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Research article

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ARTICLE INFO

ABSTRACT

Pregnancy is known to be associated with an increased demand for insulin that is normally compensated by an increased beta cell mass and insulin secretion. Recent studies have suggested enhanced beta cell function during pregnancy in women with type 1 diabetes (T1D). To explore the possible mechanisms behind enhanced beta cell function during pregnancy in women with T1D we investigated the impact of circulating factors in serum from nine women from each group of pregnant women with and without T1D, after pregnancy and non-diabetic non-pregnant women on rat islet cell proliferation and apoptosis, and on T-lymphocyte activation. In addition, circulating levels of pancreatic hormones and selected cytokines and adipokines were measured. Rat islet cell proliferation was higher in serum from pregnant women with T1D ($p < 0.05$) compared to T1D women after pregnancy. Apoptosis in INS-1E cell was lower ($p < 0.05$) in serum from pregnant women with T1D compared to T1D women after pregnancy. T-lymphocyte cell (Jurkat) proliferation was reduced by serum from pregnant women without T1D only ($p < 0.05$). Higher C-peptide levels and lower levels of ghrelin, IL-6, MCP-1, IL-8 and adipsin were observed in pregnant women with T1D compared to T1D women after pregnancy. In conclusion, the improved beta cell function in women with T1D during pregnancy may be due to lower levels of proinflammatory cytokines and/or higher levels of pregnancy-associated growth factors.

1. Introduction

Human pregnancy is characterized by an increase in insulin resistance that is compensated by an increase in insulin secretion to maintain euglycemia [1]. Type 1 diabetes (T1D) is characterized by very low levels of insulin production, but a few patients with up to 50 years duration of diabetes maintain residual functional beta cells [2]. In some women with T1D C-peptide levels were found to increase during pregnancy suggesting augmented insulin synthesis [3, 4, 5]. These findings indicate that recovery of pancreatic beta cell function occurs during pregnancy in pre-existing autoimmune conditions and this may be a key therapeutic target.

In rodents, reversible increase in beta cell mass occurs during pregnancy through hypertrophy, increased replication and decreased apoptosis of the beta cells [6]. Also in pregnant women, an increased beta cell mass has been described [7, 8] but the increased number of beta cells

seems to be due to neogenesis of beta cells from putative progenitor cells rather than by proliferation of existing beta cells [7]. We have recently found evidence for neogenesis of beta cells in mice during pregnancy by detection of neurogenin-3 (*Ngn3*), a marker of endocrine progenitors, expression in the pancreas [9]. In rodents after delivery, beta cell mass returns to non-pregnancy levels through increased beta cell apoptosis, decreased proliferation and reduced beta cell size [6]. During pregnancy, placental lactogen and prolactin play a central role in the adaptation of beta cell mass in rodents and possibly also in human pregnancy [10].

During pregnancy, T-helper 1 (Th1) lymphocytes undergo apoptosis through a Fas-mediated pathway and become less abundant, while T-helper 2 (Th2) lymphocytes become more abundant [11]. This may lead to an improvement in pre-existing autoimmune diseases such as thyroid autoimmune diseases, systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis [12, 13, 14, 15]. Circulating placental–derived factors such as progesterone, 17β-estradiol, placental lactogen, prolactin,

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chorionic gonadotrophin, and interleukin 4 have been implicated in the shift from Th1 towards Th2 immunity during pregnancy [13, 15].

The overall objective of this study was to investigate whether serum from pregnant women with and without T1D can induce rat beta cell proliferation and inhibit apoptosis and to search for possible mechanisms involved in the reversal of autoimmunity.

2. Materials and methods

2.1. Participants

As part of another study [3], blood samples were collected from nine women with clinical T1D at 33 (32–34) gestational weeks (type 1 diabetic pregnant) and within five days after delivery (type 1 diabetic controls). Seven of the nine samples were collected after delivery were from the same women as during pregnancy. Diabetes duration was 22 years (range 6–28) and HbA1c 6.0% (range 5.8–6.7) at the time of blood sampling in pregnancy. Nine non-diabetic pregnant women with uncomplicated pregnancies (non-diabetic pregnant) had blood sampled at gestational week 34 (32–38). Blood samples were also obtained from nine non-diabetic non-pregnant women (non-diabetic controls). All participants gave written informed consent. The research protocol was approved by the regional committees for ethics and science and by the Danish Data Protection Agency. The main clinical characteristics of the subjects are given in Table 1.

