenic mechanisms involving both innate and adaptative responses.[7–9]

In this sense, leukocyte populations including T-cell subpopulations, uterine Natural killer cells, decidual macrophages and dendritic cells, as pro-implantatory mediators are collectively called BIEFs (blastocyst implantation essential factors) contribute to regulating this network.[7–12]

In particular, macrophages constitute 20–30% of decidual immune cells, which are activated in an alternative pathway with wound-healing and tolerogenic abilities.[13,14] In fact, apoptotic trophoblast, smooth muscle and endothelial cells will be efficiently removed by decidual macrophages to prevent a deleterious inflammatory response. Therefore, the immediate clearance of apoptotic cells induces a immunosuppressant/regulatory phenotype of decidual macrophages, producing IL-10 and TGFβ.[15,16] Accordingly, an exacerbate generation of apoptotic bodies and/or an ineffective clearance of them might contribute to a deregulation of inflammatory response during implantation and may be an underlying cause of pregnancy complication.[17]

Celiac disease is an autoimmune disorder triggered by gluten ingestion characterized by a complex clinical pattern that can involve several extra-digestive organs.[18] Reproductive health can be affected in untreated celiac women, and sometimes gyneco-obstetric complications are the only manifestation of disease[19,20] including menstrual cycle disorders, low fertility, early miscarriages, preterm birth, intrauterine growth restriction and low birthweight.[21–26]

Patients with celiac disease display autoantibodies against tTG (tissue transglutaminase EC 2.3.2.13 or TG2), the specific autoantigen of CD and their detection is widely used as a diagnostic indicator.[27]

tTG plays several biological functions in many tissues, some of them as tissue repair mediated by transamidating activity, while others are independent of its catalytic function.[28] At the cell surface, tTG is expressed in association with $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins acting as fibronectin co-receptors involved in cell adhesion and migration[29,30] by promoting integrin clustering and cell signaling.[31] In addition, the promotion of the clearance of dying cells is a novel function described for surface tTG expressed in murine[32–34] and human macrophages.[35] Specifically, tTG binds MFG-E8 (milk fat globulin-EGF factor 8), a protein known to bridge $\beta_3$ integrins to apoptotic cells and mediate the uptake of dying cells.[33]

tTG is present at the embryo–maternal interface,[36–38] and in placenta, tTG plays a role in cross-linking fibronectin and supporting cell adhesion in association with the syncytiotrophoblast microvillous membrane[38] and stabilizes the particulate material sheded from human placenta.[39]

As tTG localizes at syncytiotrophoblast, the primary interface between maternal and fetal tissue is exposed to maternal blood and the autoantibodies against tTG developed during untreated disease could interfere with its biological functions. Data from our group and others[40–43] suggest that these autoantibodies may have a pathogenic role in gyneco-obstetric complains.

Here, we analyzed if circulating autoantibodies against tTG from patients with CD could display direct effects through their interaction with surface tTG expressed on trophoblast cells inducing tissue damage and interfering in the clearance of trophoblast cells apoptotic bodies.

## Materials and methods

### Patients

Serum samples were obtained from 10 women with recently confirmed diagnoses of celiac disease and history of gyneco-obstetric problems (infertility, early miscarriages and short breastfeeding) (mean: 33.4 years, age range: 29–47). All patients had small bowel biopsy-proven CD. All patients had anti-tTG IgA, while one of them also had IgG-specific antibodies. Nine serum samples were obtained from healthy non-pregnant women who had two or more previous normal pregnancies with no miscarriages (mean age 32.6 years, range 26–42 years) and used as control sera. All sera were stored at −20°C until use.

Patients were recruited from the Uruguayan Celiac Association and local hospital (Hospital Maciel); serum samples were used with permission from the Ethical Committee of the Faculty of Medicine, University of the Republic, Uruguay, and the informed consent of each participant was obtained.

### Determination of anti-tTG Antibodies

Levels of tTG-specific IgA and IgG antibodies in sera from patients were determined by enzyme-linked immunosorbent assay using Quanta Lite® h-tTG IgA and Quanta Lite® h-tTG IgG ELISA according to the

manufacturer's instructions (INOVA Diagnostics, San Diego, CA, USA).

### Immunostaining of Placental Tissue Sections

Sections of normal term placenta (kindly provided by Dr. G. Acosta) were dewaxed, rehydrated and boiled for 20 min in 10 mm sodium citrate, pH 6.0. Sections were pre-treated with normal goat serum and then incubated overnight at 4°C with serum samples from women with celiac disease or controls (dil. 1:50); 4E1 MAb (kindly provided by Dr. F. Chirdo)[44] was used in parallel as a positive control (1:100). After washes, quenching of endogenous peroxidase activity was performed with 3% $H_2O_2$ in PBS for 20 min. Sections were then incubated for 1 h at room temperature with either goat anti-human IgA–horseradish peroxidase (HRP) or goat anti-mouse immunoglobulin–HRP (1:100). The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride 10 mg/mL in TBS with 0.03% $H_2O_2$ for 15 min; slides were counterstained with Mayer's hematoxylin and mounted. For every assay, negative controls with no primary antibody were included.

