Circulating CD56⁺ Cells of Diabetic Women Show Deviated Homing Potential for Specific Tissues During and Following Pregnancy

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Abstract

Background—Human uterine natural killer (uNK) cells, the dominant lymphocytes in early pregnancy decidua, are important for spiral arterial remodelling. UNK cells are thought to arise from circulating CD56^{bright} NK cells that egress into decidualizing endometrium. Both incomplete spiral arterial modification and aberrant NK cell function have been linked with pre-eclampsia, a syndrome more prevalent in diabetic women. Previous *in vitro* studies showed that changes in decidual endothelium induced by type 1 diabetes reduce its interactions with circulating leukocytes. We hypothesized that diabetes additionally has direct effects on circulating CD56⁺ NK cells that impair their decidual homing potential.

Methods—Serial blood samples were collected from control, type 1 diabetic (T1D) and type 2 diabetic (T2D) pregnant women throughout and after pregnancy. *In vitro* adhesion under shear forces was used to assay the functional capacity of circulating leukocytes and of CD56⁺ cells to adhere to endothelium in cryostat sections of gestation day (gd)7 normal mouse decidua, pancreas and lymph node.

Results—Fewer CD56⁺ cells from diabetic versus control women adhered to normal decidual endothelium. The CD56⁺ cell/total cell adhesion ratio was also lower in diabetics. More diabetic CD56⁺ cells adhered to pancreatic endothelium and their proportion was greater than for controls. Neither absolute nor proportional adhesion of CD56⁺ cells to lymph node endothelium differed between diabetics and controls.

Conclusions—The CD56⁺ cell adhesion patterns of T1D and T2D women differ from those of non-diabetic women and support the hypothesis that diabetes impairs mechanisms that could be used by CD56⁺ cells for egress into decidua.

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AUTHOR'S ROLES

AVCS conducted leukocyte analysis, adhesion assay cell counts, data analyses and drafted the manuscript; SDB contributed to the design of the experiments, adhesion assay cell counts and contributed to data analyses and manuscript preparation; HR organized patient recruitment; BAC and GNS contributed to design of the experiments and to writing of the manuscript.

Keywords

decidua; pancreas; ITGA; SELL; diabetes; uterine Natural Killer cells

INTRODUCTION

Uterine Natural killer (uNK) cells are a unique, abundant, decidua-associated, cell type found early in the pregnancies of women and mice (Croy *et al.*, 2006; Lash *et al.*, 2010; Zhang *et al.*, 2010). UNK cells are strong promoters of endometrial angiogenesis and, in mice, are essential for initiation of remodelling of decidual spiral arteries, the major maternal nutrient supply vessels of implantation sites (Hu *et al.*, 2010; Li *et al.*, 2001; Quenby *et al.*, 2009; Zhang *et al.*, 2010). Roles for human uNK cells in early triggering of pregnancy-associated, spiral arterial remodelling are now recognized (Hazan *et al.*, 2010; Smith *et al.*, 2009). In humans, remodelling of spiral arteries is considered critical for complication-free pregnancy (Romero *et al.*, 2010). Both incomplete spiral arterial modification, and aberrant NK cell numbers, functions, or ratios to other immune cell types have been linked with pre-eclampsia (PE), fetal growth restriction (IUGR), and with recurrent pregnancy losses (Hiby *et al.*, 2004; Ledee *et al.*, 2008; Quenby *et al.*, 2009; Rieger *et al.*, 2009). This trio of pathologies occurs more frequently in diabetic than in healthy women, even in the presence of good glycemic control (Middleton *et al.*, 2010).

Whether human CD56^{bright} uNK cells arise from uterine progenitors or from circulating CD56^{bright} or other hematopoietic cells that home to implantation sites is not well defined. Recent reports strongly indicate that at least a significant proportion, if not all, mature human uNK cells arise from extra-uterine progenitors (Kitaya, 2008; Ledee *et al.*, 2008; Lee *et al.*, 2010; Male *et al.*, 2010; Manaster *et al.*, 2008). Adoptive transfer studies clearly show that progenitors of murine uNK cells home to the uterus (Chantakru *et al.*, 2002; Peel, 1989; Zhang *et al.*, 2009) and subsequently proliferate *in situ*. The adoptive cell transplantation approach also established that, in mice, uNK cells are the only cells able to initiate spiral arterial remodelling and that this is achieved by uNK cell-produced interferon gamma (ifng) (Ashkar *et al.*, 2000; Guimond *et al.*, 1998).