2.2. Blood samples

Blood samples were collected in the non-fasting state between 9 a.m. and 2 p.m. Blood samples were drawn into the Vacuette serum clot activator tube from an antecubital vein and centrifuged at 3000 g. Once a blood clot was formed, samples were separated into aliquots and stored at -80 °C until analysis.

2.3. Biomarker analysis

Serum circulating levels of insulin, C-peptide, ghrelin, adiponectin, leptin, adipsin, IL-6, MCP-1, IL-8, TNFα and IP-10 analyses were performed using multiplex suspension arrays (Bio-Plex, Cat. No. M5002JFT0D, ZB0000003I and 171A7002M) on a Luminex 100 analyzer (Bio-Rad Laboratories, Copenhagen, Denmark) according to the manufacturer's instructions.

2.4. Animals

All animal experiments were conducted under a protocol (J.nr. 2008/561–1515) approved by the Ethical Committee for Animal Experiments in Denmark. Animals were purchased from Taconic (Ry, Denmark).

2.5. Isolation of neonatal rat islets

Islets were isolated from neonatal (3–5 days old) rats as previously described [16]. Following isolation, islets were pre-cultured for 5–7 days in RPMI 1640 glutamax-1 medium (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL), in an atmosphere of air

with 5% CO_2 at 37 °C. The islets were dispersed into single cells by treatment with Accutase and 0.5 mM EDTA in PBS (Innovative Cell Technologies Inc, San Diego, USA) for 3 min at 37 °C. After incubation the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged for 3 min at 8000 rpm, followed by incubation in RPMI 1640 glutamax-1 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 1% of human serum albumin and placed in an atmosphere of air with 5% CO_2 at 37 °C for 15 min, and centrifuged as above. The supernatant was discarded, and the pellet was re-suspended in RPMI 1640 glutamax-1 medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 1% human serum albumin.

2.6. In vitro proliferation of neonatal rat islets cells

Neonatal rat islet single cells (2×10^4 cells/100 µl) were placed into a 96 well cell culture plate coated with 100 µl of 1 µg/ml of poly-L-lysine (Sigma-Aldrich, Denmark) for 2 h at room temperature and washed with 200 µl of PBS stored at 4 °C. The cells were incubated in 100 µl of RPMI 1640 glutamax-1 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µl of 10 µCi/ml [3H]thymidine (Amersham radioactive centre, UK) and 10% human serum to obtain the final concentration of 5% and placed in an atmosphere of air with 5% CO_2 at 37 °C. Cells were harvested after 72 h onto filter paper (BSI, UK, Cat. No. 78-115-05) using a semi-automated harvester Automash-2000 (Dynetech Laboratories, VA, USA). The filter papers were dried, cut into discs and then placed in tubes containing 2 ml of optiphase Hisafe 2 scintillation fluid (PerkinElmer, MA, USA). The radioactivity was measured using a Beckman scintillation counter.

2.7. T-lymphocyte proliferation

The Jurkat cell line has often been used as a prototypical for human T-lymphocytes to study multiple events in T-lymphocytes biology including a critical process in effective adaptive immune response [17]. Jurkat cells were suspended in RPMI 1640 glutamax-1 medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum. Jurkat cells (1×10^4 cells/100µl) were seeded into a 96 well cell culture plate followed by addition of 100 µl 40% serum from pregnant women to each well to obtain a final concentration of 20% and then cultured for 48 h in an atmosphere of air with 5% CO_2 at 37 °C. In the end, 10 µl of 10 µCi/ml [3H]thymidine was added to the culture medium for the last 4 h. Incorporation of thymidine was measured as described above using a Beckman scintillation counter.

2.8. INS-1E cell apoptosis

INS-1E (insulinoma cells) cells are generally accepted models for primary rat beta cells with glucose responsive insulin release [18, 19]. A total of 2×10^4 cells/well were seeded on 96-well poly-L-lysine coated plates and cultured in 10% RPMI 1640 glutamax-1 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum and 0.1% 2-mercaptoethanol (Sigma-Aldrich, Denmark) for 2 days at 37 °C. The medium was removed, and then the cells made quiescent by 0.5% fetal bovine serum for 24 h. After 24 h, the culture medium was removed, and cells were stimulated incubated with 20% serum from pregnant women for 16 h. Apoptosis was measured by

Table 1. Clinical characteristics of non-diabetic controls (ND-C), non-diabetic pregnant (ND-P), pregnant women with T1D (T1D-P) and T1D Controls (T1D-C). Data are shown as median (range). T1D-C samples were collected 5 days after delivery.

