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Anti-tissue transglutaminase antibody inhibits apoptotic cell clearance by macrophages in pregnant NOD mice

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ABSTRACT

Autoimmunity is a feature of celiac disease (CD) with tissue transglutaminase (tTG) as a major autoantigen. A correlation between gynecological-obstetric disorders in CD patients and the presence of circulating antibodies anti-tTG that inhibited tTG activity was reported. Serum anti-tTG antibodies were detected in a non-obese diabetic (NOD) mouse model of type I insulin-dependent diabetes mellitus and Sjögren's syndrome, two comorbid states with CD. Since pregnancy complications have been described in NOD mice, we evaluated the ability of anti-tTG antibodies to affect the functions of tTG relevant to the normal course of an early pregnancy like extracellular matrix assembling and apoptotic cell phagocytosis by macrophages. Circulating IgG antibodies against tTG were detected in NOD mice with titers that decreased at early pregnancy; interestingly, the *in vitro* transamidating activity of tTG was reduced by NOD serum samples. Particularly, anti-tTG antibody inhibited apoptotic cell phagocytosis by peritoneal macrophages from pregnant NOD mice that express the enzyme on surface. Evidence provided support for a role for anti-tTG antibodies through reduced transamidating activity and reduced apoptotic cell clearance by the macrophages of pregnant NOD mice.

1. Introduction

Pregnancy is a tightly regulated process, where systemic and local mechanisms act in synchronicity to allow the maternal immune system to tolerate the fetus. Especially at the early post-implantation stage, intense tissue remodeling and apoptosis of trophoblast cells occur in a homeostatic immunosuppressant microenvironment. Various immune cell populations contribute to this anti-inflammatory milieu, with macrophages taking center stage owing to their high functional plasticity (Mosser and Edwards, 2008; Nagamatsu and Schust, 2010). In particular, macrophages bearing an alternative activated profile participate in wound healing processes and the silent clearance of apoptotic cells (Abrahams et al., 2004; Fest et al., 2007; Straszewski-Chavez et al., 2005).

The outcome of pregnancy may be impaired by several autoimmune conditions; celiac disease (CD) is a multifactorial disease (Garrote et al., 2008) with an incidence reaching 1% in western countries (Fasano et al., 2003; Dube et al., 2005) and increasing in prevalence worldwide (Cataldo and Montalto, 2007). Women appear to be preferentially affected (Bardella et al., 2005) and associated reproductive disorders have been extensively reported (Ozgor and Selimoglu, 2010; Soni and Badawy, 2010); however, the molecular mechanisms involved remain unknown.

Tissue transglutaminase (tTG; EC 2.3.2.13) is the specific autoantigen in CD (Dieterich et al., 1997) and specific

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IgA and/or IgG antibodies against tTG are often produced during active disease; several data suggest that these autoantibodies might play a role in the development of extraintestinal disorders associated with CD (Lindfors et al., 2010). In line with this, we have recently reported a significant correlation between the presence of gyneco-obstetric disorders in celiac women and the ability of serum to affect the *in vitro* tTG transamidation activity – an acyltransfer reaction between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine (Sonora et al., 2011).tTG is localized to the endometrium and placenta at both the intracellular and extracellular compartments (Kabir-Salmani et al., 2005) where it can play biological roles in the remodeling of extracellular matrix, cell death, clearance of apoptotic cells and cell migration among others; some of these functions are mediated by its classical transamidation activity while others are independent of its catalytic functions (Fesus and Piacentini, 2002). tTG in particular has been involved in the clearance of apoptotic cells by macrophages and mice deficient for tTG showed an impaired phagocytic capacity (Toth et al., 2009).