Our previous studies addressing mechanisms that might regulate homing of circulating human uNK precursor cells to decidua found that endocrine events at the time of the LH surge increase the ability of circulating CD56^{bright} cells to interact *in vitro* with endothelium present in tissue sections of decidualized uterus from normal mice (van den Heuvel *et al.*, 2005b; van den Heuvel *et al.*, 2008). Further, this interaction was remarkably reduced in infertile cycles and in embryo transfer recipients who failed to sustain their pregnancies (van den Heuvel *et al.*, 2005a; van den Heuvel *et al.*, 2008). The defined lymphocyte-endothelial cell interactions were localized to decidua basalis and they increased in frequency as decidua matured (i.e. when simultaneous assays of adhesion were conducted on sections of gestation day (gd)6 to gd10 deciduas) (Chantakru *et al.*, 2003a; Hatta *et al.*, 2009). L-selectin (SELL) and alpha 4 integrin (ITGA) were identified in blocking studies as the molecules supporting the functional, shear-force resistant interactions between viable blood NK cells and decidual

endothelium. Proteomic studies confirmed that there is a gain in ITGA in human CD56^{bright} blood NK cells after ovulation (Peralta *et al.*, 2008).

In subsequent investigations, we addressed the effect of type 1 diabetes (T1D) on the capacity of decidual endothelium to bind lymphocytes. Adhesion of lymphocytes from normal women under shear-force to gd7 decidualized uterus from hyperglycemic and normoglycemic non obese diabetic (NOD) mice was assessed (Burke *et al.*, 2007). Decidual endothelium from the hyperglycemic mice was dramatically less able to bind to CD56⁺ NK cells (Burke *et al.*, 2007). This indicated that diabetes had modified the addressins and/or adhesion counter-receptors on decidual endothelial cells. In the same study, splenocytes from hyperglycemic NOD mice were also found to be less adhesive than splenocytes from normoglycemic NOD mice to decidual endothelium from a normoglycemic mouse. This indicated that hyperglycemia also modified the function of mouse lymphocyte homing receptors (Burke *et al.*, 2007).

The insights from studies using hyperglycemic NOD mice combined with the clinical complications seen in pregnant diabetic women (T1D, type 2 diabetes (T2D) and gestational) (Evers *et al.*, 2004; Hanson and Persson, 1993; Hsu *et al.*, 1996) led us to postulate that circulating CD56^{bright} NK cells are themselves impaired in uterine homing potential by diabetes. We anticipate that this impairment would be a mechanism to compound the relative deficit in uNK cells that would result from diabetes-induced changes to decidual endothelium, further limit spiral arterial remodelling and thus contribute to diabetic pregnancy complications. Altered homing potential might be induced through metabolic effects which induce changes to receptors or to their mobility in NK cell membranes, or by the pancreas acting as a decoy tissue that diverts the uterine recruitment of CD56^{bright} NK cells, particularly in T1D. A number of mouse studies indicate that islet-homed NK cells make essential contributions to progression of T1D (Brauner *et al.*, 2010; Gur *et al.*, 2010; Ogasawara *et al.*, 2003; Rodacki *et al.*, 2007; Xia *et al.*, 2010).

To address our hypothesis, we serially assayed the functional capacity of circulating leukocytes and CD56⁺ cells from pregnant and post partum T1D, T2D and normal women to adhere to endothelium in gd7 cryostat sections of normal mouse decidua, pancreas and peripheral lymph nodes. Peripheral nodes were used as a positive control tissue because they contain high endothelial venules reactive in the adhesion assay and as a baseline representative peripheral tissue, remote from primary diabetic disease processes. The CD56⁺ cell adhesion patterns of T1D and T2D women deviated from those of non-diabetic women and support the hypothesis that, in diabetes, circulating CD56⁺ cells are reduced in potential for homing to decidualizing uterus.