	ND-C (n = 9)	ND-P (n = 9)	T1D-P (n = 9)	T1D-C (n = 9)
Maternal age (years)	23 (22–36)	31 (26–43)	34 (28–39)	34 (28–39)
Pre-pregnancy BMI (kg/m^2)	20.8 (19.4–29.0)	22.5 (18.7–26.2)	25.0 (18.4–32.1)	24.2 (18.4–32.1)
Gestational age (weeks)	-	34 (32–38)	33 (32–34)	-
Duration of T1D (years)	-	-	22 (6–28)	19 (6–28)

examination of the presence of cytoplasmic histone-associated DNA fragments according to the manufacturer's instructions using Cell Death Detection ELISA[PLUS] kit (Roche, Indianapolis, IN, USA).

2.9. Statistical analysis

In vitro results were given as mean ± SEM. Biomarker Data was reported in box and whisker plot with median and 25–75% percentile, and minimum and maximum and all individual values. All the serum biomarker assays were performed in two individual assays with an average intra-assay CV ≤ 10% and inter-assay CV ≤ 15%. Significant differences between non-diabetic controls, non-diabetic pregnant, T1D pregnant and T1D controls were made by unpaired t-test and p-values ≤ 0.05 were considered as statistically significant. Statistical analysis was performed using Microsoft Excel 365 and GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

Serum-induced neonatal rat islet single cell proliferation was higher with serum from T1D pregnant (T1D-P) and non-diabetic pregnant (ND-P) compared to T1D controls (T1D-C) and non-diabetic controls (ND-C) respectively (p < 0.05, p < 0.005 and Figure 1A). Apoptosis of INS-1E cell was lower with serum from T1D pregnant and non-diabetic pregnant compared to T1D controls and non-diabetic controls respectively (p < 0.05, p < 0.0005 and Figure 1B). Serum-induced reduction in Jurkat cell proliferation was observed with serum from non-diabetic pregnant compared to non-diabetic controls but not with serum from T1D pregnant or from T1D controls women (p < 0.05 and Figure 1C).

C-peptide levels were higher in T1D pregnant compared to T1D controls (p < 0.05 and Figure 2). The C-peptide levels were higher in non-diabetic pregnant compared to non-diabetic controls (p < 0.05). Ghrelin levels were lower in T1D pregnant and non-diabetic pregnant compared to T1D controls and non-diabetic controls (p < 0.05 and p < 0.005). There were no statistically significant differences in insulin levels of non-diabetic pregnant compared to T1D pregnant due to insulin treatment of T1D women (data not shown).

IL-6 levels were lower in T1D pregnant compared to the T1D controls (p < 0.05) whereas IL-6 levels were higher in non-diabetic pregnant compared to non-diabetic controls (p < 0.005). Levels of MCP-1 and IL-8 both were lower during pregnancy compared to non-diabetic controls and T1D controls (MCP-1: both p < 0.0005; IL-8: p < 0.05 and p < 0.0005).

Lower levels of adiponectin were found in non-diabetic pregnant compared to T1D pregnant and non-diabetic controls (both p < 0.05). Leptin levels were higher in non-diabetic pregnant compared to non-

diabetic controls (p < 0.05). Adipsin levels were lower in T1D pregnant and non-diabetic pregnant compared to T1D controls and non-diabetic controls (both p < 0.05).

In this study, there was no statistically significant difference in levels of TNFα and IP-10 between the groups studied (data not shown).

4. Discussion

The improvement of the beta cell function during pregnancy of women with T1D, reflected in the increased C-peptide levels, can be achieved by several mechanisms.

