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Differential actions of glycodelin-A on Th-1 and Th-2 cells: A paracrine mechanism that could produce the Th-2 dominant environment during pregnancy

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Running Title: Differential actions of GdA on Th-1/Th-2 cells

Key terms: Glycodelin, T-helper cells, Th-1/Th-2, cell death, Fas, extracellular signal-regulated kinases

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Abstract

BACKGROUND: The maternal-fetal interface has unique immunological response towards the implanting placenta. It is generally accepted that a T-helper type-2 (Th-2) cytokine prevailing environment is important in pregnancy. The proportion of Th-2 cells in the peripheral blood and decidua is significantly higher in pregnant women than in nonpregnant women in the first trimester. Glycodelin-A (GdA) is a major endocrine-regulated decidual glycoprotein thought to be related to feto-maternal defense. Yet the relationship between its immunoregulatory activities and the shift towards Th-2 cytokine profile during pregnancy is unclear. **METHODS:** GdA was immunoaffinity purified from human amniotic fluid. T-helper, T-helper type-1 (Th-1) and Th-2 cells were isolated from peripheral blood. Viability of these cells was studied by XTT assay. Immunophenotyping of CD4/CD294, cell death and GdA-binding were determined by flow cytometry. The mRNA expression, surface expression and secretion of Fas/Fas ligand (FasL) were determined by qPCR, flow cytometry and ELISA, respectively. The activities of caspase-3, -8 and -9 were measured. The phosphorylation of extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinase were determined by Western blotting. **RESULTS:** Although GdA bound to both Th-1 and Th-2 cells, it had differential actions on the two cell types. GdA induced cell death of the Th-1 cells but not the Th-2 cells. The cell death was mediated through activation of caspase-3, -8 and -9 activities. GdA up-regulated the expression of Fas and inhibited the ERK activation in the Th-1 cells, which might enhance the vulnerability of the cells to cell death caused by trophoblast-derived FasL.
CONCLUSION: The data suggest that GdA could be an endometrial factor that contributes to enhancing the Th-1/Th-2 shift during pregnancy.
Introduction

Placenta is genetically a fetal semiallograft in the maternal body, and mechanisms have evolved to suppress the maternal immune response in the uterine tissue during pregnancy (Trowsdale and Betz, 2006). One of these mechanisms is change in the decidual leukocyte population (Luppi, 2003). The altered population of immune cells at the maternal-fetal interface not only allows the mother to tolerate the fetus but also to interact with the trophoblasts, thereby creating an environment that is favorable for fetal development (Luppi, 2003). Contrary to their abundance in the peripheral blood, T-cells represent a minor population of immune cells in early decidua (Loke et al., 1995), partly due to apoptosis of the leukocytes. The trophoblast cells express Fas ligand (FasL), which induces apoptosis of the Fas-expressing leukocytes (Runic et al., 1996; Green and Ferguson, 2001). Other proposed mechanisms for the causing T-cells a minority population in the deciduas include inhibition of T-helper cells proliferation by indoleamine 2,3-dioxygenase from antigen-presenting cells (Mellor et al., 2002), ligation of the inhibitory programmed death ligand 1 on uterine T cells (Guleria et al., 2005), and selective enrichment of decidual natural killer cell (Bulmer and Lash, 2005).

Despite the reduction of T-cell population in the decidua, considerable amount of T-cells are present around the extravillous trophoblasts, decidual stroma, endometrial gland and decidual vessels (Vassiliadou and Bulmer, 1998; Michimata et al., 2002). T-helper cells are classified into T-helper type 1 (Th-1) and Th-2 according to the cytokines they secrete (Mosmann et al., 1986). It
is generally thought that successful pregnancy is a Th-2 type cytokine predominant phenomenon. The percentage of peripheral blood (Saito et al., 1999b) and decidual (Michimata et al., 2002) Th-2 cells is significantly higher in pregnant women than in nonpregnant women in the first trimester. The shift from the production of inflammatory Th-1 cytokines towards Th-2 type cytokines promotes immune protection of the trophoblasts (Dealtry et al., 2000; Michimata et al., 2002; Straszewski-Chavez et al., 2005). Pregnancy loss is associated with increased Th-1/Th-2 cytokine ratio (Daher et al., 2004). However, several Th-1 cytokines such as IFN-γ and TNF-α have shown to be important in uterine vascular remodelling and implantation (Chaouat, 2007), suggesting that the Th1/Th2 paradigm for pregnancy may be too simplistic.

Glycodelin-A (GdA) is an immunosuppressive glycoprotein abundantly expressed in the decidualized endometrium (Seppala et al., 2002; Seppala et al., 2007). It induces apoptosis of lymphocytes (Lee et al., 2009) and monocytes (Tee et al., 2008), skewing of T-cell response towards Th-2 phenotype (Mishan-Eisenberg et al., 2004), and modulates the activities of natural killer cells (Lee et al., 2010), B-cells (Yaniv et al., 2003), and dendritic cells (Scholz et al., 2008). Recently, GdA was demonstrated to suppress the cytolytic activity of CD8+ T-cell (Soni and Karande, 2010). The immunosuppressive activities of GdA are believed to be related to fetomaternal defense (Clark et al., 1996). The serum and decidual concentration of GdA peaks around week 10 of pregnancy, consistent with a role in survival of the fetoplacental unit (Seppala et al., 2002). Decreased maternal serum glycodelin is associated with early spontaneous abortion (Salim et al., 2007) and recurrent...
miscarriage (Dalton *et al.*, 1998).