The non-obese diabetic (NOD) mouse is a useful model for examining the immunoregulatory abnormalities that lead to autoimmune cell destruction and type I or insulin-dependent diabetes mellitus (T1DM) (Wicker et al., 2005). At the prediabetic stage, it models several features of Sjögren's syndrome (SS) (Delaleu et al., 2011). A loss of tolerance in the B cell compartment is one of the earliest indicators of the overt autoimmune process (Yu et al., 2000) with an active autoantibody repertoire observed as early as the 4th week of age with broad pathological potential (Thomas et al., 2002). Interestingly, the involvement of tTG has been associated with the disturbance in cellular homeostatic mechanisms in both SS-like and T1DM autoimmune stages (Di Sabatino et al., 2012).

Signs of pregnancy impairment were reported in NOD mice at the pre-diabetic and diabetic stages. A decline in litter size and increased resorption rates associated with local regulatory T cell and NK cell defective activity were reported (Burke et al., 2007; Lin et al., 2009; Roca et al., 2009). NOD mice spontaneously produce antibodies against tTG (Sblattero et al., 2005); however, the association between autoantibodies and reproductive complications in these animals has not been reported so far. In particular, since the direct role of anti-tTG antibodies in the phagocytic function of NOD mice macrophages has not yet been studied, and that the phagocytosis of apoptotic cells is one of the central functions of macrophages during early pregnancy, here we aimed to analyze the presence of anti-tTG antibodies in NOD mice serum and their ability to interfere with phagocytosis of apoptotic cells by the macrophages of pregnant NOD mice.

2. Materials and methods

2.1. Animals

Normally cycling NOD and BALB/c mice of 16 weeks of age were mated with NOD and BALB/c males (syngeneic mating) and gestational day 0 was indicated by a vaginal plug. NOD and BALB/c female mice were bred and maintained at the Central Animal Care Facility of the School of Exact and Natural Sciences, University of Buenos Aires (FCEyN-UBA). Mice were maintained on a 12:12 h light–dark schedule and fasted overnight with water *ad libitum* before being used. They were tested routinely for blood glucose levels and considered to be prediabetic as their values of serum glucose on two occasions over a 24-h period did not differ significantly from those of control mice (0.9 ± 0.1 g/l, $n = 38$). NOD mice, either normally cycling or at 9 days of gestation, were used for blood extraction, peritoneal macrophage isolation, and implantation site immunostaining. All studies were conducted according to standard protocols of the Animal Care and Use Committee of the FCEyN-UBA.

2.2. Anti-tTG antibodies determination in mice sera

Serum samples from NOD and BALB/c mice were diluted 1:100 in PBS–Tween 20 (0.05%) BSA 1% and the assay was carried out according to our previous work, but using appropriate dilutions of horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibodies (Sigma–Aldrich) (Sonora et al., 2011).

2.3. Total serum IgG quantifications in mice sera

Total IgG antibody titers were determined by capture ELISA using commercial reagents and the reciprocal endpoint technique. Briefly, 96-well plates were coated with goat anti-mouse IgG antibodies and appropriate serial dilutions of each sample were incubated in the same plate. After goat anti-mouse IgG conjugate incubation, the assay was completed as described above.

The reciprocal dilution value corresponding to an arbitrary OD value (0.3) was obtained from the dilution curve of each sample to report total IgG antibody levels (IgG titer).

2.4. tTG transamidating activity assay

The measurement of guinea pig tTG transamidating activity is based on the crosslinking of 5-(biotinamido)-pentylamine substrate (Pierce) into immobilized human fibronectin used as an acceptor protein. The assay was carried out according to our previous work (Sonora et al., 2011). To evaluate the effect of serum on transglutaminase activity of tTG, the amine substrate was added after incubating the serum samples from both BALB/c and NOD mice for 20 min at 37 °C and washings.

Each duplicate assay was performed in parallel with the competitive specific inhibitor monodansylcadaverine at 250 μM as a control. tTG activity in the presence of serum is indicated as relative activity, expressed as a percentage of the basal activity obtained without the addition of serum (relative activity = $OD_{serum} \times 100/OD_{basal}$).

