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ORIGINAL ARTICLE *Early pregnancy*

Circulating CD56^{+} cells of diabetic women show deviated homing potential for specific tissues during and following pregnancy

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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 that is more prevalent in diabetic women. Since previous *in vitro* studies have shown that changes in decidual endothelium induced by type 1 diabetes (T1D) reduce its interactions with circulating leucocytes, 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, T1D and T2D pregnant women throughout and after pregnancy. *In vitro* adhesion under shear forces was used to assay the functional capacity of circulating leucocytes 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 compared with 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.

Key words: decidua / pancreas / diabetes / uterine natural killer cells / pregnancy

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 (Li *et al.*, 2001; Quenby *et al.*, 2009; Hu *et al.*, 2010; Zhang *et al.*, 2010). Roles for human uNK cells in early triggering of pregnancy-associated, spiral arterial remodelling are now recognized (Smith *et al.*, 2009; Hazan *et al.*, 2010). In humans, remodelling of spiral arteries is considered critical for a 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 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 glycaemic 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; Manaster *et al.*, 2008; Male *et al.*, 2010; Lee *et al.*, 2010). Adoptive transfer studies clearly show that progenitors of murine uNK cells home to the uterus (Peel, 1989; Chantakru *et al.*, 2002; 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 (Guimond *et al.*, 1998; Ashkar *et al.*, 2000).

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, 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, 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.*, 2003; 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 an increase 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 hyperglycaemic and normoglycaemic non-obese diabetic (NOD) mice was assessed (Burke *et al.*, 2007). Decidual endothelium from the hyperglycaemic 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 hyperglycaemic NOD mice were also found to be less adhesive than splenocytes from normoglycaemic NOD mice to decidual endothelium from a normoglycaemic mouse. This indicated that hyperglycaemia also modified the function of mouse lymphocyte homing receptors (Burke *et al.*, 2007).

The insights from studies using hyperglycaemic NOD mice combined with the clinical complications seen in pregnant diabetic women [T1D, type 2 diabetes (T2D) and gestational] (Hanson and Persson, 1993; Hsu *et al.*, 1996; Evers *et al.*, 2004) 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 (Ogasawara *et al.*, 2003; Rodacki *et al.*, 2007; Brauner *et al.*, 2010; Gur *et al.*, 2010; Xia *et al.*, 2010).

To address our hypothesis, we serially assayed the functional capacity of circulating leucocytes and CD56^{+} cells, from pregnant and post-partum diabetic women (T1D and T2D) and from 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 represent a baseline 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 in the ages of 21−40 years 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 ($\sim$12, 18−20 and 33−35 weeks' gestation) and after 6 weeks post partum at scheduled clinic visits. At the time of the post-partum sample donation, 37% of the patients reported breastfeeding. 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, Fullarton, CA, USA) or with 7-amino-4-chloromethylcourmarin (CMAC; Molecular Probes, Invitrogen, Burlington, ON, Canada) as previously reported (van den Heuvel *et al.*, 2005b). Patients completed a data collection form that was available at each visit during the 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 leucocyte adhesion under shear force to frozen mouse tissue sections

Two sections (12 μm mid-sagittal) from each of the three 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 for 30 min). For some leucocyte 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 for 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 leucocytes and of Cy-5-tagged CD56$^+$ cells were enumerated in 25 high powered fields (HPFs) (x 400) under fluorescent microscopy. Counts were made by one or two individuals (A.V.C.S. and S.D.B.), who, in preparatory experiments, scored samples equivalently. For dual 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 were called the CD56$^+$ cell ratio. Because the adhesion results for T1D and T2D were similar (not shown), pooled adhesion data representing all of the diabetic patients are presented.

Statistical analysis

Maternal and delivery characteristics were analysed using one-way analysis of variance (ANOVA) with Bonferroni's post-test. Adhesion assay cell counts are presented as means ± SEM and were analysed 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 analysed using the Prism 4.03 Statistical Software package (GraphPad, San Diego, CA, USA).