The mechanisms that generate the Th-2 cytokine-rich environment during pregnancy are not fully known. We hypothesized that GdA has differential actions on Th-1 and Th-2 cells, contributing to the Th1/Th2 shift. Therefore, the objectives of this report were to study the actions of GdA on Th-1/Th-2 cell ratio, and to compare the actions of GdA on the two cell types in terms of cell death, Fas/FasL expression and intracellular signaling.

**Materials and Methods**

**Purification of glycodelin from human amniotic fluid, seminal plasma and cumulus matrix**

The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster approved the protocol of this study. Glycodelin isoforms including glycodelin-A, glycodelin-S (GdS) and glycodelin-C (GdC) were purified from amniotic fluid, seminal plasma and cumulus matrix, respectively, by affinity chromatography using monoclonal anti-glycodelin antibody (Clone F43-7F9) as described (Riittinen *et al.*, 1989; Lee *et al.*, 2009). In brief, the collected samples were diluted with tris-buffered saline (TBS, pH 7.4) and 0.1% Triton X-100 in a ratio of 1:3-1:5 was added. They were loaded onto anti-glycodelin column, which was then washed successively by TBS, 1M NaCl with 1% isopropanol, 10 mM ammonium acetate with 0.1% isopropanol, pH 5 and TBS. Glycodelin was eluted by 20 mM CaCl₂ with 0.1% trifluoroacetic acid. The eluted GdS and GdC were further purified with anion-exchange Mono-Q (GE Healthcare)
column by AKTA purifier 10 (GE Healthcare). Deglycosylated glycodelin was prepared by

denaturation of GdA in 0.1% β-mecaptoethanol before incubation with 0.5 mU N-Glycosidase F at

37°C for 24 hours (Lee et al., 2009). The concentrations of glycodelin were determined by a protein

assay kit (Bio-Rad, Hercules, USA).

Isolation of human peripheral T-helper cells and enrichment of Th-2 cells

Human non-pregnant female peripheral blood was obtained from the Hong Kong Red Cross.

Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation was used to isolate

the PBMCs. The contaminated red blood cells and the adherent cells were removed by the red blood

cell lysing buffer (0.084% NaHCO₃, 0.83% NH₄Cl and 0.003% ethylenediaminetetra-acetic acid)

and by adhesion to plastic culture flask, respectively. T-helper cells (CD3⁺CD4⁺) were isolated by

negative immuno-magnetic separation using CD4⁺ T-cell isolation kit II (Miltenyi Biotec Inc.,

Bergisch Gladbach, Germany). The purity of the CD3⁺CD4⁺ cells increased to 90-95% after

processing.

Th-2 cells were then positively selected by the anti-CD294 (Chemoattractant receptor of Th-2

cells, CRTH-2) MicroBead Kit (Miltenyi Biotec Inc.). The purity of the CD4⁺CD294⁺ Th-2 cells

was >85% after processing (Supporting information Figure S1). The cell population that did not

bind to the anti-CD294 antibody column (CD4⁺CD294⁻) was considered as an enriched Th-1 cell

preparation. To verify that the enriched Th1 and Th2 cells were producing polarised cytokines we
have analyzed cytokines in the culture supernatant by ELISA. The isolated Th-2 cells secreted
significantly (P<0.05) less IL-2, IL-8 and IFN-γ (Th-1 cytokines) and more IL-10 (Th-2 cytokine)
than that of the enriched Th-1 cells as determined by ELISA (Supporting information Table ST1).
The cells were resuspended in 10% fetal bovine serum supplemented RPMI 1640 medium (Sigma,
St. Louis, MO).

Immunophenotyping of T-helper cells

Cells (5x10^5) were treated with different concentrations (0.01-1 μg/mL) of GdA in 500 μL of
culture medium for 48 hours before the immunophenotyping. In brief, treated cells were
successively washed twice with PBS and once with 1% BSA containing 0.1% sodium azide in PBS.
The cells were then incubated with anti-CD4-FITC (T-helper cell marker); anti-CD294-PE (Th-2
cell marker); anti-Fas-FITC; anti-FasL-PE and PE/FITC-conjugated mouse isotypic control (BD
Biosciences, San Jose, CA) in 1% BSA and 0.1% sodium azide in PBS. The cells were analyzed by
a flow cytometer using 525 nm and 575 nm band pass filters and the results were evaluated by the
WinMDI 2.8 (The Scripps Research Institute Cytometry Software, http://facs.Scripps.edu/software.html). The non-viable cells were removed by gating with forward
scatter/side-scatter.

Cell viability assay
Cells (3x10^4) were incubated with 0.001-1 μg/mL of GdA, GdS, GdC or deglycosylated glycodelin in 100 μL of culture medium for 36 hours before cell viability determination. Cell viability was determined by the XTT assay (Roche Diagnostics Co., Basel, Switzerland). In brief, freshly prepared XTT labeling mixture (50 μL) was added to the cell culture 12 hours before the end of the experiment. The absorbance was measured at 450 nm with λ correction at 595 nm. The cell viability was expressed as Suppression Index = (Absorbance of treated cells - Absorbance of blank)/(Absorbance of control - Absorbance of blank) × 100%.