MATERIALS AND METHODS

Participant recruitment and blood sampling

T1D, T2D and age-matched normal control women between the ages of 21–40 years old were recruited in their first trimester (11–13 weeks) of gestation) of a singleton pregnancy from Kingston General Hospital (Kingston, Ontario, Canada). Exclusion criteria included a previous diagnosis of hypertension, medication currently being taken to treat hypertension,

polycystic ovarian syndrome, cancer and/or the diagnosis of an autoimmune disease other than T1D. Patients were informed of all potential risks and signed consent forms before participating in the study. This study was approved by Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

Blood was collected into acid citrate dextrose (ACD) tubes once per trimester (approximately 12, 18–20 and 33–35 weeks gestation) and after six weeks post-partum at scheduled clinic visits. 37% of the patients reported breastfeeding at the time of the post partum sample donation. Six additional subjects were recruited to give a single blood sample for function blocking studies. Samples were blind encoded and sent to the research laboratory where lymphocytes were immediately isolated by density gradient centrifugation. For flow cytometry (Beckman Coulter FC 500 flow cytometer with CXP Software (Beckman Coulter, Mississauga, ON, Canada), cells were labelled with anti-CD3-Cy7 (Beckman Coulter) and anti-CD56-Cy5 to enumerate CD3⁺CD56^{bright} and CD3⁺CD56^{dim} NK cells per 100,000 cells (as per (Borzychowski *et al.*, 2005)). For adhesion assays 30×10^6 cells were needed. These were labelled with either anti-CD56-Cy5 (1:10; Beckman Coulter, Iotest, Fullerton, CA, USA) or with CMAC (Molecular Probes, Invitrogen, Burlington, ON, Canada) as previously reported (van den Heuvel *et al.*, 2005b). Patients completed a data collection form at each visit that was available during trial summary analysis.

Mice and tissue dissections

C57BL/6J female and male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), paired and checked each morning for a copulation plug. Gd0 was assigned to the morning of plug detection. Female mice were euthanized using tri-bromo-ethanol (250 mg/kg) followed by cervical dislocation on gd7 under protocols approved by Queen's University's Animal Care Committee. Uteri, pancreas and subcutaneous lymph nodes were dissected, embedded in OCT Cryomatrix (Shandon, Pittsburgh, PA, USA) and stored for use as substrate tissues at -80°C for a maximum interval of 10 days. Uterine draining and mesenteric lymph nodes were excluded from the lymph node pool.

Human leukocyte adhesion under shear force to frozen mouse tissue sections

Two sections (12 μm midsagittal) from each of the 3 mouse organs were melted onto individual positively charged microscope slides (Fisher Scientific, Mississauga, ON, Canada) immediately before use. They were transferred to a rotating (80 rpm) table held at 4°C and either 15×10^6 cells/mL CD56-Cy5-labelled or 15×10^6 cells/mL CMAC-labelled cells suspended in a volume of 300 μL RPMI were overlain onto the sections. Rotation was then increased (112 rpm; 30 min). For some leukocyte samples, function-blocking antibodies (a mixture of anti-human CD62L (eBioscience, San Diego, CA, USA) and anti-CD49d (Immunotech, Beckman Coulter, Mississauga, Canada) monoclonal antibodies) were employed as previously described (van den Heuvel *et al.*, 2005b). Sections were rinsed to remove non-adherent cells, fixed 30 min. in 4% neutral buffered paraformaldehyde (Sigma-Aldrich, Oakville, Ontario, Canada), rinsed and mounted with coverslips using AquaPolymount (PolySciences Inc., Washington, PA, USA) (Stamper and Woodruff, 1976). Numbers of adherent CMAC-tagged total leukocytes and of Cy-5-tagged CD56⁺ cells were enumerated in 25 high powered fields (HPF) ($\times 400$) under fluorescent microscopy. Counts

were made by 1 or 2 individuals (AVCS and SDB), who, in preparatory experiments, scored samples equivalently. For dually counted samples, a mean of the counts was used in statistical analysis. Adhering Cy-5-tagged CD56⁺ cells are referred to as CD56⁺ cell adhesion and the ratios of Cy-5-tagged CD56⁺ cells/CMAC⁺ cells as CD56⁺ cell ratio. Because the adhesion results for T1D and T2D were similar (not shown), pooled adhesion data are presented representing all of the diabetic patients.

Statistical analysis

Maternal and delivery characteristics were analyzed using one-way ANOVA with Bonferroni's post-test. Adhesion assay cell counts are presented as means $\pm$ SEM and were analyzed using two-way ANOVA with Bonferroni's post-test. A one-way ANOVA with Bonferroni's post-test was then performed on results that showed significance in the two-way ANOVA analysis to more accurately determine where statistical differences occurred in the data comparisons. A *P* value < 0.05 was considered statistically significant. Serial data were analyzed using the Prism 4.03 Statistical Software package (GraphPad, San Diego, CA).