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-pregnancy, during pregnancy 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 nine serially studied T1D, two developed PE in the third trimester. While patients were initially recruited into the longitudinal study with the goal of donating all four samples (one per trimester plus post-partum), this was not always possible. A complete set of samples was collected from nine patients (five controls and four T1D). Out of 22 potential samples for each of the four time points, 20 were collected for the first trimester, 18 for the second trimester, 16 for the third trimester and 11 for the post-partum period. Maternal demographics, obstetrical history and delivery information for the serial blood donors are summarized in Table I. Means from the three patient 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, birthweight (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 h 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 two 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, five of the T1D were Class C, while the remaining four T1D (which included the two PE patients) were Class D. The Class D T1D did not have significantly different values for the clinical parameters across pregnancy compared with the Class C T1D patients but they all delivered prematurely and had the lowest birthweights out of the T1D and control groups. Furthermore, the offspring from three of the Class D T1D patients (including one of the PE patients) were sent to the neonatal intensive care unit 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 I shows mean blood

Table I Maternal and delivery demographics of control, T1D and T2D patient groups.
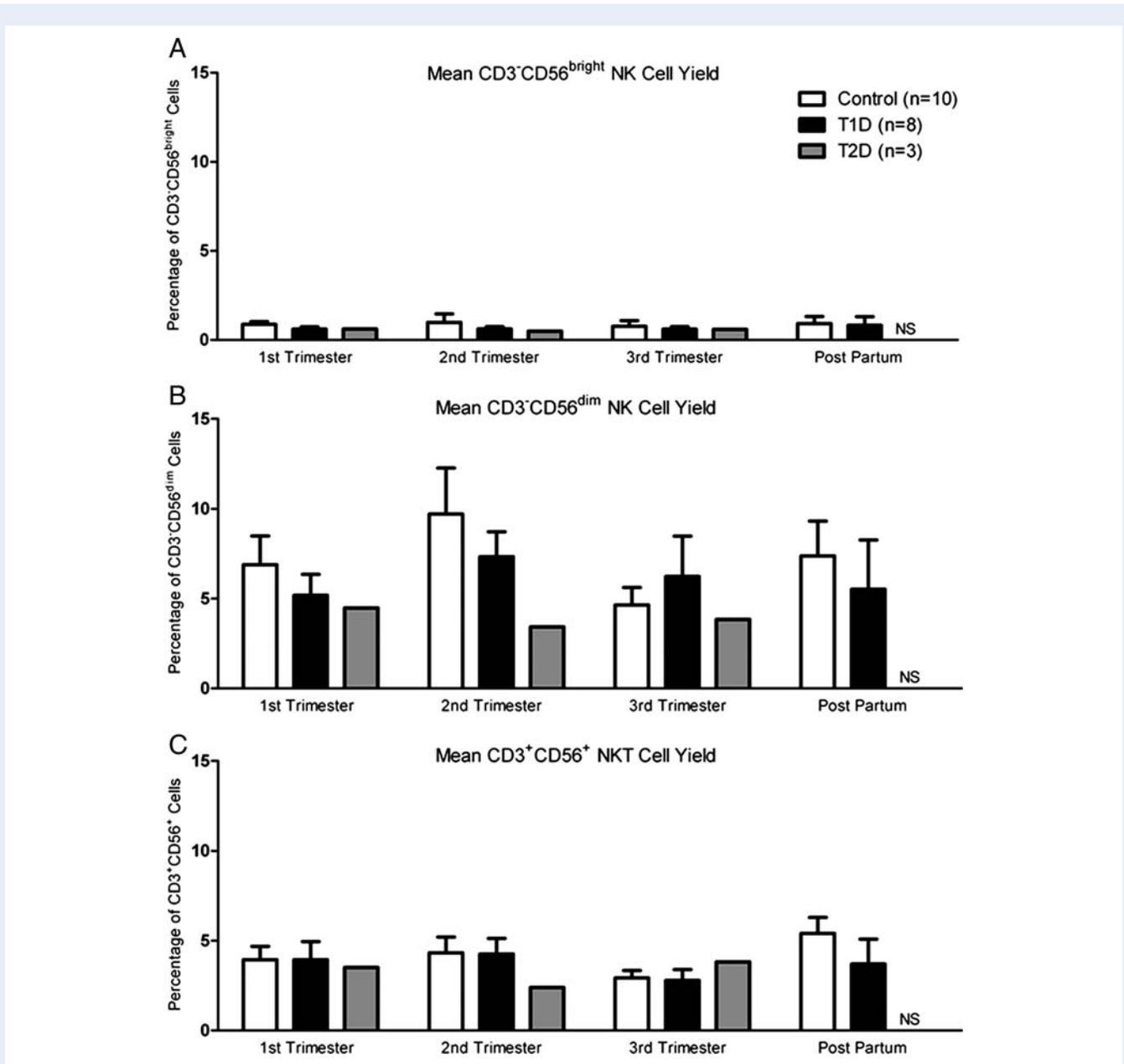
	Control (n = 10)	Type 1 diabetic (n = 9)	Type 2 diabetic (n = 3)
Age (years)	28.0 ± 1.6	26.7 ± 1.5	35.0 ± 1.0
Obstetrical history			
Gravida	1.7 (1–3)	2.6 (1–7)	2.5 (2–3)
Term deliveries	0.7 (0–2)	0.2 (0–1)	1.5 (1–2)
Preterm deliveries	0.1 (0–1)	0.3 (0–2)	0.5 (0–1)
Abortions	0.1 (0–1)	1.0 (0–4)	0.0 (0–0)
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, birthweight of newborn; SVD, spontaneous vaginal delivery; C-section, Caesarean section.