Cell death analysis

Cells (5x10^5) were treated with 0.01-1 μg/mL of GdA in 500 μL of culture medium for 48 hours. Apoptotic and necrotic cell deaths were determined by flow cytometry using Yo-Pro®-1 and propidium iodide dye (Invitrogen, Carlsbad, CA). The treated cells were washed twice with PBS, incubated with Yo-Pro®-1 (1 μL) and propidium iodide (1 μL) in 1 mL PBS for 15 minutes, and analyzed immediately by flow cytometer using the 525 nm and 610 nm band pass filters. The data were analyzed by WinMDI 2.8.

Determination of Fas/FasL mRNA and secreted sFas/sFasL

Cells (5x10^5) were treated with GdA (0.01-1 μg/mL) in 500 μL of culture medium for 48 hours. The QuickPrep RNA extraction kit (Stratagene, La Jolla, CA) was then used to extract total RNA.
from the cells. RNA was reverse transcribed using the TaqMan reverse transcription reagent kit
(Applied Biosystems, Foster City, CA) and multiscrypt reverse transcriptase. qPCR was performed
using the TaqMan PCR core reagent kit (N8080228, ABI Biosystems). In brief, cDNA sample (1 µL)
was mixed with 2x TaqMan universal PCR master mix, Fas or FasL target primers and probe (ABI
Biosystems) and 18S internal control primers and probe (ABI Biosystems) in a 96-well reaction
plate (ABI Biosystems). The reactions were performed in triplicates. PCR was performed at 50°C
for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1
minute in an ABI 7500 system (ABI Biosystems). The Ct values of the Fas and FasL experiments
was <35 and that of 18S was <20, respectively. The relative quantification value (RQ) was
calculated by $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The data was present as relative
expression = (RQ of glycodelin-treated cells - RQ of negative control)/(RQ of control cells - RQ of
negative control).

The levels of soluble Fas (sFas) and FasL (sFasL) in the culture supernatant of the treated cells
were measured by ELISA according to the manufacturer’s protocol (Bender Medsystem®,
Burlingame, CA). Briefly, the microwell coated with sFas or FasL monoclonal antibodies were
washed twice with 300 µL of wash buffer. One hundred microlitres of culture medium or standard
was then added to the well, washed and incubated with biotin-conjugated detector antibodies for 2
hours at 37°C. The unbound material was washed away and streptavidin-HRP (100 µL per well) was
added. Color development was performed using 100 µL of 3,3′,5,5′-tetramethylbenzidine as
chromogen. The reaction was stopped by the addition of 2 M sulphuric acid (1000 μL/well) and the absorbance was measured immediately at 450 nm with λ correction at 595 nm in an ELISA plate reader (Infinite F200, Tecan, Männedorf, Switzerland).

Caspase-3, -8 and -9 activity assays

Cells (1x10⁶) were treated with different concentrations (0.01-1 μg/mL) of GdA in 500 μL of culture medium for 48 hours before the caspases activity assay. Caspase activities were determined with the use of synthetic substrates of caspase-3 (Z-DEVD-R110, Invitrogen), caspase-8 (IETD-pNA, Invitrogen) and caspase-9 (Ac-LEHD-pNA, Millipore). Cells were washed with PBS and lysed with 50 μL of cell lysis buffer provided by the assay kit at 4°C for 1 hour. The cell lysate (50 μL) was then mixed with different caspase substrates in 50 μL of reaction buffer and incubated in dark according to the manufacturer’s protocols. The fluorescence intensity or absorbance was measured immediately after incubation. Caspase activities were expressed as relative activity (%) = (Absorbance of glycodelin-treated cells - Absorbance of blank)/(Absorbance of control cells - Absorbance of blank) × 100%.

Western blot analysis of MAPK/ERK activation

The action of GdA on the MAPK/ERK activation in T-helper cells (1x10⁶) was determined by western blot analyses after treatment of GdA (1 μg/mL) for 6 hours followed by the
phytohaemagglutinin (5 μg/mL) stimulation for 30 minutes. Cells were lysed with CytoBluster™ protein extraction reagent (Novagen®, Darmstadt, Germany) at 4°C for 2 hours in the presence of a cocktail of protease inhibitors (Calbiochem, San Diego, CA). The protein lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for Western blot analysis of the components of the MAPK pathways using anti-ERK, anti-phospho-ERK, anti-p38 (BD Biosciences), anti-phospho-p38, anti-JNK and anti-phospho-JNK antibodies (Cell signaling, MA). Density of the protein bands were measured by Quantity One software (Bio-Rad) and the density values are present as relative quantities = (Density of glycodelin treated cells)/(Density of control).

**GdA binding assay**

GdA was labeled with Alexa Flour®488 according to the manufacturer’s protocol (Invitrogen). Cells (5x10⁵) were incubated with the labeled GdA (1 μg/mL) for 2 hours. Unbound GdA was removed by washing with PBS twice. The samples were then re-suspended in 500 μL of PBS and analyzed by flow cytometry. The fluorescence signals were measured using 525 nm band pass filter and the data were analyzed by WinMDI 2.8 software.

**Data Analysis**

All values were expressed as mean ± SEM. For all experiments, the non-parametric ANOVA on Rank test for comparisons was used to identify differences between groups. If the data were
normally distributed, parametric Student’s t-test or non-parametric Mann Whitney U test were used where appropriate as the post-test. The data were analyzed by SigmaStat 2.03 (Jandel Scientific, San Rafael, CA) with a P-value less than 0.05 was considered as significant.