2.5. Immunostaining of NOD endometrium tissue sections

Sections of pregnant uteri from 16-week-old NOD mice at day 9 of gestation were fixed in 4% paraformaldehyde,

pretreated with normal goat serum and then incubated overnight at 4 °C, with a serum sample from BALB/c mice (1:100) or tTG-specific mouse IgG monoclonal antibody 2G3 (1:100) as a positive control (Di Niro et al., 2005), kindly provided by Dr. F. Chirdo from the University of La Plata, Argentina. The assay was carried out according to our previous work (Sonora et al., 2011), but using appropriate dilutions of biotinylated goat anti-mouse immunoglobulin (Thermo Scientific) and peroxidase-conjugated streptavidin.

2.6. tTG expression in NOD mice peritoneal macrophages by RT-PCR and immunofluorescence

Resident nonstimulated macrophages were isolated from the peritoneal cavity of pregnant NOD and BALB/c mice at day 9 with ice-cold HANKS, washed thoroughly and suspended in RPMI-10% BFS before plated in 24-well plates as previously described (Larocca et al., 2011). Macrophage cell suspension (>85% F4/80+ by flow cytometry) from each individual pregnant mouse was analyzed separately for the expression of tTG by RT-PCR or immunofluorescence or used in phagocytosis experiments. For RT-PCR, RNA was isolated with TRIzol® reagent (Life Technologies), cDNAs were generated from 1 μg of RNA using a MMLV reverse transcriptase, RNAse inhibitor, and oligodT kit (Promega). Forward and reverse primers (tTG: Forward: *CACACCACAGGAGAAGAGCGAAGG*; Reverse: *ATCCAGTCCACCACATCAGCGTGG*. GAPDH: Forward *TGATGACATCAAGAAGGTGGTGAAG*, Reverse: *TCCTTGGAGGCCATGTAGGCCAT*) were used at the following conditions: 94 °C for 10 min, 35 cycles of 94 °C for 20 s, 66 °C for 20 s, 72 °C for 20 s, and 72 °C for 10 min. PCR products were detected as in previous work (Roca et al., 2009) and results expressed as arbitrary units normalized to GAPDH expression. Immunofluorescence for the assessment of tTG protein expression and localization was performed on macrophages plated on glass coverslips into 24-well plates, fixed with methanol and incubated with monoclonal antibody anti-tTG (2G3; 1:60 in PBS–Tween) after blocking with BSA 0.5% and revealed with an anti-mouse FITC (1:100; Sigma–Aldrich).

2.7. Phagocytosis of apoptotic thymocytes by NOD macrophages

Apoptotic thymocyte phagocytosis by pregnant NOD mice macrophages was carried out as previously described (Larocca et al., 2011). NOD mice thymocytes were induced to apoptosis with 1×10^{-8} M dexamethasone for 4 h and then added to a monolayer of macrophages isolated from each pregnant NOD mouse in a 1:5 ratio (2.5×10^5 macrophages: 1.25×10^6 thymocytes). After incubation for 90 min and washing, cells were stained with hematoxylin and eosin and counted. Before the addition of apoptotic thymocytes, macrophages were incubated for 30 min with monoclonal anti-tTG antibody (2G3; 1/60) or normal BALB/c mouse serum (1/30) as a negative control. Results were expressed as percentage of phagocytosis and phagocytic index.

2.8. Statistical analysis

Mann–Whitney test and Wilcoxon matched pairs signed rank test were used to compare experimental data obtained from independent or paired samples, respectively. Spearman test was used to evaluate the correlation among experimental data. Statistical calculations were performed using the GraphPad Prism Software version 5.00 Demo. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Levels of anti-tTG antibodies in pregnant and normally cycling NOD mice serum and inhibition of enzyme transamidating activity

First, we analyzed the levels of anti-tTG antibodies in pre-diabetic NOD female mice sera compared with BALB/c mice and pregnant NOD mice under normal breeding conditions.