### Cell Lines

The trophoblast cell line Swan-71 (derived from telomerase-mediated transformation of a 7-week cytotrophoblast isolate described by Straszewski-Chavez[45]) was cultured in complete Dulbecco's modified Eagle's medium (DMEM), 10% FBS (Gibco™ Invitrogen Corporation, Grand Island, NY, USA). It was used to evaluate the antibodies effect on apoptosis, proliferation, migration and clearance of apoptotic cells.

The THP1 cell line was obtained from ATCC and maintained at $2x10^5$ cells/mL in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. THP1 cells ($2 \times 10^5$/mL) were differentiated using 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St Louis, MO, USA) for 3 days. Then, PMA-containing media was removed, and cells were incubated for a further 5 days in fresh RPMI 1640 (10% FBS, 1% L-glutamine).

### tTG Expression in Swan-71

*Western blot analysis*

Cell extracts from Swan-71 (obtained by lysis with 50 mM Tris–HCl, 0.1% Triton-X-100, 1 mM EDTA, 2-mercapto ethanol in the presence of proteinase inhibitors) and guinea pig tissue transglutaminase were subjected to SDS–PAGE in 12% polyacrylamide gel. Proteins were then transferred to nitrocellulose which was subsequently blocked with 5% skimmed milk in PBS. Membranes were probed with human serum samples (dil. 1:1000 in PBS-1% BSA-0.05% Tween-20), or tTG-specific MAb (TG100, MS-RB-060-PCL, Thermo Fisher Scientific, Fremont CA, USA) diluted 1:4000 followed by incubation with appropriate dilutions of HRP-conjugated rabbit anti-human IgG (Dako, Denmark), HRP-conjugated rabbit anti-mouse IgG (Thermo Fisher Scientific) or a secondary antibody alone as a negative control. Immunoblots were developed with the Super Signal chemiluminescence system (Pierce, Rockford, IL, USA).

*Flow cytometry*

Surface tTG expression in the Swan-71 cell line was analyzed by labeling it with tTG-specific MAb TG100 (1 μg/$10^5$cels) and respective isotypic control followed by FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich).

To assess the binding of human tTG-specific antibodies from patients with celiac disease, cells were incubated with serum samples from women with celiac disease or controls (dil. 1:10 in 1% PBS-BSA) followed by FITC-conjugated anti-human IgA antibodies (1:100; Sigma-Aldrich).

Ten thousand events were acquired in a FACS Aria II cytometer®, and results were analyzed using WinMDI 2.8 (free software http://facs.scripps.edu/software.html).

### Scratch Assay

An injury was generated using a pipette tip in confluent Swan-71, cultures seeded over fibronectin, and wound healing was assessed by microscopy at different times (4, 8 and 24 h) in the presence of TG100 MAb (10 μg/mL), celiac or control sera (dil. 1:50).

Results were expressed as the ratio between the initial length of injury and the optimal incubation time for each experimental condition.

### Migration Assays

Migration assays were performed with Swan-71 cells in transwell systems (BD Falcon cell culture inserts). Cells were seeded in 8-μm-pore inserts ($25 \times 10^4$ cells/insert) which were then set in a 24-well plate

containing the DMEM medium with FBS 20% as chemo-attractant stimuli and were allowed to migrate in the absence or presence of the TG100 MAb (10 μg/mL), and after 24 and 48 h, the cell migration rate to the lower compartment was quantified by microscopy after DAPI staining of the insert membrane.

### Proliferative Response

Swan-71 cells at 70% of confluence in a 96-well flat-bottom plate were cultured for 72 h in the presence of CD or control sera. Then, cells were pulsed for 18 h with 1 μCi/well of methyl-[$^3$H]-thymidine [$^3$H]TdR (NEN, Boston, MA, USA). Trophoblast cells were then lysed and harvested on glass fiber filters using a Packard Filtermate cell harvester (Packard Instruments, LaGrange, IL, USA). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). Tests were conducted in triplicate, and results were expressed as proliferation index (PI) defined as the ratio between mean cpm values for each experimental condition (CD or control serum) and control wells (culture medium).