Results

GdA increased the proportion of Th-2 cells in the T-helper cell population

T-helper cells (CD3⁺CD4⁺) were isolated by negative immuno-magnetic separation and treated with different concentrations of GdA. The non-viable cells were removed by gating with forward scatter/side-scatter. In the viable population, the treatment significantly increased the proportion of CD4⁺CD294⁺ cells (Th-2 cells) after 48 hours (Figure 1). Treatment with 1 µg/mL of GdA increased the percentage of Th-2 cells from 1.05±0.07% (Control) to 1.34±0.08% (P<0.05). As a result, the ratio of viable CD4⁺CD294⁻ (Th-1) cells to CD4⁺CD294⁺ (Th-2) cells decreased from 94.33 to 72.56 (P<0.05). The change in Th1/Th2 ratio could be due to a decrease in Th-1 cells or an increase in Th-2 cells. Therefore, we studied the action of GdA on the two isolated sub-populations.

GdA induced cell death of the Th-1 cells but not the Th-2 cells

GdA at concentrations ≥0.1 µg/mL significantly decreased (P<0.05) the viability of the isolated T-helper cells as demonstrated by the XTT assay (Table 1). The suppression index of the cells treated with 1 µg/mL of GdA was 53.67±8.94% (P<0.05), which was significantly lower than that of...
Consistently, GdA dose-dependently induced both apoptosis and necrosis of T-helper cells (Figure 2). The viability of the cells treated with 1 µg/mL of GdA decreased from 91.33±1.05% (Control) to 69.34±4.81% (P<0.05), whereas the proportion of apoptotic and necrotic cells increased from 3.56±0.16 (Control) to 15.74% (P<0.05) and from 5.08±0.67% (Control) to 14.69±2.09% (P<0.05), respectively. Differentially glycosylated glycodelin isoforms, glycodelin-S and glycodelin-C, and deglycosylated glycodelin had no effects on the viability and cell death of the T-helper cells.

Positive selection process for CD294+ was used to isolate Th-2 cells from the total T-helper cells. The selection process decreased the viability of the Th-2 cells (~80%), which was lower than that of the Th-1 cells (~90%). Similar reduction in Th-1 cell viability was observed after incubating the cells with CD45 microbeads for positive selection (Supporting information Figure S2). GdA did not affect cell death of the isolated Th-2 cells. This was in striking contrast to that of the Th-1 sub-population, the viability of which was significantly (P<0.05) reduced from 90.13 ± 0.90% to 68.82 ± 4.58% as a result of increases in apoptotic and necrotic cell death after GdA (1 µg/mL) treatment (Figure 2, Table 2).

GdA upregulated the Fas expression in isolated T-helper cells and Th-1 cell

The effects of GdA on Fas/FasL expression were analyzed by quantitative polymerase chain
reaction, flow cytometry and ELISA (Table 2). GdA dose-dependently increased the mRNA and surface expression of Fas. At 1 μg/mL of GdA, the Fas mRNA expression was significantly (P<0.05) upregulated by 2.34±0.62 fold in isolated T-helper cells, and the cell surface Fas expression was also significantly (P<0.05) increased from 34.10±1.41% (Control) to 45.26±2.17%. In contrast, GdA did not affect the FasL expression and sFas secretion in the isolated T-helper cells. The level of sFasL in the culture medium was low and barely detectable.

The effect of GdA on Fas expression was also determined in the enriched Th-1 and Th-2 cells by flow cytometry (Table 2). GdA treatment (1 μg/mL) significantly enhanced (P<0.05) the Fas expression of Th-1 cells from 33.17±3.80% to 54.78±3.97, but had no effect on the Th-2 cells.

GdA enhances the caspase activity in isolated T-helper cells and Th-1 cells

The caspase-3, -8 and -9 activities of T-helper cells were investigated using specific substrates (Table 3). As compared to the controls without treatment, 1 μg/mL of GdA significantly (P<0.05) increased the caspase-3, -8 and -9 activity to 151.25±24.66%, 120.10±11.85% and 130.34±10.75%, respectively of the control values. GdA had no significant effect on the expression level of non-activated pro-caspase-3, -8 and -9 as determined by Western blotting analysis.

In the enriched Th-1 cell sub-population, incubation with 1 μg/mL GdA significantly increased (P<0.05) the caspase-3 and caspase-9 activities to 163.89±8.18% and 133.72±10.24% of the control values. Although the caspase-8 activity was increased to 119.11±6.81%, the difference did not reach
GdA had no effect on caspase-3, -8 and -9 expression in the Th-2 cells.

**GdA suppresses ERK activation in isolated T-helper cells and Th-1 cells**

The expression and activation by PHA of ERK, p38 and JNK were determined by Western blot analysis. GdA dose-dependently reduced the phosphorylated-ERK level, but not that of phosphorylated-JNK nor phosphorylated-p38 in the T-helper cells (Figure 3A). GdA at a concentration of 1 μg/mL significantly reduced the levels of the 42 kDa and the 44 kDa phosphorylated-ERK to 0.48±0.11 and 0.61±0.09 (P<0.05) respectively, as compared to the control without treatment. GdA treatment did not affect the expression or phosphorylation of p38 or JNK.