Our results show that sera from nonpregnant NOD mice have a significantly higher concentration of IgG antibodies against tTG than age-matched BALB/c mice determined by ELISA (medians 0.767 *vs.* 0.414, respectively, $P = 0.019$; Fig. 1A). In contrast, there were no significant differences in total IgG between pregnant pre-diabetic NOD and BALB/c mice (Fig. 1B). We could not detect IgA anti-tTG antibodies in any of the mice groups. When comparing the level of anti-tTG antibodies in sera from pregnant and nonpregnant pre-diabetic NOD mice, we observed lower levels of IgG anti-tTG antibody titers at day 9 of gestation (medians 0.409 *vs.* 0.767, respectively, $P = 0.020$; Fig. 1A) whereas no changes were found in total IgG (Fig. 1B). Anti-tTG antibody levels in pregnant BALB/c mice were similar to those in nonpregnant BALB/c mice (not shown).

We next analyzed the effect of NOD sera containing specific antibodies on transamidating activity *in vitro*, since this activity is necessary for many processes including extracellular matrix remodeling (Belkin, 2011) and TGF-β1 incorporation to the extracellular matrix in order to be activated (Verderio et al., 1999). As can be seen in Fig. 1C, NOD mice sera decreased transamidating activity (23–54% of decrease from basal activity), whereas sera from control BALB/c mice did not modify or increase the *in vitro* enzyme activity. The competitive specific inhibitor monodansylcadaverine used as a control decreased transamidating activity an average of 70% from basal activity (data not shown). There was no significant correlation between the effect determined on enzyme activity and IgG anti-tTG antibody concentrations in mice sera (Spearman's test).

3.2. Anti-tTG antibodies recognize pregnant uterus structures

Based on the reduced levels of serum autoantibodies against tTG in pregnant NOD mice on day 9 of gestation compared with nonpregnant NOD mice, we hypothesized that they could have been absorbed into the pregnant uterus; thus, we explored whether maternal–placental interface structures can specifically bind anti-tTG

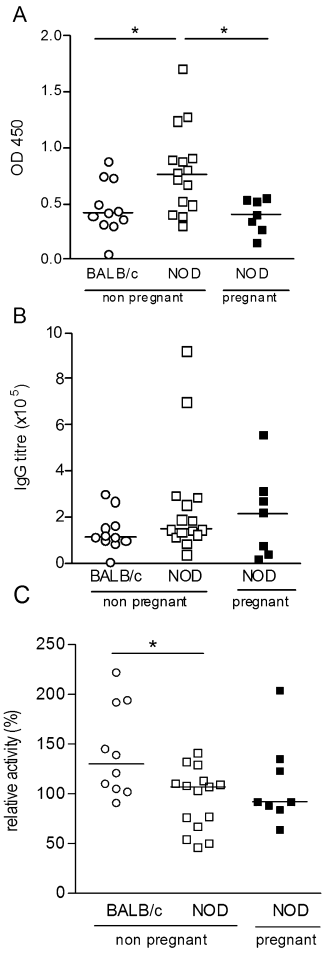

Fig. 1. Anti-tTG antibody determination in mice sera. Levels of specific IgG against tTG determined by ELISA are shown for 16-week-old BALB/c and NOD mice under nonpregnant conditions and for pregnant NOD mice on day 9 of gestation (A). Titers of total IgG antibodies are shown for each individual (B). *In vitro* transamidating tTG activity was evaluated under basal conditions and in the presence of 1:100 diluted mice serum. Relative activity (%) = OD serum × 100/basal OD is indicated for each sample (C). *Statistically significant difference between groups is indicated ($P < 0.05$, Mann–Whitney test).

antibodies. Slices of viable implantation sites from NOD mice on day 9 of gestation were incubated with an anti-tTG monoclonal antibody (2G3) or BALB/c mice as a negative control. Fig. 2A and B shows specific labeling of basal cytoplasm of epithelial cells and uterine glands by the monoclonal anti-tTG antibody. Also, small cells within the endometrial stroma were positive for 2G3 immunostaining. There was no label with BALB/c control mice antibodies (Fig. 2C).