### Apoptosis Assay

Swan-71 basal apoptosis was induced by fetal bovine serum (FBS) deprivation, and the effect of antibodies on the apoptosis rate was evaluated in parallel with TG100 MAb (25 μg/mL) and sera dilutions from controls or women with celiac disease (1:20). After 24 h, apoptosis was assessed by double staining with FITC-labeled annexin-V and propidium iodide (PI) following the manufacturer's recommendations (Immunotech, Marseille, France). The apoptosis rate was defined as the percentage of apoptotic cells (annexin-V positive); results for each experimental condition were expressed as the fold increase in relation to basal condition (FBS deprivation).

### Phagocytosis Assay

PMA differentiated THP-1 cells were used to study the effect of antibodies on the phagocytosis of apoptotic Swan-71 cells as a target. Swan-71 cells were labeled with CFSE, and apoptosis was induced with 4 μM camptothecin treatment for 12 h and controlled with IP/annexin-V staining as described above. Phagocytosis assays using differentiated $1 \times 10^5$ THP-1 cells and CFSE-stained apoptotic Swan-71 cells (ratio 1:1) were performed in the presence of TG100 MAb (10 μg/mL) and sera dilutions from controls or women with celiac disease (1:50) for 2 h at 37°C. As a phagocytosis control, co-cultures were performed at 4°C. Cells were stained with a phycoerytrin-conjugated anti-CD45 AcMo (BD Pharmingen), and the percentage of double-positive CD45-CFSE cells determined by flow cytometry was used as the percentage of THP-1 cells that ingested apoptotic cells.

### Analysis of tTG–MFG-E8 Binding by ELISA

Microtiter plates (Maxisorb, Nunc) were incubated with 10 μg/mL solution of guinea pig tTG (Sigma-Aldrich) in PBS, overnight at 4°C. After blocking with 1% PBS-BSA, the wells were incubated with rabbit anti-tTG polyclonal antibody (RB-060-PCL; Thermo Fisher Scientific) at serial dilutions (1:500–1:2000) for 1 h at 37°C followed by incubation with recombinant human MFG-E8 (R&D Systems, MN, USA) in several concentrations (850–6250 pg/mL). Bound MFG-E8 was subsequently detected by specific MAb (MAB27671; R&D Systems, MN, USA). Finally, bound anti-MFG-E8 antibody was detected with HRP-conjugated goat anti-mouse diluted 1:5000 (Thermo Fisher Scientific) and developed with $H_2O_2$-TMB substrate solution. Assays were performed in duplicate, and the media for each condition is indicated. Results are expressed as relative binding (%) in relation to the signal obtained without anti-tTG antibody.

### Statistical Analysis

The Mann–Whitney test was applied to compare data from different groups, and the Spearman test was used to analyze the correlation between specific antibodies titers and each studied effect.

GraphPad Prism4 software (GraphPad, San Diego, CA, USA) was used and; significance was determined at $P < 0.05$.

## Results

### tTG is Expressed in Swan-71 Cells and in Term Placenta

In a first step, we evaluated tTG surface expression on Swan-71 cells, a cytotrophoblast cell line from

7 weeks of gestation, and in human term placental tissue. We could observe that reference MAb TG100 and celiac patient sera recognized tTG on Swan-71 detected by FACS analysis and also confirmed by Western blot. Fig. 1(a) shows a representative flow cytometry profile of tTG staining in Swan-71 cells by TG100 MAb and serum from a patient with celiac disease. Fig. 1(b) shows the immunoreactive band of tTG in the extract from trophoblast cells after incubation with TG100 MAb or CD serum. Moreover, when we evaluated tTG expression on term placental tissue by immunohistochemistry using the serum from a celiac woman with high anti-tTG IgA titers, we observed a strong staining of the syncytiotrophoblast surface with a similar pattern as that obtained with the anti-tTG MAb (4E1), characterized by a non-uniform mark distribution (Fig. 1c: ii and i, respectively).

### Anti-tTG MAb and Celiac Women Sera Delayed Injury Healing and Migration

As the early implantation period is characterized by tissular remodelation and tTG contributes to injury healing, we investigated the effect of celiac sera on injury healing and cell migration of trophoblast cells. Similar size wounds were introduced on monolayers of Swan-71 cell line incubated in the presence of sera from healthy or celiac women with gyneco-obstetric complications. After 8 h, a measurable decrease of injury length was observed in all samples and as depicted in Fig. 2(a), sera from women with celiac disease displayed significantly delayed injury healing compared with control sera ($*P = 0.037$ Mann–Whitney test). Fig. 2(b) shows injury healing in the presence of a representative control serum, celiac serum or the TG100 MAb as a positive control.