RESULTS

Patient characteristics

A total of 10 control, 9 T1D, and 3 T2D women were recruited for serial blood donation in their first trimester of pregnancy. T1D women maintained blood glucose control pre-, during, and post-pregnancy through the use of an insulin pump. T2D women used an insulin pump for the duration of pregnancy but returned to their pre-pregnancy oral anti-diabetic drugs after delivery. Of the 9 serially studied T1D, 2 developed pre-eclampsia (PE) in the third trimester. While patients were initially recruited into the longitudinal study with the goal of donating all 4 samples (1 per trimester plus post partum), this was not always possible. A complete set of samples was collected from 9 patients (5 controls and 4 T1D). Out of 22 potential samples for each of the 4 time points, 20 were collected for first trimester, 18 for second trimester, 16 for third trimester and 11 for post-partum. Maternal demographics, obstetrical history, and delivery information for the serial blood donors are summarized in Table I. Means from the 3 patients groups (control, T1D and T2D) are presented for factors such as maternal age, gravida (total number of times the patient had been pregnant), number of term/preterm deliveries, abortions and living children. Gestational age at delivery, birth weight (g) of the newborn and method of delivery (spontaneous vaginal delivery or Caesarean section) are also presented.

Data for blood pressure, blood glucose levels, HbA1C percentages and urine protein/24 hour results were collected from clinical blood work sheets for each trimester. Means across pregnancy were calculated for control, T1D and T2D patients and the results are summarized in Table II. Third trimester results from the 2 PE patients are also presented in Table II. All of the T1D had abnormal blood glucose and HbA1C levels. Using the White Classification of Diabetic Pregnancies, 5 of the T1D were Class C while the remaining 4 T1D (which included the 2 PE patients) were Class D. The Class D T1D did not have significantly different values for the clinical parameters across pregnancy compared to the Class C T1D patients but they all delivered prematurely and had the lowest birth weights out

of the T1D and control groups. Furthermore, the offspring from three of the Class D T1D (including one of the PE) were sent to the neonatal intensive care unit (NICU) after delivery.

Circulating CD56⁺ cell numbers across pregnancy

To determine whether diabetes alters the numbers of CD56⁺ cells in the circulation of pregnant women, CD3⁻CD56^{bright}, CD3⁻CD56^{dim} and CD3⁺CD56⁺ cells were enumerated. Figure 1 shows mean blood CD56⁺ cell yields from control, T1D and T2D patients across pregnancy. As expected, CD3⁻CD56^{dim} NK cells were more abundant than CD3⁻CD56^{bright} NK (~10 fold) and CD3⁺CD56⁺ NKT (~2 fold) cells. Neither pregnancy nor diabetes significantly altered numbers of circulating CD3⁻CD56^{bright} and CD3⁺CD56⁺ cells in our cohort. CD3⁻CD56^{dim} cells appeared to be less frequent in pregnant T2D than in healthy women in all trimesters but availability of only 2 samples at each time point precluded statistical analysis.

Homing potential of CD56⁺ cells in normal women

To establish the pattern for homing potential of CD56⁺ cells to decidua, pancreas and peripheral lymph node in normal pregnancy, adhesion data from control patients was used. Numbers of CD56⁺ cells adhering to endothelium for each tissue showed no statistical variation over pregnancy although absolute numbers of adhering cells were different for each tissue (not shown). The lowest number of adhering cells was seen for pancreas, consistent with reports of limited lymphocyte-endothelial cell interactions in non-activated pancreatic vessels, which are lined with undifferentiated squamous endothelium (Chantakru *et al.*, 2003a).