3.3. tTG expression in pregnant NOD macrophages

On the basis that tTG is required to form an efficient engulfing portal for the clearance of apoptotic cells by macrophages, and that this process is essential in normal pregnancy, we next explored tTG expression on the surface of NOD macrophages. Fig. 3A shows that resting peritoneal macrophages isolated from pregnant NOD mice expressed tTG. More than 90% of macrophages expressed the enzyme with a major surface membrane localization as revealed by immunofluorescence. Besides, we investigated whether enzyme expression was modified by pregnancy in peritoneal macrophages. Fig. 3B shows that the macrophages of day 9 pregnant NOD mice expressed significantly higher levels of tTG than nonpregnant, age-matched NOD mice. Interestingly, when BALB/c mice macrophages were analyzed for tTG expression, a high level of expression was found even in nonpregnant mice and their levels were similar to those of NOD pregnant macrophages (not shown).

3.4. Inhibitory effect of anti-tTG antibodies on the phagocytosis of apoptotic cells by NOD mice macrophages

To address if anti-tTG antibodies could impair phagocytosis of apoptotic cells by resting peritoneal macrophages of pregnant NOD mice, we used the anti-tTG monoclonal antibody. Fig. 4A shows that when macrophages from pregnant NOD mice expressing tTG on their surface were pre-incubated with anti-tTG antibody, they reduced their capacity to phagocyte apoptotic cells. In particular, both the ability to engulf apoptotic bodies (percentage phagocytosis) and the avidity to engulf more than one apoptotic body (phagocytic index) of the macrophages of pregnant NOD mice was significantly reduced by the anti-tTG antibody (Fig. 4A and B, respectively). Fig. 4C shows representative microphotographs of apoptotic cell engulfment by NOD macrophages. The inhibitory effect of the anti-tTG antibody on phagocytosis was significantly higher than the <5% inhibition of phagocytosis shown by a normal mouse serum (1:30) used as a control for the different NOD mice macrophages tested (data not shown).

4. Discussion

Reproductive health is often compromised in untreated celiac women (Ozgor and Selimoglu, 2010). Recurrent early miscarriages (Ciacci et al., 1996) and premature and low-birth-weight babies (Martinelli et al., 2000) have been reported, but the mechanisms underlying these pregnancy complications are still unknown.

Tissue transglutaminase, the main autoantigen of CD, is a multifunctional enzyme (Belkin, 2011) that is widely expressed in most tissues, including the human maternal–fetal interface (Robinson et al., 2006; Kabir-Salmani et al., 2005). It controls cell and tissue homeostasis through its involvement in proliferation, terminal differentiation, and apoptosis (Fesus and Piacentini, 2002; Rossin et al., 2012). At the cell surface, tTG is involved in adhesive and signaling functions acting as a co-receptor through its association with $\beta_1/\beta_3/\beta_5$ integrins, thus playing a central role in cell adhesion and migration (Akimov and Belkin, 2001). Extracellular tTG contributes to the stabilization of the extracellular matrix and tissue repair provided its high affinity interaction with fibronectin (Upchurch et al., 1991) and owing to transamidating activity, tTG also mediates TGF-β1 incorporation into the extracellular matrix in order to facilitate its activation (Verderio et al., 1999).