To investigate whether the delayed injury healing observed in the presence of CD patient sera was caused by interference in the trophoblast cell migration due to anti-tTG antibodies, we performed migration assays using transwells system (pore 8 μm) with Swan-71 cells seeded in the upper compartment in the absence or presence of TG100 MAb; trophoblast cells were allowed to migrate to the lower compartment containing 20% FBS as chemo-attractant, and after 48 h, the trophoblast cells in lower compartment were quantified. We observed about a 15% decrease in the migration rate of trophoblast cells in

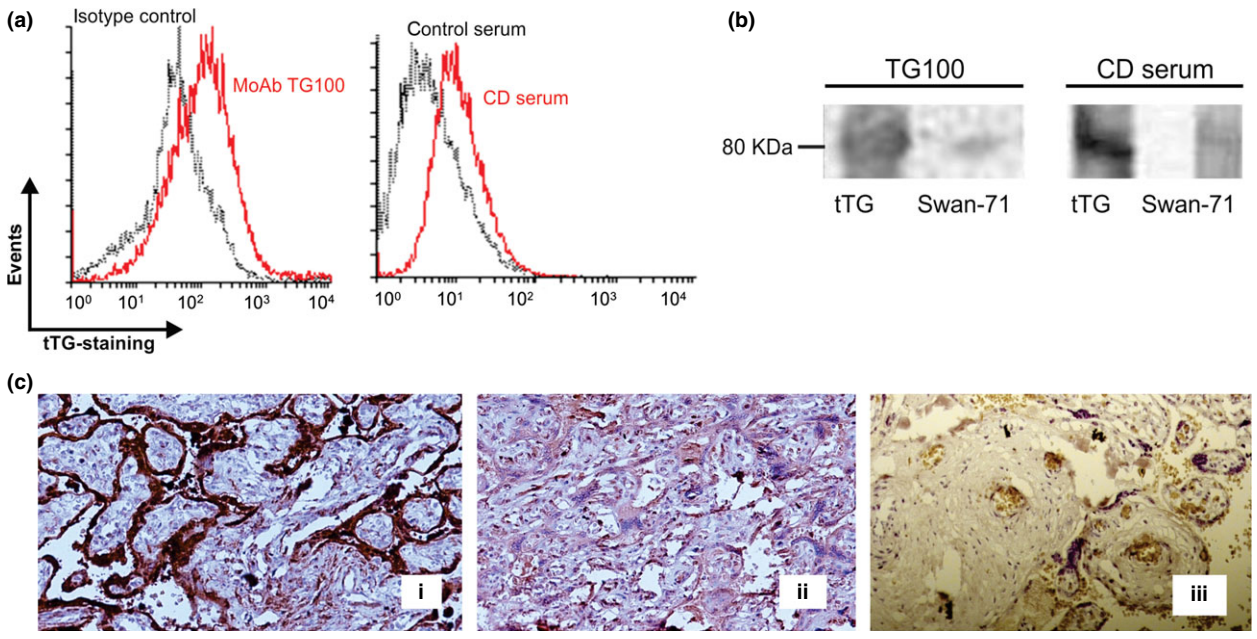


**Fig. 1** Tissue transglutaminase (tTG) is expressed in Swan-71 cells and in term placenta. tTG expression on Swan-71 cells was evaluated by FACS analysis (a) and Western blot (b) with reference MAb TG100 and a representative CD serum sample. Immunohistochemical patterns of term placental tissue is shown at 200× magnification (c). Reference anti-transglutaminase MAb (4E1) staining of syncytiotrophoblast is shown (i), serum from a celiac patient with history of obstetric problems showing binding of IgA at the syncytial surface (ii) and negative serum control (iii).