For control women, the proportion (ratio) of CD56⁺ cells amongst total adhering cells was greater for decidua than for pancreas or lymph node, particularly in second trimester (Figure 2). When considered as a ratio against total adhered cells, about 85% of the population adherent to decidual endothelium was CD56⁺, a ratio invariant over pregnancy and the post partum ($P > 0.05$). The proportion of CD56⁺ cells adhering to pancreatic endothelium was ~50% and did not change across the 4 time points studied ($P > 0.05$). This ratio was a statistically different to decidua in second trimester ($P < 0.05$). The proportion of CD56⁺ cells amongst cells adhering to lymph node was intermediate between decidua and pancreas, except at its lowest point in second trimester when the proportion was similar to pancreas and differed from decidua ($P < 0.05$). The proportion of CD56⁺ cells adhering to lymph node endothelium was itself stable through pregnancy and post partum ($P > 0.05$). These data suggest that in healthy women, CD56⁺ cell interactions with endothelial cells in decidua, pancreas and lymph node are stable between week 11 of pregnancy and term and occur at frequencies similar to those seen in random (i.e. male or not menstrual cycle monitored female) samples (Chantakru *et al.*, 2003b; Frey *et al.*, 1998).

Homing potential of CD56⁺ cells in pregnant diabetic women

In Figure 3, mean numbers of CD56⁺ cells from diabetic donors that adhere to endothelium in gd7 decidualized uterus, pancreas and lymph node are compared with the mean numbers of adherent cells from control donors. Patient source had a statistically significant effect on absolute (Figure 3A) and proportional (Figure 3B) adhesion of CD56⁺ cells to decidual

endothelium ($P < 0.01$ and $P < 0.0001$ respectively). Fewer CD56⁺ cells from diabetics adhered to decidua compared to controls and their proportion amongst adhering cells had dropped approximately 50%. The effect of diabetes on CD56⁺ cell adhesion to decidual endothelium was not consistent for each trimester ($P < 0.05$). The greatest difference between control and diabetic decidual adherent CD56⁺ cell counts was in second trimester.

The effect of patient type on cell adhesion to pancreatic endothelium was statistically significant ($P < 0.001$, Figure 3C) with diabetes elevating adhesion. Differences in the proportion of adhering CD56⁺ to total cells (Figure 3D) were also significant for patient type ($P < 0.001$) with the ratio being ~2 fold greater for diabetics. These results provide a clear pattern predicting a gain in CD56⁺ cell homing potential to pancreas occurring simultaneously with a loss in CD56⁺ homing potential for decidua during diabetic pregnancy. Additionally, the gain in post partum pancreatic ratio of CD56⁺ cells suggests that there may be a single circulating pool of tissue homing NK cells.

Mean numbers of CD56⁺ cells adherent to peripheral lymph node endothelium are shown for diabetic and control patients in Figure 3E. There was no statistical difference between control and diabetic adherent cell counts ($P > 0.05$). The CD56⁺ to total cell ratio for cells adhering to lymph node (Figure 3F) was not affected by patient type ($P > 0.05$). Thus, the fundamental ability of NK cells from diabetic women to interact with endothelium in a tissue remote from disease is not significantly altered by pregnancy or in the postpartum.

Homing potential of CD56⁺ cells in pre-eclamptic T1D

Diabetic women are at increased risk for PE and this occurred in 2/9 T1D pregnancies in this study. To assess whether PE further altered the functional homing potential of CD56⁺ cells, adhesion data from these subjects were examined versus non PE T1D (n=7) (not shown). The small PE sample size precluded statistical analysis. The number of PE CD56⁺ cells adherent to decidual endothelium appeared to be similar to T1D and stable during pregnancy but was higher post partum for one PE patient. PE CD56⁺ cell adhesion to the pancreatic endothelium was variable. It seemed greater than in non-PE T1D in first trimester for one PE patient, greater in second trimester for the other PE patient and similar to non-PE T1D in third trimester for both patients. The PE patient with elevated post partum CD56⁺ cell adhesion to decidual endothelium also had elevated post partum adhesion to pancreatic but not lymph node endothelium. CD56⁺ cell adhesion to lymph node endothelium was variable but not elevated compared to non-PE T1D.

Blocking adhesive function of diabetic CD56⁺ cells

To determine whether CD62L and CD49d, the molecules regulating the interactions between CD56^{bright} cells and endothelium in normal pregnant women (Chantakru *et al.*, 2003a; van den Heuvel *et al.*, 2005b) were altered by diabetes, adhesion assays were performed in the presence of function blocking antibodies to these molecules. CD56⁺ cells from 6 non-PE patients (1 each of first, second and third trimester of T1D and T2D) were studied. The mean number of adherent CD56⁺ cells decreased 75% for all 3 of the gd7 murine tissues when the pooled blocking antibodies were present (data not shown). This result suggests that

diabetes does not alter the major molecular pathways used by CD56⁺ cells for egress from the circulation.