Table II Clinical characteristics of control, T1D (including PE) and T2D patients across pregnancy and of PE 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$0.6)	NM	NM
Urine protein/24 h (mg/dl)	4.0 ($\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 T1D. Hb, haemoglobin; 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 h are 5−20 mg/dl.

Figure 1 Mean NK cell yields 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 and (**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.

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 ($\sim$10-fold) and CD3$^+$CD56$^+$ NKT ($\sim$2-fold) cells. Neither pregnancy nor diabetes significantly altered numbers of circulating CD3$^-$CD56^{bright} and CD3$^+$CD56$^+$ cells in our cohort. CD3$^-$CD56^{dim} cells appeared to be less frequent in pregnant T2D than in healthy women in all trimesters but the availability of only two 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., 2003).

For control women, the proportion (ratio) of CD56$^+$ cells among total adhering cells was greater for decidua than for pancreas or lymph node, particularly in the second trimester (Fig. 2). When considered as a ratio against total adhered cells, $\sim$85% of the population adherent to decidual endothelium was CD56$^+$, a ratio invariant over pregnancy and the post-partum period ($P > 0.05$). The proportion of CD56$^+$ cells adhering to pancreatic endothelium was $\sim$50% and did not change across the four time points studied ($P > 0.05$). This ratio was statistically different to decidua in the second trimester ($P < 0.05$). The proportion of CD56$^+$ cells among cells adhering to lymph node was intermediate between decidua and pancreas, except at its lowest point in the 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 (Frey et al., 1998; Chantakru et al., 2003).

## Homing potential of CD56+ cells in pregnant diabetic women

In Fig. 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 (Fig. 3A) and proportional (Fig. 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 with controls and their proportion among adhering cells had dropped $\sim$50%. The effect of diabetes on CD56$^+$ 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 the second trimester.