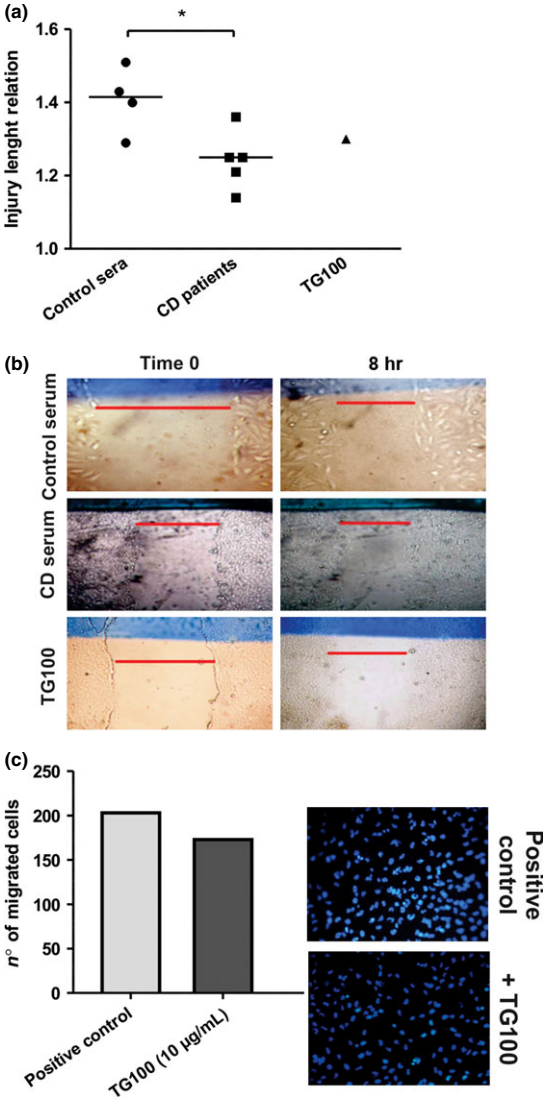


**Fig. 2** Anti-tissue transglutaminase (tTG) MAb and celiac women sera delayed injury healing and migration of trophoblast cells. (a) Similar-sized wounds were introduced on monolayers of Swan-71 cells, and the length was measured initially and after 8 h. Results are expressed as the ratio between those measures in the presence of control sera, celiac patient sera (both dil. 1:50) and anti-tTG MAb (triplicate assay). Horizontal bars indicate medians (*$P$ = 0.037 Mann–Whitney test). (b) Representative pictures of injury healing length after 8 h in the presence of a representative control serum, celiac serum or the TG100 MAb as a positive control (magnification 100×). (c) Migration assays were performed with Swan-71 cells in transwell systems. Cells were seeded in the upper compartment in the absence or presence of the TG100 MAb (10 µg/mL) using FBS 20% as chemo-attractant stimuli in the lower compartment. The left panel shows a quantitative analysis of trophoblast cell migration to the lower compartment in the absence or presence of TG100 MAb, and the right panel shows representative pictures of Swan-71 cells after 48 h of migration from one representative experiment (magnification 100×).

the presence of TG100 MAb (Fig. 2c, left panel), and representative pictures of Swan-71 cells after 48 h of migration in one representative experiment are shown in Fig. 2(c, right panel).

### Celiac Sera Interfere in the Proliferation Rate and Promote Apoptosis of Trophoblast Cells

As cell surface tTG is a cue for survival and proliferation,[28] we investigated CD sera effects on trophoblast cells. Therefore, we quantified trophoblast cell proliferation in the presence of sera obtained from patients with CD or controls. As depicted in Fig. 3(a), sera from patients with CD significantly reduced the thymidine uptake in comparison with control sera (*$P$ = 0.004, Mann–Whitney test).

Taking into account the lower proliferation levels of trophoblast cells in the presence of CD sera, we wondered if autoantibodies could affect their survival and promote apoptosis. Therefore, Swan-71 cells were cultured in the absence or presence of sera from patients with CD or controls, and we evaluated the apoptosis rate by annexin-V/PI staining after 24 h of starvation. We could observe that CD patient sera significantly increased the apoptosis levels in comparison with that observed for control sera with comparable protein content of each serum (fold increase median 1.7 for CD sera and 0.8 for controls; *$P$ = 0.011 Mann–Whitney test) as shown in Fig. 3(b); in fact, the apoptotic levels observed with some CD sera were similar to those observed with TG100 MAb that induced a 2.7-fold increase of apoptotic cells in comparison with basal apoptosis. Interestingly, among CD sera analyzed, 6 of 8 correspond to women with previous miscarriages. Fig. 3(c) shows illustrative FITC annexin-V/PI dot plots of Swan-71 cells for each experimental condition.

### tTG MAb Interfere in the Clearance of Apoptotic Trophoblast Cells Through a Mechanism Involving MFG-E8 Interaction

At the maternal–placental interface, the effective removal of apoptotic cells by phagocytes is an essential process which prevents the lysis and release of self-antigens and paternal alloantigens.[2,4,5] Therefore, we evaluated whether the anti-tTG antibodies interfere in the phagocytosis process of trophoblast apoptotic bodies. For that purpose, we used the PMA-treated THP1 cell line that resemble macrophages in morphology and differentiation properties,[46] and we confirmed tTG expression by