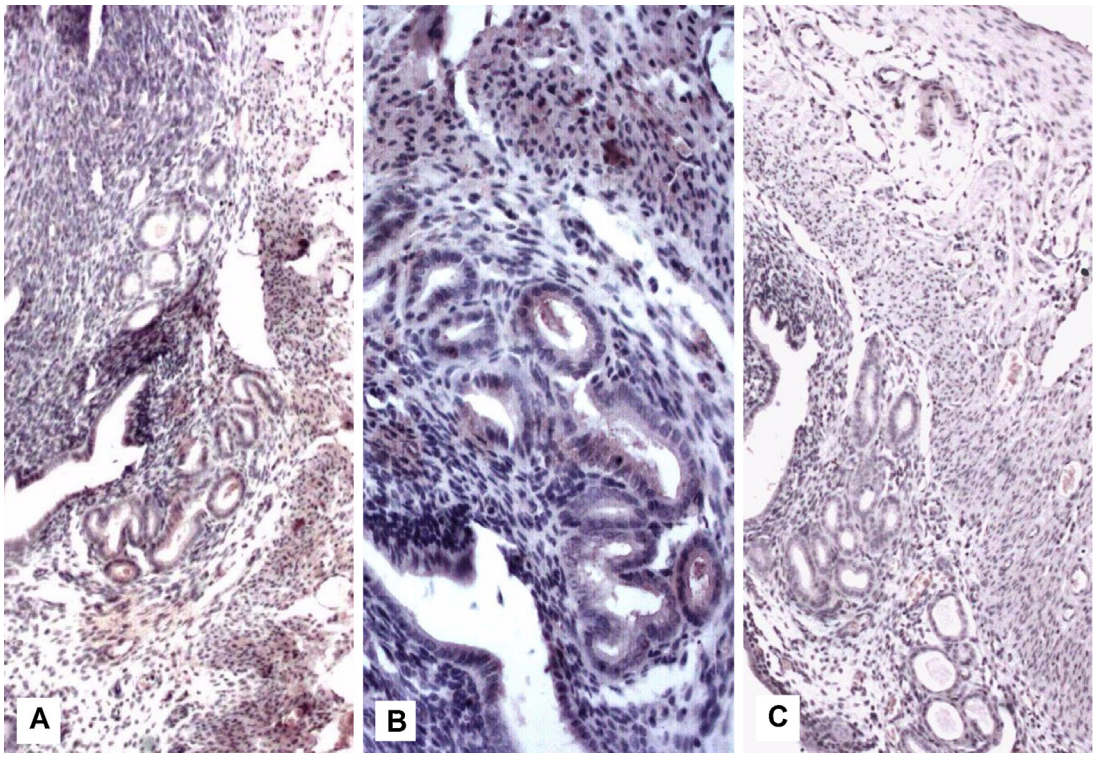

Fig. 2. Anti-tTG antibodies bind to uterine structures. Histological sections of NOD pregnant endometrium were incubated with anti-tTG monoclonal antibody 2G3 (A, B) or BALB/c serum (C). NOD mice implantation sites isolated on day 9 of gestation were fixed for immunohistochemistry studies as described in Section 2. Photographs are representative of at least three experiments with different NOD mice uterine slices. A, C (100×); B (400×).

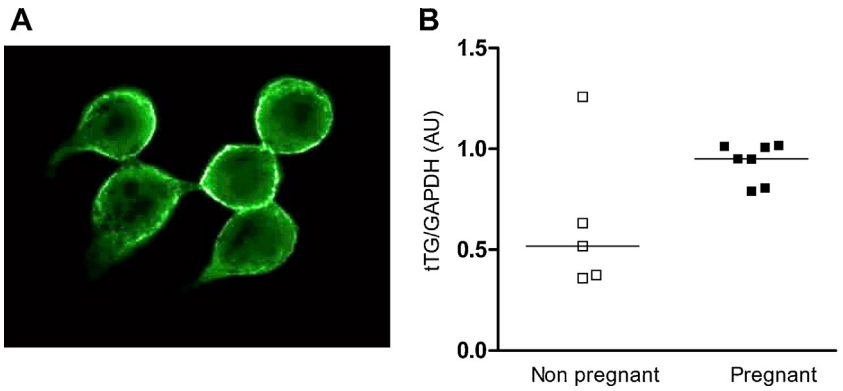

Fig. 3. tTG expression in the peritoneal macrophages of NOD mice by immunofluorescence and RT-PCR. Resting peritoneal macrophages isolated from pregnant NOD mice were incubated with anti-tTG monoclonal antibody 2G3 (1:60) and anti-mouse immunoglobulin FITC-conjugated antibody (1:100) representative microphotograph (1000×) (A). tTG expression levels determined by RT-PCR are shown for 16-week-old NOD mice that were either nonpregnant or pregnant on day 9 of gestation and results shown are median values as described in Section 2 (B).