GdA suppressed (P<0.05) the phosphorylated-ERK level in the PHA-stimulated Th-1 cells but not the Th-2 cells (Figure 3B). Treatment with 1 μg/mL of GdA decreased (P<0.05) the expression of phosphorylated-p42 and p44 ERKs in the Th-1 cells to 0.58±0.09 and 0.57±0.10, respectively, but had no effect on the non-phosphorylated ERK.

**GdA had similar binding on Th-1 and Th-2 cells**

The binding of fluorescent labeled GdA on Th-1 and Th-2 cells was determined by flow cytometry (Supporting information Figure S3). The percentage of Th-1 cells with bound GdA (91.17±0.90%) was not significantly different from that of Th-2 cells (93.77±0.84%), indicating that the binding of GdA to these cells was similar.
Discussion

The maintenance of pregnancy requires a Th-2 cytokine dominant environment (Dealtry et al., 2000; Michimata et al., 2002). Dysregulation of Th-1 and Th-2 cells is associated with implantation failure and recurrent pregnancy loss (Daher et al., 2004). The ratio of Th-1/Th-2 cells decrease drastically in the peripheral blood and early decidua of pregnant women when compared with the nonpregnant one (Saito et al., 1999a; Saito et al., 1999b; Michimata et al., 2002). Such a change has been proposed to be resulted from selective modulation of differentiation, chemoattraction, and proliferation of Th-2 cells and death of Th-1 cells. GdA stimulates the Th-2 type cytokine shift in T-cells (Mishan-Eisenberg et al., 2004). However, the specific mechanisms of action are still unclear. This study provides evidence for a novel mechanism of GdA in shifting the Th-1/Th-2 balance, i.e. differential actions on Th-1 and Th-2 cells in terms of selective induction of cell death, concomitant with increased expression of Fas in the Th-1 cells.

Cytotoxic action of GdA on T-cells has been reported (Sundarraj et al., 2008; Lee et al., 2009). In this study, both the T-helper and enriched Th-1 cells responded similarly to GdA-mediated cell death. It is because the major population in the peripheral blood T-helper cells was the Th-1 cells, and Th-2 cells only constitute a minor proportion ranging from 0.4-6.5% (Nagata et al., 1999). The cytotoxic action of GdA on Th-1 cells is glycan-dependent, consistent with the reported contribution of the glycosylation of GdA to its binding to (Ish-Shalom et al., 2006) and induction of cell death in
lymphocytes (Lee et al., 2009). On the other hand, GdA has no effect on the isolated Th-2 population.

The percentage of peripheral blood Th-2 cells is significantly higher in pregnant women than in nonpregnant women in the first trimester of pregnancy (Saito et al., 1999a). The dosage (0.1-1 μg/ml) of GdA exerting its biological activities in this study is within the concentration range of GdA (0.2-1.2 μg/ml) in the peripheral blood of women in their first trimester of pregnancy (Seppala et al., 2002), suggesting that the observations could be physiologically relevant. Due to the high abundance of GdA in the first-trimester decidual tissue, it is possible that GdA may possess similar actions on decidual T-cells. However, experimental evidence on this possibility is still lacking.

GdA increased apoptotic and necrotic cell death of the Th-1 subpopulation. Previous studies had reported differential effects of apoptotic and necrotic cells in modulating the activities of other immune cells. For example, the presence of apoptotic, but not the necrotic T-cells, up-regulates IL-10 production from macrophages (Chung et al., 2007). The development of dendritic cells is also affected by apoptotic T-cells (Newton et al., 2003). Therefore, the changes in apoptotic and necrotic T-cell population induced by GdA may further modulate the immune response of pregnant women.

The results of this study showed that GdA-induced cell death in both T-helper cells and Th-1 cells are associated with the increase in caspase-3, -8, -9 activities. This suggests that both mitochondrial-independent and mitochondrial-dependent pathways are involved. Previous studies showed that both native and recombinant GdA induced apoptosis in T-cells through the
mitochondrial-dependent pathway as indicated by influx of mitochondrial membrane calcium ion and involvement of caspase-9 and Bcl-2 activities (Sundarraj et al., 2008). There are no reports on the involvement of mitochondrion-independent pathway in GdA-mediated cell death of lymphocytes, whereas both recombinant glycodecin and native GdA have been reported to induce cell death of monocytes via caspase-8 pathway (Tee et al., 2008).

A novel observation in this study is the differential induction of GdA on Fas expression in T-cells; it was enhanced by GdA in the Th-1, but not the Th-2 cells. Fas/FasL pathway regulates clonal deletion of T-cells at the fetomaternal interface (Coumans et al., 1999; Jerzak and Bischof, 2002), as well as in some other immunologically privileged sites, such as the anterior chamber of the eye and the testis (Green and Ferguson, 2001). The trophoblasts express FasL (Runic et al., 1996), while the T-helper cells produce both Fas and FasL (Ramsdell et al., 1994). Binding of FasL to Fas receptor induces trimerization of the Fas receptor, which activates the Fas-associated death domain and the caspase cascade leading to apoptosis. Most importantly, the Th-1 cells express more Fas receptors than the Th-2 cells do, and they are more susceptible to Fas/FasL-induced cell death (Roberts et al., 2003). Thus, the up-regulation by GdA of Fas receptor in the Th-1 cells may further increase vulnerability of these cells to death-induction by the FasL derived from the trophoblasts or lymphocytes.