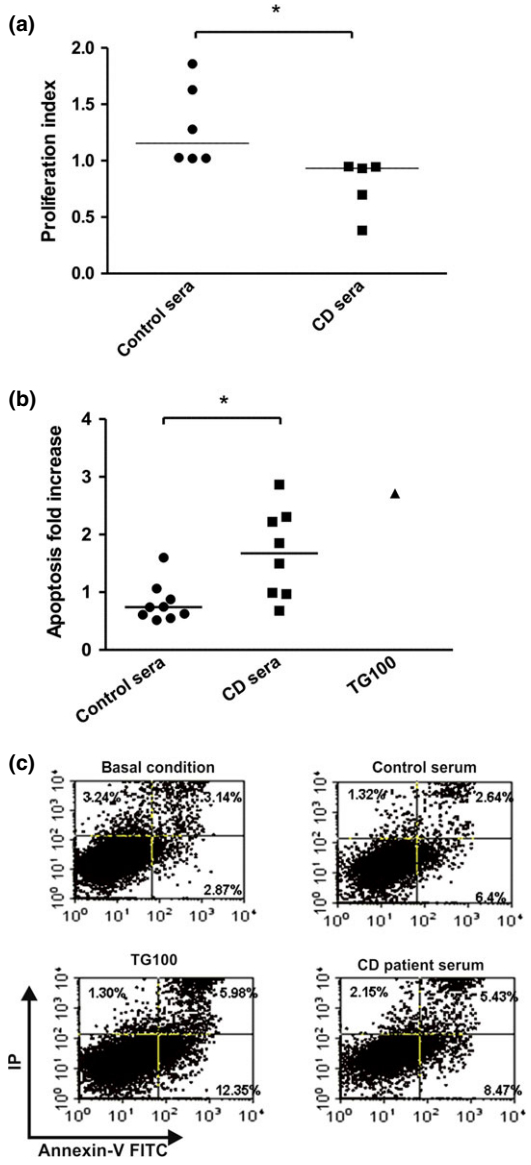


**Fig. 3** Celiac sera interfere with the proliferation and induce apoptosis of trophoblast cells. (a) Swan-71 cells at 70% of confluence in a 96-well flat-bottom plate were cultured for 72 h in the presence of control or CD patient sera. Then, cells were pulsed for 18 h with [$^3$H]TdR, and incorporated radioactivity was measured (cpm). Tests were conducted in triplicate, and results were expressed as proliferation index (PI) defined as the ratio between mean cpm values for each experimental condition (CD or control serum) and control wells (culture medium). Horizontal bars indicate medians (*$P$ = 0.004, Mann–Whitney test). (b) Swan-71 cells were cultured in the presence of control sera ($n$ = 9), CD patient sera ($n$ = 8) or TG100 MAb (triplicate assay) and the apoptosis levels quantified by IP/annexin-V staining. Results are expressed as the apoptosis fold increase. Horizontal bars indicate medians (*$P$ = 0.011 Mann–Whitney test). (c) Illustrative dot plots of Swan-71 cells in the presence of control serum, CD serum or TG100 MAb.

flow cytometry (data not shown) as was previously reported.[47]

For the phagocytosis assays, differentiated THP-1 cells were co-cultured with apoptotic Swan-71 bodies stained with CFSE as target in the presence or absence of CD patient sera, control sera or TG100 MAb. As depicted in Fig. 4(a), we observed a decrease in the percentage of CD45+CFSE+ cells in those cultures performed in the presence of CD patient sera in comparison with those performed with control sera. In fact, the TG100 MAb reduced the phagocytosis process in comparison with controls (median 23.2% versus 28.4%; TG100 21.4%). Fig. 4(a), lower panel shows illustrative dot plots of differentiated THP-1 cells after phagocytosis of CFSE apoptotic trophoblast bodies in the presence of CD serum, control serum or TG100 and a control of the phagocytosis process performed at 4°C.

As the present results suggest that antibodies against tTG disturb the phagocytosis process which is critical to maintaining immune homeostasis at the placental–maternal interface, we investigated a potential mechanism of interference. In this sense, MFG-E8 is the engulfment-related molecule best linked to clearance of apoptotic cells,[33,48] and according to our results, it is also produced in significant amounts by differentiated THP-1 cells (0.4–1.0 ng/mL), and hence, we then investigated whether anti-tTG antibodies interfere with the interaction between tTG and MFG-E8. For that purpose, an ELISA was performed in which a rabbit anti-tTG polyclonal antibody in different dilutions was added to tTG-coated plates, and then, MFG-E8 (850 or 6250 pg/mL) was incorporated to assess whether the interaction between tTG and MFG-E8 could be disturbed by specific antibodies. As show in Fig. 4(b), anti-tTG antibodies significantly reduced the binding of MFG-E8–tTG in comparison with those assays performed in the absence of antibodies and the interference of MFG-E8 and tTG binding seems to be dose dependent, as an additional control MFG-E8 was added to determine the adherence to the plate in the absence of tTG.