DISCUSSION

Endothelial cells are gate keepers between circulating leukocytes and tissue compartments. Shear forces from flowing blood marginalize leukocytes destined for extravasation. Activation and engagement of addressin and adhesion molecules such as SELP and ITGA result in leukocyte rolling and slowing along endothelial surfaces, firm adhesion and either trans-endothelial or inter-endothelial cell exodus of leukocytes from a vessel into tissue (Butcher *et al.*, 1999). The functional capacity of addressin and adhesion molecules on viable leukocytes can be assessed in *in vitro* adhesion assays to cryostat sections of the organs of interest. This approach maintains optimal, microanatomical relationships in the target tissue and has been directly correlated with *in vivo* homing potential to tumors (Burd *et al.*, 1998; Evans, 2000). Cross species conservation of homing receptors permits use of mouse tissue substrates to address human leukocyte homing potential in a rigorously-controlled, genetically-defined manner. It is also important because storage of substrate tissues quickly (< 2 week) leads to loss of endothelial cell function, precluding use of archived human tissue. This assay has been important in defining mechanisms recruiting lymphocytes to the pancreas in T1D and in identification of the LH surge as a time at which CD56⁺ cells have an elevated capacity to interact with decidual endothelium in fertile women (van den Heuvel *et al.*, 2005a; van den Heuvel *et al.*, 2008).

Data collected in the current study are the first to define the baseline gestational pattern for the interactions between viable, normal blood CD56⁺ cells and endothelial cells in decidua, pancreas and subcutaneous lymph nodes. These interactions are stable for normal pregnancies. Our data are consistent with a previous report (van den Heuvel *et al.*, 2008) in which adhesion to normal mouse decidual endothelium by lymphocytes from infertile women who became pregnant while receiving acetylsalicylic acid, IVIG and heparin was studied. In that study, CD56^{bright} cell adhesion to decidual endothelium was stable between weeks 3–32 of gestation and similar to values in the proliferative and secretory phases of the menstrual cycle. Gains in adhesive interactions were only seen at week 2 of gestation and at the LH surge of a previous menstrual cycle (van den Heuvel *et al.*, 2008). The latter finding in these treated patients is consistent with the gain in adhesion at the LH surge seen in fertile, healthy women (van den Heuvel *et al.*, 2005c). Because our study did not address pregnancies prior to week 3 of gestation and used random menstrual cycle days for postpartum sampling, stability of CD56⁺ cell adhesion to decidual endothelium would be predicted. Of note, in the study of embryo transfer recipients, van den Heuvel *et al.* reported that neither the menstrual cycle nor pregnancy altered the adhesive functions of CD56^{dim} or NKT cells with decidual endothelium. In the current study, CD56⁺ cells were not separated as dim vs. bright. Conventional fluorescence microscopy can visually distinguish between CD56^{bright} and CD56^{dim} subsets (Chantakru *et al.*, 2003b; Frey *et al.*, 1998) but this is improved with the assistance of computer digital analysis (Frey *et al.*, 1998) which we did not employ. Our protocol design did not include T cell labelling but, based upon the previous studies, we anticipate that most of the non-labelled adherent cells we observed were T cells. Stably adherent CD56⁺ NKT cells would have been the only T cell subset counted. For each

tissue, the proportion of bound leukocytes expressing CD56⁺ was different. Decidual endothelium engaged the highest proportion of CD56⁺ cells when second trimester blood samples were used.

This study clearly shows that diabetes is associated with changes in the homing potential of CD56⁺ cells for some but not all organs. Dramatic, inverse changes were seen for decidua (reduction) and pancreas (gain), while there were no changes in homing interactions with endothelial cells of lymph node. These data support the hypothesis that diabetes (T1D and T2D) reduces the ability of CD56⁺ cells to interact with decidual endothelium. This finding was consistent for CD56⁺ cells from women in all three trimesters of pregnancy and for the same women post partum. The elevated adhesion of CD56⁺ cells to pancreas in all trimesters suggests that in addition to decreased ability to interact with decidual endothelium, CD56⁺ cells will be reduced in decidua by a second mechanism, preferential or decoy recruitment to pancreas. Elevated pancreatic adhesion by CD56⁺ cells from T2D women was initially surprising as T2D, unlike T1D, is not characterized by elevated immune cell infiltration of pancreatic islets. However, as T2D progresses, it can be accompanied by islet inflammation with beta cell destruction. The similar CD56⁺ cell adhesion patterns of T1D and T2D pregnant women suggest long standing type 2 disease with islet involvement.