4.1. Expansion of the beta cell mass

In rodent pregnancy, the beta cell adaptation mainly occurs by proliferation of the existing beta cells due to increased levels of placental lactogen and prolactin that have been shown to stimulate rodent beta cell proliferation and insulin production in vitro [10, 20, 21]. In the present study, serum from both T1D pregnant and non-diabetic pregnant induced proliferation of neonatal rat islet cells to the same extent indicating that the pregnancy-associated beta cell stimulating factor(s) are present in T1D. Circulating levels of placental lactogen increase proportionally with increasing gestational age in both pregnant women with T1D and non-diabetic pregnant [22]. During human pregnancy, an increase in the beta cell mass may rather be due to neogenesis than proliferation [7, 8]. We have found that serum from non-diabetic pregnant can induce *Ngn3* expression in pancreas from fetal rats in vitro [9] suggesting that circulating factors are involved in promoting beta cell formation during pregnancy. We have identified a number of peptides in serum from healthy pregnant women that may be involved in the increase in beta cell mass [23]. Recently we found that certain integrins are involved in the beta cell adaptation to pregnancy in the rat [24].

Apoptosis in INS-1E cells was lower both with serum from T1D pregnant and non-diabetic pregnant compared to T1D controls and non-diabetic controls. In vivo studies in non-diabetic pregnant rodents have shown similar findings [6]. The reduction in apoptosis supports an increased viability and beta cell expansion during pregnancy. This may also be due to an increase in circulating levels of lactogenic hormones during pregnancy as they have anti-apoptotic effects in beta cells [25, 26, 27]. However, no significant changes in apoptosis of beta cells in pancreatic sections from pregnant women were observed [7] suggesting that beta cell expansion in humans pregnancy occurs by neogenesis. Fetal factors like dlk-1 (Pref-1, FA1) and trefoil factors (TFF-3 in rat and human) that are abundant in the maternal circulation may contribute to the transient increase in beta cell formation in both mother and child [28, 29].
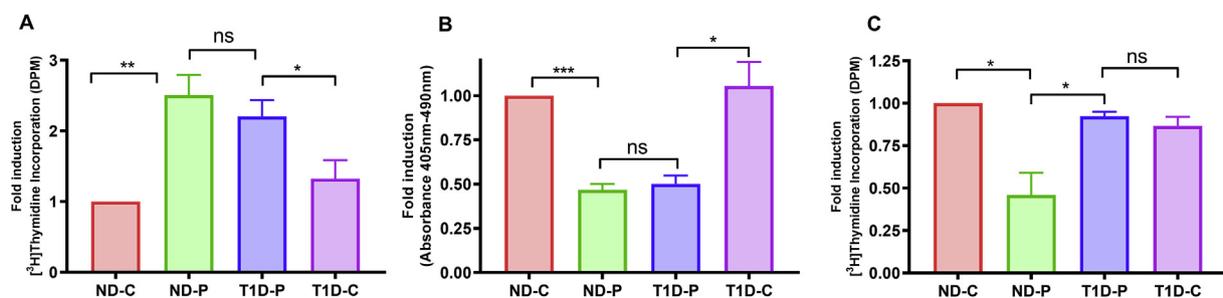