## Discussion

The presence of specific antibodies against tTG is a feature of celiac disease, while patients are not under a gluten-free diet. A pathogenic role of these autoantibodies on the reproductive health of women has been postulated because: (i) tTG participates in

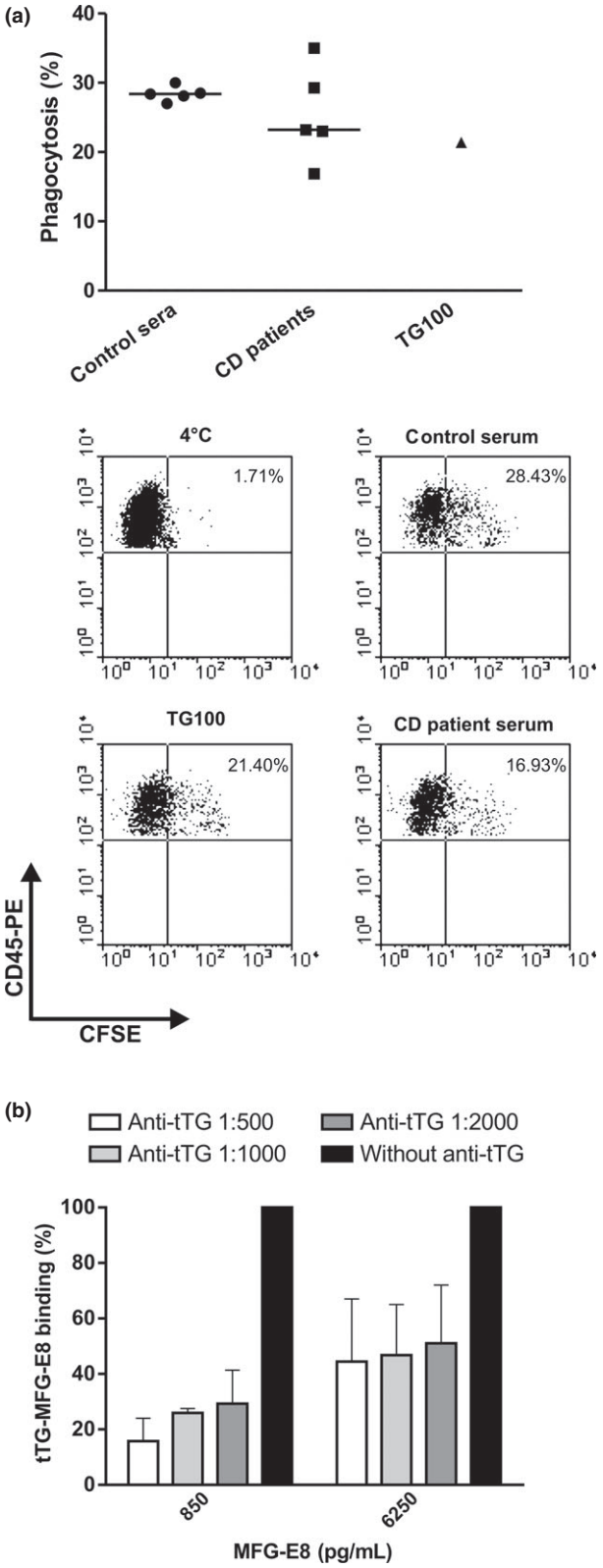


**Fig. 4** Tissue transglutaminase (tTG) MAb interfere with the clearance of apoptotic trophoblast cells through a mechanism involving tTG–MFG-E8 interaction. (a) Phagocytosis assay. Differentiated THP-1 cells in the presence of PMA were co-cultured with apoptotic Swan-71 bodies CFSE-stained in the presence of control sera, CD patient sera or TG100 MAb (triplicate assay). The percentage of THP-1 cells that had phagocyted apoptotic Swan-71 cells was determined by FACS analysis counting the frequency of CD45+CFSE+ cells. CD patient sera reduced the frequency of CD45+CFSE+ cells in comparison with control sera (median: 23.2% versus 28.4%; TG100 21.4%). The lower panel shows illustrative dot plots of differentiated THP-1 cells after phagocytosis of CFSE apoptotic trophoblast bodies in the presence of control serum, CD serum or TG100. (b) Anti-tTG antibodies effect on tTG–MFG-E8 interaction. Rabbit anti-tTG antibody at several dilutions (1:500; 1:1000 and 1:2000) was incubated in plate sensitized with tTG and MFG-E8 (850 or 6250 pg/mL). Results are expressed as relative binding (%) in relation to the signal obtained without anti-tTG antibody. Assays were conducted in duplicate, and results were expressed as mean + S.E.M.

endometrial physiological processes throughout the menstrual cycle, during decidualization and implantation[36] and controls several physiological processes that maintain tissue homeostasis[49,50] and (ii) tTG expressed in placenta is accessible to circulating antibodies as the enzyme is exposed in the outer face of the syncytiotrophoblast microvillous membrane and cytotrophoblast.[38,39,41]

In this sense, we have recently reported an association between gyneco-obstetric complications and anti-tTG antibodies titers in women with CD.[40] The aim of the present work is to study the effects of autoantibodies on several processes involved in implantation (survival, migration and proliferation of trophoblast cells) and homeostasis (clearance of apoptotic cells) that could be mediated by surface tTG. As tTG localizes to the syncytial microvillous surface of human placenta,[38] we used Swan-71 cells as a model and for the first time describe the expression of tTG on these trophoblastic cells using a commercial MAb and show that it can be the target of antibodies in the sera from celiac women with a history of obstetric complaints (Fig. 1).