In this study, we further demonstrate the suppressive effect of GdA on ERK activation in the Th-1 cells. ERK activation is important in T-cell activation, homeostasis and cytokine secretion.
(Dong et al., 2002). It has been correlated with Th-1 response (Borovskiy et al., 2002), and regulation of the expression of Th-1 cytokines including IL-1 (Wang et al., 2004) and IFN-\(\gamma\) (Mainiero et al., 1998) in different cell types. The ERK activation in T-cells also inhibited the cells to Fas-mediated apoptosis (Holmstrom et al., 2000). Therefore, apart from inducing Fas expression, GdA treatment may sensitize Th-1 cells to Fas receptor-mediated apoptosis by suppressing ERK activation in the cells, as has been shown in primary peripheral T-cells after inhibition of ERK signaling (Holmstrom et al., 2000). It is of interest that GdA also suppresses ERK activation in spermatozoa (Yeung et al., 2009) and trophoblast cells (Lam KKW and Chiu PCN, unpublished data), suggesting that ERK may have a central role in GdA signaling in different biological systems. Consistently, the lack of cytotoxic activity of GdA on Th-2 cells is associated with absence of ERK suppression in these cells.

The observation of similar binding of GdA onto Th-1 and Th-2 cells suggests that GdA binding alone does not contribute to the differential response of Th-1 and Th-2 cells to GdA treatment. However, the observation does not exclude the possible presence of different GdA receptors with the same affinity in the two cell types. CD45 has been proposed as a possible GdA receptor in T-cells (Rachmilewitz et al., 2003). Differential expression of the CD45RC isoform in CD4\(^+\) T-cells sub-populations is associated with differences in cytokine production upon stimulation; the CD45R\(^{\text{high}}\) sub-population produces mainly type-1 cytokines including IL-2, while the CD45R\(^{\text{low}}\) sub-population produces IL-17, IL-10 and Th-2 cytokines (Ordonez et al., 2009). Alternatively, the
two cell types may have the same GdA receptor but different intracellular signaling leading to the observed differential action of GdA. The current data cannot distinguish between these possibilities.

We concluded that GdA may involve in shifting the Th-1/Th-2 ratio in the peripheral blood and decidua by selectively reducing the Th-1 cell population, both directly through induction of cell death of Th-1 cells and indirectly through enhancing the expression of Fas and suppression of ERK activation in the Th-1 cells, thereby enhancing their vulnerability to cell death induced by trophoblast-derived FasL. Overall, the present data uncover the mechanisms in part by which GdA contributes to immunoprotection of the fetoplacental unit during human pregnancy.

Acknowledgments

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Figure legends

**Figure 1 Effect of GdA on T-helper type-1 and type-2 cells population.** T-helper cells (5x10^5) were incubated with 0.01, 0.1 and 1 μg/mL of GdA for 48 hours. Cells were stained with CD4-FITC and CD294-PE for immunophenotyping of Th-1 and Th-2 cells. Data are mean ± SEM, N=9, * P<0.05, P values are shown for significant differences as compared to control.

**Figure 2 Effect of glycodelins on cell death of T-helper, Th-1 and Th-2 cells.** T-helper, Th-1 and Th-2 cells (5x10^5) were incubated with 0.01, 0.1 and 1 μg/mL of GdA, -S, -C and deglycosylated glycodelin (De-Gd) for 48 hours. Viable, necrotic and apoptotic cells were quantified by bivariate Yo-Pro®-1/PI flow cytometry. Cells without stain were viable. Cells labeled with Yo-Pro®-1 only were apoptotic cells. Cells labeled with Yo-Pro®-1 and PI were necrotic cells. Data are mean ± SEM, N=5 (T-helper cells), N=9 (Th-1 and Th-2 cells), * P<0.05, P values are shown for significant differences as compared to control.

**Figure 3 Effect of GdA on ERK activation in T-helper, Th-1 and Th-2 cells. (A) T-helper, (B)** Th-1 and Th-2 cells (1x10^6) were incubated with 1 μg/mL of GdA for 6 hours followed by PHA stimulation for 30 minutes. Protein expressions were determined by Western blotting. Representative blots are shown. The ERK/pERK protein bands were measured by densitometry. Data are mean ± SEM, N=4. * P<0.05, P values are shown for significant differences as compared to control.
density values are present as relative quantities = (Density of glycodelin treated cells)/(Density of control).
Table 1. Effect of Glycodelins on viability of T-helper cells in XTT assay.

<table>
<thead>
<tr>
<th>Glycodelin (μg/mL)</th>
<th>GdA</th>
<th>GdS</th>
<th>GdC</th>
<th>De-Gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>88.98 ± 6.23</td>
<td>100.07 ± 1.54</td>
<td>102.22 ± 1.08</td>
<td>106.60 ± 2.42</td>
</tr>
<tr>
<td>0.01</td>
<td>70.16 ± 11.38</td>
<td>99.39 ± 1.60</td>
<td>102.01 ± 1.75</td>
<td>103.18 ± 2.64</td>
</tr>
<tr>
<td>0.1</td>
<td>54.62 ± 9.26 *</td>
<td>101.56 ± 2.30</td>
<td>103.28 ± 2.03</td>
<td>100.57 ± 2.66</td>
</tr>
<tr>
<td>1</td>
<td>53.67 ± 8.94 *</td>
<td>105.92 ± 3.94</td>
<td>104.94 ± 3.30</td>
<td>105.61 ± 6.16</td>
</tr>
</tbody>
</table>

T-helper cells (3x10^4) were incubated with 0.001, 0.01, 0.1 and 1μg/mL of GdA, -S, -C and deglycosylated glycodelin (De-Gd) for 48 hours. XTT labeling mixture was added 12 hr before measurement. Data are mean ± SEM, N=4, * P<0.05, P values are shown for significant differences as compared to control. Suppression index (%) = (Absorbance of Gd - Absorbance of blank)/(Absorbance of control-Absorbance of blank) × 100%
Table 2. Effect of GdA on Fas/FasL mRNA expression of T-helper, Th-1 and Th-2 cells.