The effect of patient type on cell adhesion to pancreatic endothelium was statistically significant ($P < 0.001$, Fig. 3C) with diabetes elevating adhesion. Differences in the proportion of adhering CD56$^+$ to total cells (Fig. 3D) were also significant for patient type ($P < 0.001$) with the ratio being $\sim$2-fold greater for diabetics. These results provide a clear pattern predicting a gain in the homing potential of CD56$^+$ cells to pancreas occurring simultaneously with a loss in the homing potential of CD56$^+$ cells for decidua during diabetic pregnancy. Additionally, the gain in the 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 Fig. 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 (Fig. 3F) was not affected by patient type ($P > 0.05$). Thus, the fundamental ability of NK cells
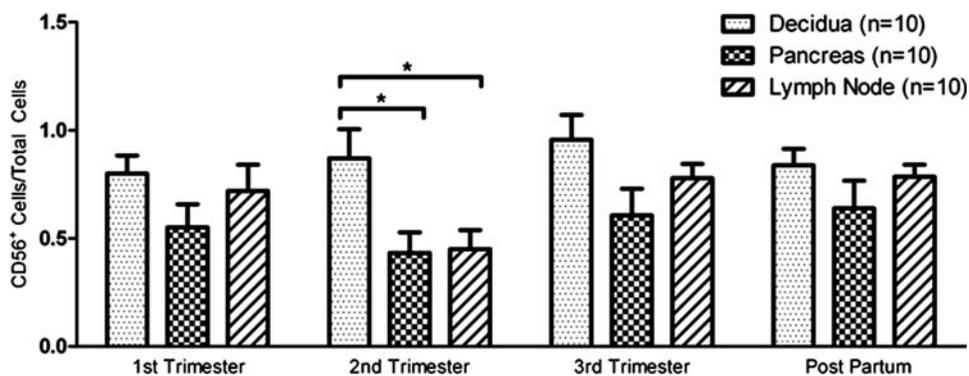
Figure 2 Adhesion pattern of CD56$^+$ cells from control patients on gd7 murine decidua, pancreas and lymph node sections. CD56$^+$ to total adherent cell ratios are presented. Results are presented as bar graphs showing means $\pm$ SEM. Significant differences are shown between tissue types at the same time point for the CD56$^+$ to total adherent cell ratio. *$P < 0.05$.
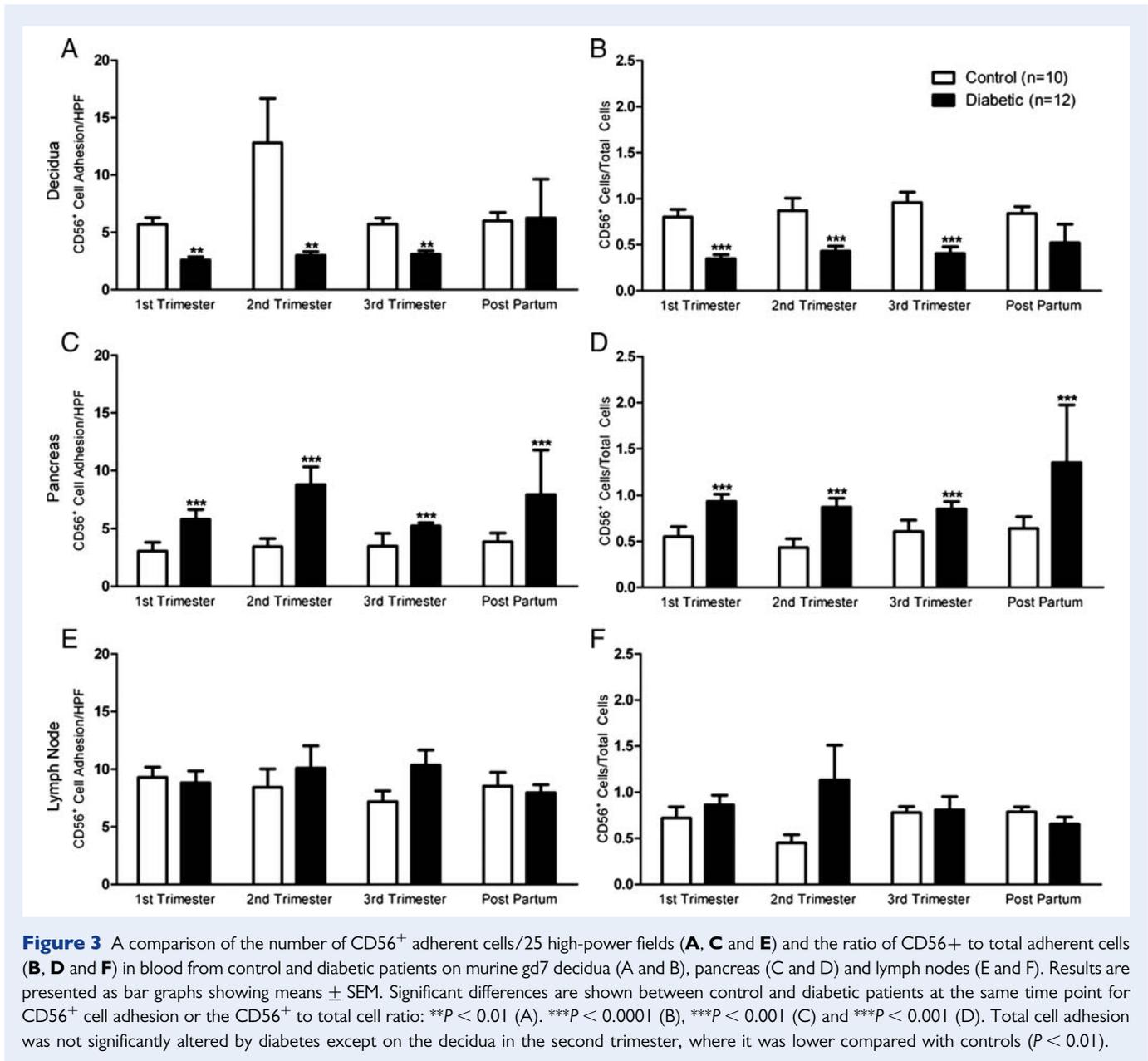
Figure 3 A comparison of the number of CD56$^+$ adherent cells/25 high-power fields (**A**, **C** and **E**) and the ratio of CD56+ to total adherent cells (**B**, **D** and **F**) in blood from control and diabetic patients on murine gd7 decidua (A and B), pancreas (C and D) and lymph nodes (E and F). Results are presented as bar graphs showing means ± SEM. Significant differences are shown between control and diabetic patients at the same time point for CD56$^+$ cell adhesion or the CD56$^+$ to total cell ratio: **$P < 0.01$ (A). ***$P < 0.0001$ (B), ***$P < 0.001$ (C) and ***$P < 0.001$ (D). Total cell adhesion was not significantly altered by diabetes except on the decidua in the second trimester, where it was lower compared with controls ($P < 0.01$).