During the peri-implantation period, the trophoblast invasive capacity depends on their ability to proliferate and migrate, here we could observe that anti-tTG reduces the proliferation and migration of trophoblast reflected by a delay in the closing of the gap injury (Fig. 2). In fact, sera from patients with CD could directly affect trophoblast survival, not only reducing their proliferation rate, but even promoting their apoptosis (Fig. 3). These data are in line with previous work performed with primary cells

isolated from term placenta,[42] but our work focuses on a first trimester trophoblast cell model and further explores the consequences of the accumulation of apoptotic trophoblasts.

Under normal conditions, apoptotic cells are quickly removed by maternal macrophages at early stages of pregnancy. The phagocytosis of the apoptotic cells elicits the release of anti-inflammatory mediators such as IL-10, TGFβ and prostaglandins *in vitro*[13–15]; moreover, this recognition actively suppresses inflammatory cytokine release *in vitro*.[51]

Therefore, the inefficient clearance of exacerbated apoptotic trophoblast cells could promote a deregulation in the maternal immune response at the maternal–placental interface conditioning the immunosuppressive effects. As a consequence, inflammation is often observed at the maternal–fetal interface as the final pathological assault in many cases of pregnancy losses, including those of unexplained etiologies.[4,52] In a previous work, we observed that PBMCs obtained from patients with recurrent pregnancy loss with immunological causes displayed an exacerbated pro-inflammatory and Th1 immune response after the interaction with Swan-71 cells, reflected by an increase in T-bet expression level and nitrite production while decreasing TGFβ and IL-10 production [52].

In addition, increased rates of apoptosis and inflammation were described in placenta from untreated celiac patients with low birthweight babies.[53]

In this context, we hypothesize that tTG expressed on the surface of phagocytes in the maternal–fetal interface can also be targeted by autoantibodies on the basis of the novel role of tTG on the clearance of apoptotic cells[33] and evidence from tTG knockout mice that revealed that the phagocytic capacity of tTG-null macrophages to engulf apoptotic cells is impaired.[32,34] In addition, we recently reported that the anti-tTG antibody spontaneously produced by the non-obese diabetic (NOD) mouse model inhibited apoptotic cell phagocytosis by peritoneal macrophages from pregnant NOD mice that express tTG on surface. Evidence provided supports a role for anti-tTG antibodies through reduced transamidating activity and reduced apoptotic cell clearance by pregnant NOD mice macrophages.[54]

We used PMA-treated THP-1 as a cell model for maternal macrophages and observed an average 18% reduction in the phagocytosis rates in the presence of serum samples from women with celiac disease in relation to sera from healthy women.

No significant correlation was observed among tTG-specific antibody titers and any of the cell functions evaluated (healing, proliferation, migration and phagocytosis); this lack of correlation can be attributable to differences in the fine specificity repertoire of antibodies among patients as tTG-specific antibodies are evolved against different functional domains of the enzyme, and each subsets of autoantibodies could differentially contribute to pathology.[55,56]

To further explore the molecular mechanism putatively involved in phagocytosis impairment, we focused on the role of tTG in establishing a link with MFG-E8 on the cell surface.[33] We first verified the presence of MFG-E8 in cultures derived from THP-1 cells, as MFG-E8 is one of the best studied engulfment-related molecules.[48] As a first approach, we explored the ability of anti-tTG antibodies to interfere with the tTG and MFG-E8 binding which could result in a decrease in the phagocytosis of the apoptotic trophoblast cells; our results show that this mechanism could be involved as the interaction between tTG and MFG-E8 was decreased in the presence of the rabbit anti-tTG polyclonal antibody. Further work is currently underway to decipher if this molecular mechanism can take place at the cellular level in the presence of specific antibodies elicited during celiac disease.

Results presented herein provide experimental evidence that the autoantibodies against tTG could promote the apoptosis of trophoblast cells, delay injury healing and interfere in the clearance of trophoblast apoptotic bodies through a mechanism putatively involving MFG-E8 interaction. The present evidence not only shows that anti-tTG autoantibodies contribute to trophoblast direct tissue damage, but they could also disrupt apoptotic trophoblast cell phagocytosis promoting a pro-inflammatory microenvironment characterized as in adverse pregnancy and obstetric outcomes.

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