<table>
<thead>
<tr>
<th>GdA (μg/mL)</th>
<th>T-helper cells</th>
<th>Surface expression (%)</th>
<th>mRNA expression (Relative expression)</th>
<th>Soluble component secretion (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T-helper cells</td>
<td>Th-1 cells</td>
<td>Th-2 cells</td>
<td>T-helper cells</td>
</tr>
<tr>
<td>Fas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>34.10 ± 1.41</td>
<td>33.17 ± 3.80</td>
<td>16.98 ± 1.54</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>41.70 ± 5.07</td>
<td>-</td>
<td>-</td>
<td>1.36 ± 0.44</td>
</tr>
<tr>
<td>0.1</td>
<td>41.89 ± 5.67</td>
<td>-</td>
<td>-</td>
<td>1.45 ± 0.27</td>
</tr>
<tr>
<td>1</td>
<td>45.26 ± 2.17 *</td>
<td>54.78 ± 3.97 *</td>
<td>19.43 ± 1.99</td>
<td>2.34 ± 0.62 *</td>
</tr>
<tr>
<td>FasL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22.50 ± 3.61</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>25.5 ± 4.48</td>
<td>-</td>
<td>-</td>
<td>0.82 ± 0.15</td>
</tr>
<tr>
<td>0.1</td>
<td>24.46 ± 4.98</td>
<td>-</td>
<td>-</td>
<td>0.87 ± 0.12</td>
</tr>
<tr>
<td>1</td>
<td>24.33 ± 4.16</td>
<td>-</td>
<td>-</td>
<td>1.80 ± 0.86</td>
</tr>
</tbody>
</table>

T-helper, Th-1 and Th-2 cells (5x10^5) were incubated with 0.01, 0.1 and 1 μg/mL of GdA for 48 hours. Fas and FasL surface expression were determined by flow cytometry. Fas and FasL mRNA expression was quantified by qPCR. sFas and sFasL secretion to the culture medium were determined by ELISA. Data are mean ± SEM, N=5, * P<0.05, P values are shown for significant differences as compared to control. The level of sFasL was undetectably low. Relative expression = (RQ of glycodelin treated cells - RQ of negative control)/(RQ of control treated cells - RQ of negative control)
Table 3. Caspase-3, -8 and -9 activities of GdA treated T-helper cells.

<table>
<thead>
<tr>
<th>GdA (μg/mL)</th>
<th>T-helper cell</th>
<th>Caspase-3</th>
<th>Th-1 cell</th>
<th>Th-2 cell</th>
<th>Caspase-8</th>
<th>Th-1 cell</th>
<th>Th-2 cell</th>
<th>Caspase-9</th>
<th>Th-1 cell</th>
<th>Th-2 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>112.25 ± 4.60*</td>
<td>-</td>
<td>-</td>
<td>115.03 ± 12.19</td>
<td>-</td>
<td>-</td>
<td>111.34 ± 11.22</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>116.83 ± 6.70*</td>
<td>-</td>
<td>-</td>
<td>111.45 ± 10.22</td>
<td>-</td>
<td>-</td>
<td>123.22 ± 8.22</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>151.25 ± 24.66*</td>
<td>163.89 ± 8.18*</td>
<td>110.33 ± 4.69</td>
<td>120.10 ± 11.85*</td>
<td>119.11 ± 6.81</td>
<td>107.40 ± 3.51</td>
<td>130.34 ± 10.75*</td>
<td>133.72 ± 10.24*</td>
<td>95.96 ± 14.91</td>
<td></td>
</tr>
</tbody>
</table>

T-helper, Th-1 and Th-2 cells (1x10⁶) were incubated with 0.01, 0.1 and 1 μg/mL of GdA for 48 hours. Activities of caspase-3, -8 and -9 were determined by caspase activity assay (N=6). Data are mean ± SEM, * P<0.05, P values are shown for significant differences as compared to control. Caspase activities were expressed as relative activity (%) = (Absorbance of glycodelin-treated cells - Absorbance of blank) / (Absorbance of control cells - Absorbance of blank) × 100%.
Control

GdA (0.01 \( \mu \) g/mL)

GdA (0.1 \( \mu \) g/mL)

GdA (1 \( \mu \) g/mL)

CD4+CD294- (Th-1): 95.53 ± 0.27%
CD4+CD294+ (Th-2): 1.05 ± 0.07%
Th-1/Th-2: 94.33 ± 6.33

CD4+CD294- (Th-1): 95.26 ± 0.39%
CD4+CD294+ (Th-2): 1.15 ± 0.08%
Th-1/Th-2: 86.42 ± 6.56

CD4+CD294- (Th-1): 95.02 ± 0.42%
CD4+CD294+ (Th-2): 1.13 ± 0.07%
Th-1/Th-2: 87.08 ± 5.99

CD4+CD294- (Th-1): 94.98 ± 0.34%
CD4+CD294+ (Th-2): 1.34 ± 0.08% *
Th-1/Th-2: 72.56 ± 4.16 *
**Figure 2.**