Figure 1. A) Proliferation of neonatal rat islet cells. Neonatal rat islet cells were stimulated with 5% pooled serum and cultured for 3 days. Cells were harvested and disintegrations per mint (DPM) of [3H]thymidine were counted. Data reported in fold induction of DPM of [3H]thymidine to non-diabetic controls of four independent experiments. **B) Apoptosis of INS-1E cells.** Apoptosis in INS-1E cells was induced with 20% of pooled serum. After 16 h of culture, apoptosis was measured by examination of the presence of cytoplasmic histone-associated DNA fragments in four independent experiments using Roche Cell Death Detection ELISA[PLUS]. The data reported in fold induction of absorbance to non-diabetic controls. **C) Proliferation of T-lymphocyte cells.** Jurkat cells were cultured for 48 h in 20% pooled serum and then [3H]thymidine was added to culture media for last 4 h and [3H]thymidine incorporation was measured as counts of disintegrations per minute (DPM) in three independent experiments. The data reported in fold induction to non-diabetic controls and data presented as mean ± SEM. *p < 0.05 **p < 0.005, ***p < 0.0005 and ns: not significant. Non-diabetic controls (ND-C), non-diabetic pregnant (ND-P), pregnant women with T1D (T1D-P) and T1D controls (T1D-C).
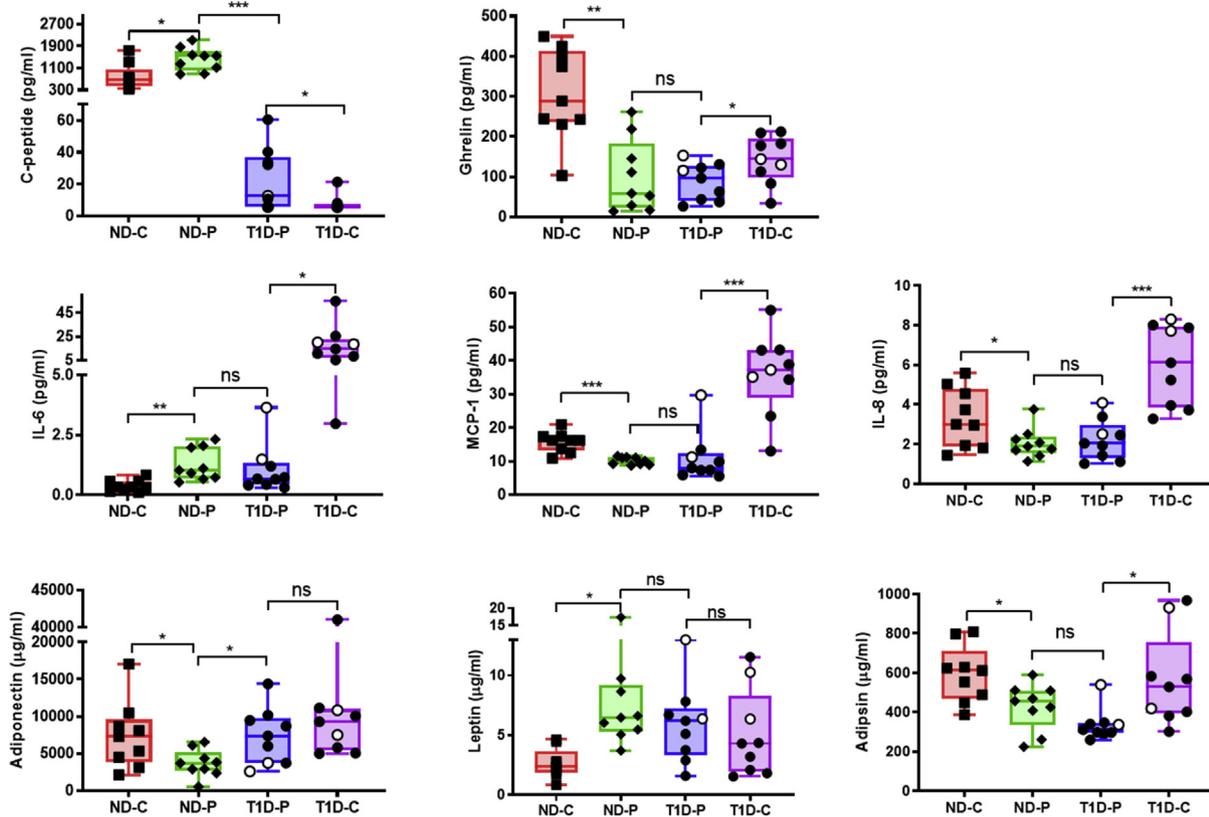
Figure 2. Circulating levels of C-peptide, Ghrelin, IL-6, MCP-1, IL-8, Adiponectin, leptin and adipsin in healthy non-pregnant, pregnant without and with T1D pregnant, and T1D women after pregnancy. Circulating pancreatic hormones, cytokine and adipokines were measured in serum from nine subjects (n = 9) in each group of non-diabetic controls (ND-C), non-diabetic pregnant (ND-P), pregnant women with T1D (T1D-P) and T1D controls (T1D-C). C-peptide levels at or below 5.7 pg/ml were set to detection limit. Data were reported in box and whisker plot with median and 25–75% percentile, and minimum and maximum with showing all individual values and p-values indicated as *p < 0.05 **p < 0.005, ***p < 0.0005 and ns: not significant. Seven of the nine women with T1D control samples collected after delivery were from the same women (filled circles) as during pregnancy and remaining two T1D control samples collected from different women (open circle).