Elevated pancreatic leukocyte-endothelial interactions are well established as contributors to disease progression in both T1D and T2D. Hyperglycemia activates islet endothelial cells, which facilitates leukocyte migration through the expression of pro-inflammatory cytokines and adhesion molecules (Calles-Escandon and Cipolla, 2001; Homo-Delarche *et al.*, 2006; Morigi *et al.*, 1998). Peripheral node addressin (PNAd) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), both endothelial cell ligands for SELL, are up-regulated in the pancreas of hyperglycemic NOD mice. When SELL or one of its ligands is blocked in young NOD mice, leukocyte infiltration to the pancreas is inhibited, which delays or prevents the development of insulinitis and, subsequently, diabetes (Faveeuw *et al.*, 1994; Friedline *et al.*, 2002; Hanninen *et al.*, 1993). T-cell adhesion to pancreatic endothelium from 20 week NOD mice with insulinitis is completely blocked in *in vitro* adhesion assays when anti-ITGA antibodies are present (Yang *et al.*, 1993). However, because the pancreas sections used in this study were from normoglycemic mice, up-regulation of endothelial adhesion molecules does not account for the preferential binding of diabetic CD56⁺ cells to pancreas. A lymphocyte-based mechanism must be sought that remains uncompromised when the CD56⁺ cells interact with lymph node endothelium.

One possibility for such a mechanism is that microparticles or soluble molecules released from inflamed islets or dying beta cells are taken up by CD56⁺ cell receptors and elevate CD56⁺ cell interactions with pancreatic endothelium. How this, plasma lipidemia or patient insulin therapy could decrease interactions with endothelium in decidua and not alter interactions with lymph node endothelium remains conceptually problematic. Integrins such as ITGA4, signal from inside out, as well as from outside in (Abram and Lowell, 2009). Both pathways involve receptor clustering that might be reduced by the rigidity that characterizes diabetic blood leukocytes. Integrins also transmit outside-in signals through cooperation with other receptors including antigen receptors. Blocking or engagement of one or more integrin-associated receptors might alter homing to tissues without high

endothelial venules such as decidua or pancreas but not affect homing to organs with high endothelial venules such as lymph node. An alternate concept is that there is only one pool of tissue homing CD56⁺ cells able to respond to circulating products of inflammation. For our study, more inflammation in the diabetic pancreas than in normal implantation sites would skew homing potential, increasing it for pancreas over decidua but have no effect on interactions with lymph node. Further study will be needed to reveal whether our findings for CD56⁺ cells from a limited number of patients describe independent or related events and to define the roles of tissue specific antigens.

Not only were absolute numbers of CD56⁺ cells adhering to decidual endothelium reduced but the proportion of CD56⁺ cells amongst bound cells fell. This shift in ratio could have clinical relevance because total leukocyte adhesion to decidual endothelium was not changed significantly by diabetes. Our data are reminiscent of the shifts between blood regulatory T (Treg) cells and Th17 cells that have been linked causally with PE. In comparison with normal pregnancy, the Treg cell compartment was significantly decreased by PE. This gave a relative but not absolute increase in the number of Th17 cells that is thought to enable IL17-based contributions to pre-eclamptic pathology (Santner-Nanan *et al.*, 2009).

CD56⁺ adherent cell counts for the 2 PE patients were variable compared to non-PE T1D patients and did not reveal whether PE further alters CD56⁺ homing potential. PE only manifests after 20 weeks of gestation and, at this time, neither patient's lymphocytes appeared to be functionally different from non-PE T1D. The pathological basis of PE is thought to arise early in pregnancy. However, from this small sample size, no change in CD56⁺ cell uterine homing potential above that seen in T1D was observed in 2nd trimester and only transient changes in pancreatic homing potential were suggested. Additional investigation into the CD56⁺ cell adhesion patterns of PE patients could clarify the effect of PE on CD56⁺ cell homing potential during pregnancy. The results of this study show that diabetic women have abnormalities in CD56⁺ cell homing potential compared to healthy women during pregnancy. In order to further elucidate how CD56⁺ cells are affected by diabetes and how this may contribute to the development of PE during pregnancy, transcriptional, proteomic and epigenetic studies will be needed to create a global profile of the functional capacity of CD56⁺ cells across diabetic gestations with and without PE.