<table>
<thead>
<tr>
<th>T-helper cell</th>
<th>Viable</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.33 ± 1.05</td>
<td>3.56 ± 0.16</td>
<td>5.08 ± 0.67</td>
</tr>
<tr>
<td>GdA 0.01 µg/mL</td>
<td>89.45 ± 0.24</td>
<td>3.98 ± 0.28</td>
<td>6.57 ± 0.48</td>
</tr>
<tr>
<td>GdA 0.1 µg/mL</td>
<td>71.82 ± 0.33 *</td>
<td>15.59 ± 0.36 *</td>
<td>12.60 ± 0.16 *</td>
</tr>
<tr>
<td>GdA 1 µg/mL</td>
<td>69.34 ± 4.81 *</td>
<td>15.74 ± 3.82 *</td>
<td>14.69 ± 2.09 *</td>
</tr>
<tr>
<td>GdS 1 µg/mL</td>
<td>92.40 ± 0.96</td>
<td>3.83 ± 0.50</td>
<td>3.65 ± 0.58</td>
</tr>
<tr>
<td>GdC 1 µg/mL</td>
<td>92.52 ± 0.80</td>
<td>3.51 ± 0.51</td>
<td>3.75 ± 0.54</td>
</tr>
<tr>
<td>De-Gd 1 µg/mL</td>
<td>92.09 ± 0.91</td>
<td>3.42 ± 0.36</td>
<td>4.33 ± 0.73</td>
</tr>
<tr>
<td>Th-1 Cell</td>
<td>90.13 ± 0.90</td>
<td>3.19 ± 0.57</td>
<td>6.68 ± 0.90</td>
</tr>
<tr>
<td>GdA 1 µg/mL</td>
<td>68.82 ± 4.58 *</td>
<td>14.44 ± 1.89 *</td>
<td>16.73 ± 3.87 *</td>
</tr>
<tr>
<td>Th-2 Cell</td>
<td>80.38 ± 1.29</td>
<td>7.86 ± 1.22</td>
<td>11.63 ± 1.55</td>
</tr>
<tr>
<td>GdA 1 µg/mL</td>
<td>76.64 ± 2.72</td>
<td>9.55 ± 1.58</td>
<td>13.89 ± 2.01</td>
</tr>
</tbody>
</table>
Figure 3.

A

<table>
<thead>
<tr>
<th>GdA (μg/ml)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-tubulin</td>
<td><img src="alpha-tubulin.png" alt="Image" /></td>
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<td><img src="alpha-tubulin.png" alt="Image" /></td>
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<tr>
<td>ERK</td>
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<tr>
<td>pERK</td>
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<td><img src="pJNK.png" alt="Image" /></td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Th-1 cell</th>
<th>Th-2 cell</th>
</tr>
</thead>
<tbody>
<tr>
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<td><img src="alpha-tubulin.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="ERK.png" alt="Image" /></td>
<td><img src="ERK.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="pERK.png" alt="Image" /></td>
<td><img src="pERK.png" alt="Image" /></td>
</tr>
</tbody>
</table>

### Densitometry (Relative quantification)

<table>
<thead>
<tr>
<th>GdA (μg/mL)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK (p42)</td>
<td>0.89 ± 0.12</td>
<td>1.02 ± 0.02</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>pERK (p42)</td>
<td>0.80 ± 0.22</td>
<td>0.60 ± 0.09 *</td>
<td>0.48 ± 0.11 *</td>
</tr>
<tr>
<td>ERK (p44)</td>
<td>0.96 ± 0.03</td>
<td>0.99 ± 0.05</td>
<td>0.97 ± 0.03</td>
</tr>
<tr>
<td>pERK (p44)</td>
<td>0.89 ± 0.11</td>
<td>0.72 ± 0.03 *</td>
<td>0.61 ± 0.09 *</td>
</tr>
<tr>
<td>p38</td>
<td>1.09 ± 0.02</td>
<td>1.11 ± 0.10</td>
<td>1.09 ± 0.09</td>
</tr>
<tr>
<td>pp38</td>
<td>1.03 ± 0.06</td>
<td>0.91 ± 0.07</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>JNK (p46)</td>
<td>1.01 ± 0.04</td>
<td>0.93 ± 0.04</td>
<td>0.94 ± 0.08</td>
</tr>
<tr>
<td>pJNK (p46)</td>
<td>Not detected</td>
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<tr>
<td>JNK (p54)</td>
<td>1.03 ± 0.04</td>
<td>1.14 ± 0.08</td>
<td>1.09 ± 0.18</td>
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<td>pJNK (p54kD)</td>
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<td>1.01 ± 0.03</td>
<td>1.07 ± 0.06</td>
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</tbody>
</table>

### Densitometry (Relative quantification)

<table>
<thead>
<tr>
<th>GdA (1 μg/mL)</th>
<th>Th-1</th>
<th>Th-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK (p42)</td>
<td>1.01 ± 0.20</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>pERK (p42)</td>
<td>0.58 ± 0.09 *</td>
<td>0.88 ± 0.13</td>
</tr>
<tr>
<td>ERK (p44)</td>
<td>1.05 ± 0.14</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>pERK (p44)</td>
<td>0.57 ± 0.10 *</td>
<td>0.83 ± 0.17</td>
</tr>
</tbody>
</table>