4.2. Suppression of the autoimmune reaction against the beta cells

Proliferation of T-lymphocyte Jurkat cells were reduced by serum from non-diabetic pregnant but not by serum from T1D pregnant suggesting that the adaptive immune reaction is not suppressed in T1D pregnancy. Previously, it was found that antibodies to GAD65 were unchanged in pregnant women with T1D [3] suggesting that no change in the Th2 activity occurs in pregnancy. However, it is still possible that there is a reduction of the specific Th1 reactivity against beta cell antigens that are not reflected by the Jurkat cells. Interestingly, not only the glycemic control in the mother is transiently improved during pregnancy but also in a period postpartum [30] as well as in the offspring. Thus it was shown that offspring of T1D women had a much lower risk of developing T1D than offspring of T1D fathers [31] suggesting tolerance to autoantigens during T1D pregnancy. Very recently it was reported that offspring of T1D mothers had a reduced CD4+ T-cells to proinsulin and insulin due to increased induction of regulatory T-cells [32]. It is suggested that the maternal hyperglycemia increases the fetal (pro)insulin production that is transmitted to the mother leading to tolerance [33]. Early maturation of the fetal beta cells may thus protect the offspring against T1D in later life [34].

4.3. Reduction of the levels of proinflammatory cytokines

The present study showed a marked change in the circulating levels of cytokines and adipokines in T1D pregnant. IL-6 levels of T1D pregnant

were markedly reduced compared with the levels T1D controls. High levels of IL-6 were also found in non-pregnant youth with T1D [35, 36, 37]. It has been reported that prolactin suppresses IL-6 release from the adipose tissue [38] that may contribute to the low level in T1D pregnant women. However, the role of IL-6 in beta cell function is still controversial as both deleterious and protective effects on beta cells have been reported [39]. During healthy pregnancy leptin levels are higher and may enhance the mobilization of maternal fat stores to increase availability and transplacental transfer of lipid substrates to the fetus as reported in several studies [36]. The leptin levels can be influenced by several factors; such as placental synthesis of leptin, maternal adipose tissue, maternal energy expenditure and metabolism [36]. The reduced levels of ghrelin during pregnancy in both non-diabetic and T1D diabetic women suggest that the appetite regulation is affected [40]. Adipsin levels were low in both T1D pregnant and non-diabetic pregnant compared to controls and high levels of circulating glucose and insulin seem to be associated with the inhibition of adipsin [41].

Total adiponectin levels in T1D pregnant were comparable to T1D controls but levels were higher than in non-diabetic pregnant. Adiponectin levels increase with progression of T1D and are associated with vascular complications [42]. Low-grade systemic inflammation and other pregnancy-associated factors may be involved in low adiponectin levels during pregnancy [35, 43, 44]. It has been shown that the adiponectin-mediated induction of IL-6, MCP-1 and IL-8 is impaired in monocytes from T1D patients [45]. As adiponectin release is inhibited by prolactin in mice [46] the observed reduction in IL-6,

IL-8 and MCP-1 during pregnancy may be mediated by the lactogenic hormones.

Reduced levels of proinflammatory cytokines in T1D pregnant women may be involved in the improvement of the beta cell function. This is supported by recent studies of islets isolated from newly diagnosed T1D patients where the insulin-containing islets were initially unable to secrete insulin in response to glucose but recovered their function after being cultured for some days in vitro [47] suggesting that suppression of the diabetogenic environment may allow recovery of the secretory function. In fact, low levels of T-cell derived proinflammatory cytokines have been shown to stimulate beta cell proliferation and insulin secretion [48].

In summary, the improved beta cell function observed during T1D pregnancy is multifactorial and associated with high levels of pregnancy-associated growth factors including lactogenic hormones and fetal factors that may promote beta cell expansion by recruitment of progenitor cells and reduce the levels of inflammatory cytokines. In addition, alterations in an immunological shift from Th1 predominance towards Th2 predominance may suppress the autoimmunity in T1D pregnancy. Identification of the factor(s) in non-diabetic pregnant that suppress the activation of T-lymphocytes may be useful in the treatment of T1D. However, further investigations on T-lymphocyte subsets including regulatory T-cells during pregnancy in women with T1D are required.

Declarations

Author contribution statement

A. Nalla: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

L. Ringholm: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

S. Sørensen: Performed the experiments.

P. Damm and E. Mathiesen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

J. Nielsen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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