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Figure 1.

Mean NK cell yield from blood samples of control, type 1 diabetic and type 2 diabetic patients. (A) CD3⁻CD56^{bright} NK cells. (B) CD3⁻CD56^{dim} NK cells. (C) CD3⁺CD56⁺ NKT cells. Results are presented as bar graphs showing means $\pm$ SEM. Statistical analysis was not performed on T2D data sets as only two samples per time point were available for this study. NS = no sample.

Figure 2.

Adhesion pattern of CD56⁺ cells from control patients on gd7 murine decidua, pancreas and lymph node sections. CD56⁺ to total adherent cell ratios are presented. Results are presented as bar graphs showing means $\pm$ SEM. Significant differences are shown between tissue types at the same time point for CD56⁺ to total adherent cell ratio. *, $P < 0.05$.

Figure 3.

A comparison of the number of CD56⁺ adherent cells/25 high power fields (A,C,E) and ratio of CD56⁺ to total adherent cells (B,D,F) in blood from control and diabetic patients on murine gd7 decidua (A, B), pancreas (C, D) and lymph node (E, F). Results are presented as bar graphs showing means $\pm$ SEM. Significant differences are shown between control and diabetic patients at the same time point for CD56⁺ cell adhesion or CD56⁺ to total cell ratio. **, $P < 0.01$ (A). ***, $P < 0.0001$ (B). ***, $P < 0.001$ (C). ***, $P < 0.001$ (D). Total cell adhesion was not significantly altered by diabetes except on decidua in the second trimester, where it was lower compared to controls ($P < 0.01$).

TABLE I

Maternal and delivery demographics of control, type 1 diabetic and type 2 diabetic patient groups.

	Control (n=10)	Type 1 Diabetic (n=9)	Type 2 Diabetic (n=3)
Age (years)	28.0 ± 1.6	26.7 ± 1.5	35.0 ± 1.0
Obstetrical History			
Gravida	1.7 (1–3)	2.6 (1–7)	2.5 (2–3)
Term Deliveries	0.7 (0–2)	0.2 (0–1)	1.5 (1–2)
Preterm Deliveries	0.1 (0–1)	0.3 (0–2)	0.5 (0–1)
Abortions	0.1 (0–1)	1.0 (0–4)	0.0 (0–0)
Living Children	0.6 (0–2)	0.3 (0–1)	1.5 (1–2)
Delivery Information			
GAD (weeks)	39.8 ± 0.8	35.9 ± 1.2	38.0 ± 0.4
BW (g)	3749.1 ± 230.8	3327.5 ± 232.6	3500.0 ± 215.0
SVD	80%	40%	50%
C-section	20%	60%	50%

Data are mean ± SEM, mean (range) or percent rate. GAD, gestation age at delivery. BW, birth weight of newborn. SVD, spontaneous vaginal delivery. C-section, Caesarean section.

TABLE II

Clinical characteristics of control, type 1 diabetic (including pre-eclamptic) and type 2 diabetic patients across pregnancy, and of pre-eclamptic patients in the third trimester.

	Control (n=10)	Type 1 Diabetic (n=9)	Type 2 Diabetic (n=3)	Pre-Eclamptic (n=2)
Blood Pressure (mmHg)	115/73 ($\pm$ 1.9/1.3)	122/73 ($\pm$ 2.7/2.0)	133/87 ($\pm$ 14.5/3.3)	142/87 ($\pm$ 10.5/8.5)
Blood Glucose (mmol/L)	NAD	8.7 ($\pm$ 1.2)	10.1 ($\pm$ 0.0)	NM
HbA1C (% of Hb)	NAD	7.9 ($\pm$ 0.6)	NM	NM
Urine protein/24 hour (mg/dL)	4.0 ($\pm$ 3.0)	63.3 ($\pm$ 27.2)	0.0 ($\pm$ 0.0)	15.0 ($\pm$ 15.0)

Data are mean $\pm$ SEM. Both PE patients are type 1 diabetic. Hb, hemoglobin. NAD, no abnormality detected. NM, not measured. Normal ranges for blood glucose (3.3–5.6 mmol/L) and HbA1C (3.6–5.0% of Hb). Trace values for urine protein/24 hour are 5–20 mg/dL.