In addition, macrophage surface tTG is involved in the clearance of apoptotic cells (Toth et al., 2009).

Immunological mechanisms of reproductive disorders in CD can be postulated on the basis of the presence of antibodies against this enzyme, which participates in endometrial physiological processes throughout the menstrual cycle, during decidualization (Signorini et al., 1988) and implantation (Kabir-Salmani et al., 2005). In line with this, we have recently reported an association of reproductive complications and anti-tTG antibody titers in women with CD (Sonora et al., 2011).

Based on reports in patients with CD and on the knowledge that pre-diabetic NOD mice spontaneously produce anti-tTG antibodies and show early pregnancy complications, we analyzed the presence of anti-tTG antibodies in pregnant NOD mice serum and its potential impact on macrophage phagocytic function during early gestation. Here, we present evidence indicating that anti-tTG antibodies are present in NOD mice sera, decrease transamidating activity, bind to the NOD pregnant endometrial cells and inhibit the phagocytosis of apoptotic cells by pregnant NOD mice macrophages that express

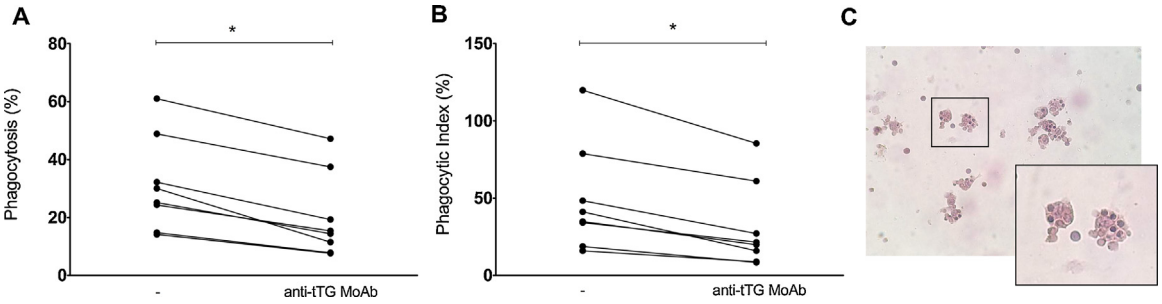

Fig. 4. Anti-tTG antibody inhibits apoptotic cell phagocytosis by the macrophages of pregnant NOD mice. Peritoneal macrophages were isolated from pregnant NOD mice on day 9 and cultured for 90 min with apoptotic thymocytes in the presence or absence of anti-tTG monoclonal antibody 2G3 as indicated in Section 2. Percentage of phagocytosis (A) and the phagocytic index (B) were calculated for each individual mouse sample. *Statistically significant differences between groups are indicated ($P < 0.05$, Wilcoxon matched pairs signed rank test). Representative microphotographs of apoptotic cell phagocytosis by NOD macrophages 100× and detail 400× (C).

tTG. These conclusions are based on the following results. First, higher concentrations of anti-tTG antibodies were found in NOD mice of 16 weeks of age compared with age-matched BALB/c mice, and these antibodies inhibit enzyme transamidating activity *in vitro*. Second, circulating anti-tTG antibodies in NOD mice sera were reduced in circulation on day 9 of gestation, reaching the normal levels seen in nonpregnant BALB/c mice. Third, tTG expression was significantly higher in macrophages of pregnant NOD mice compared with nonpregnant mice, and the monoclonal anti-tTG antibody impaired the phagocytosis of apoptotic cells by pregnant NOD macrophages, an effect that was not seen with a normal mouse serum.