from diabetic women to interact with endothelium in a tissue remote from disease is not significantly altered by pregnancy or in the post-partum period.

Homing potential of CD56+ cells in PE T1D

Diabetic women are at an increased risk for PE and this occurred in two of the nine T1D pregnancies in this study. To assess whether PE further altered the functional homing potential of CD56$^+$ cells, adhesion data from these subjects were compared with that of non-PE T1D patients ($n = 7$) (data 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 that of other T1D patients and was 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 the first trimester for one PE patient, greater in the second trimester for the other PE patient and similar to non-PE T1D in the 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 with that of 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., 2003; 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 six non-PE patients (one each of the first, second and third trimester of T1D and T2D) were studied. The mean number of adherent CD56$^+$ cells decreased >75% for all three 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 leucocytes and tissue compartments. Shear forces from flowing blood marginalize leucocytes destined for extravasation. Activation and engagement of addressin and adhesion molecules such as SELL and ITGA result in leucocyte rolling and slowing along endothelial surfaces, firm adhesion and either trans-endothelial or inter-endothelial cell exodus of leucocytes from a vessel into tissue (Butcher et al., 1999). The functional capacity of addressin and adhesion molecules on viable leucocytes 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 tumours (Burd et al., 1998; Evans, 2000). Cross species conservation of homing receptors permits the use of mouse tissue substrates to address human leucocyte 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 the 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, 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 during 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., 2005b). Because our study did not address pregnancies prior to Week 3 of gestation and used random menstrual cycle days for post-partum 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 versus bright. Conventional fluorescence microscopy can visually distinguish between CD56^{bright} and CD56^{dim} subsets (Frey et al., 1998; Chantakru et al., 2003) 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 leucocytes 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 nodes. 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 a decreased ability to interact with decidual endothelium, CD56$^+$ cells will be reduced in the 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 leucocyte–endothelial interactions are well established as contributors to disease progression in both T1D and T2D. Hyperglycaemia activates islet endothelial cells, which facilitates leucocyte migration through the expression of pro-inflammatory cytokines and adhesion molecules (Morigi et al., 1998; Calles-Escandon and Cipolla, 2001; Homo-Delarche et al., 2006). 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 hyperglycaemic NOD mice. When SELL or one of its ligands is blocked in young NOD mice, leucocyte infiltration to the pancreas is inhibited, which delays or prevents the development of insulitis and, subsequently, diabetes (Hanninen et al., 1993; Faveeuw et al., 1994; Friedline et al., 2002). T-cell adhesion to pancreatic endothelium from 20-week NOD mice with insulitis 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 normoglycaemic 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 lipidaemia or patient insulin therapy, could decrease interactions with endothelium in the 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 leucocytes. 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 nodes. 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 the pancreas over the 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 leucocyte 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 a 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 PE pathology (Santner-Nanan et al., 2009).

CD56$^+$ adherent cell counts for the two PE patients were variable compared with 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 the CD56$^+$ cell uterine homing potential above that seen in T1D was observed in the second trimester and only transient changes in the pancreatic homing potential were suggested. Additional investigation into the CD56$^+$ cell adhesion patterns of PE patients could clarify the effect of PE on the CD56$^+$ cell homing potential during pregnancy.

The results of this study show that diabetic women have abnormalities in CD56$^+$ cell homing potential compared with healthy women during pregnancy. 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.

Authors' roles

A.V.C.S. conducted leucocyte analysis, adhesion assay cell counts and data analyses, and drafted the manuscript; S.D.B. contributed to the design of the experiments, conducted adhesion assay cell counts and contributed to data analyses and manuscript preparation; H.R. organized patient recruitment; and B.A.C. and G.N.S. contributed to the design of the experiments and to the writing of the manuscript.

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