The fact that tTG expressed in endometrial structures can be recognized by specific autoantibodies could have implications in the outcome of pregnancy. The decrease in tTG-specific antibodies in NOD mice serum at day 9 of pregnancy suggests a rapid *in vivo* recognition and clearance from circulation. In line with this, immunohistochemistry studies indicated that tTG-specific monoclonal antibodies recognize this epitope on different structures of the pregnant uterus of NOD mice. Moreover, since tTG transamidating activity is involved in wound healing and tissue remodeling and here we showed that anti-tTG antibody sera reduced transamidating activity of the enzyme *in vitro*, it is conceivable that these antibodies might play a pathogenic role in the impaired pregnancy score of NOD mice. In line with this, low levels of anti-tTG antibodies found in control BALB/c mice serum inhibited neither tTG transamidation activity nor bound uterine structures.

Inhibition of tTG enzyme activity and function by specific antibodies has been reported in different *in vitro* assays altering endothelial permeability, angiogenesis, and cell differentiation and proliferation (Caja et al., 2011), arguing that anti-tTG antibodies could be involved in pathogenesis. In particular, macrophage surface tTG appears to be involved in the clearance of apoptotic cells by promoting efficient signaling in the phagocytic cell, acting as a β3 integrin co-receptor in macrophages (Toth et al., 2009). In the absence of tTG, integrin β3 does not adequately recognize apoptotic cells, with the consequent impairment in the formation and stabilization of engulfing portals (Toth et al., 2009). Of note, $tTG^{-/-}$ mice presented a defective clearance of apoptotic cells and developed autoimmunity in an age-dependent manner (Szondy et al., 2003).

The inhibition of phagocytosis of apoptotic cells by the macrophages of pregnant NOD mice shown here supports tTG antibodies playing a pathogenic role during early pregnancy. The effect was consistently found in all individually tested macrophages of pregnant NOD mice, whereas phagocytosis was not modified by normal mice serum, suggesting the specificity of tTG recognition on macrophages. On the other hand, the evidence that tTG is increased in pregnant compared with nonpregnant NOD mice suggests that probably macrophage tTG is involved in different processes occurring at the maternal–placental interface other than the silent clearance of apoptotic cells. In fact, our results are consistent with previous observations on the upregulation of tTG expression during macrophage differentiation. In those studies, tTG accumulated rapidly and reversibly in mouse peritoneal macrophages cultured in mouse serum or plasma or previously primed *in vivo* with thioglycolate, but not when they were stimulated with pro-inflammatory bacterial stimuli (Murtaugh et al., 1983). Interestingly, the expression of tTG markedly increased in macrophages during differentiation from monocytes (Murtaugh et al., 1983; Mehta et al., 1985) and this effect has been associated with an enhanced migration on fibronectin (Mehta et al., 1985; Akimov and Belkin, 2001). Consistently, the down-regulation of surface tTG and its functional inhibition by blocking antibodies significantly decreased adhesion and spreading of monocytic cells as well as reducing the migration of myeloid cells (Akimov and Belkin, 2001). It is probable that more than one of these mechanisms could be operating *in vivo* in early pregnancy in NOD mice, particularly the adequate formation of engulfing portals by the blockade of macrophage tTG by autoantibodies might play a significant role. Moreover, on the knowledge that a constant level of about 20–30% of macrophages are found in deciduas throughout pregnancy and that this requires the permanent migration of monocytes from circulation to the pregnant uterus where they differentiate to macrophages (Fest et al., 2007; Mor and Cardenas, 2010), and considering the reported up-regulation of tTG during monocyte to macrophage differentiation, the increased tTG expression

in the macrophages of pregnant NOD mice might reflect a pregnancy-associated state of activation.

To our knowledge, this report describes for the first time that tTG-binding antibodies present in the serum of NOD mice can limit tTG activity and that the specific recognition of tTG expressed on pregnant NOD mice macrophages interfere with the phagocytosis of apoptotic cells. Our data provide new evidence to support a possible link between anti-tissue transglutaminase antibodies and impaired reproductive scoring reported in NOD mice.

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