The phenotype of decidual CD56+ lymphocytes is influenced by secreted factors from decidual stromal cells but not macrophages in the first trimester of pregnancy.

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Abstract

During the first trimester of pregnancy the decidua is comprised of decidual stromal cells (DSC), invading fetal trophoblast cells and maternal leukocytes, including decidual natural killer (dNK) cells and macrophages. dNK cells are distinct from peripheral blood NK cells and have a role in regulating trophoblast invasion and spiral artery remodelling. The unique phenotype of dNK cells results from the decidual environment in which they reside, however the interaction and influence of other cells in the decidua on dNK phenotype is unknown. We isolated first trimester DSC and decidual macrophages and investigated the effect that DSC and decidual macrophage secreted factors have on CD56+ decidual lymphocyte receptor expression and cytokine secretion (including dNK cells). We report that DSC secreted factors induce the secretion of the cytokines IL-8 and IL-6 from first trimester CD56+ cells. However, neither DSC nor decidual macrophage secreted factors changed CD56+ cell receptor expression. These results suggest that secreted factors from DSC influence CD56+ decidual lymphocytes during the first trimester of pregnancy and therefore may play a role in regulating the unique phenotype and function of dNK cells during placentation.

Keywords: decidua, natural killer cells, macrophages, pregnancy, first trimester, stromal cells

Abbreviations:

cAMP, 8 Bromo-cyclic AMP; dNK, decidual natural killer; DSC, decidual stromal cell; EVT, extravillous trophoblast; IGFBP1, insulin-like growth factor binding protein; MPA, medroxyprogesterone17-acetate; pb, peripheral blood; PRL, prolactin; VSMC, vascular smooth muscle cell

1. Introduction

In the first trimester of pregnancy the decidua is rich with maternal leukocytes, made up of decidual NK (dNK) cells, decidual macrophages, T cells, a small number of B cells and dendritic cells and other innate lymphoid cells (ILCs) (Bulmer and Johnson, 1984, Bulmer et al., 1991, Vacca et al., 2015). In the post-ovulatory mid-secretory phase of the menstrual cycle decidualisation recruits NK cells to the uterus and local proliferation occurs (Sojka et al., 2018). This cell population reaches a peak in the late secretory phase and continues to accumulate in pregnancy, comprising 70% of the decidual leukocyte population by the end of the first trimester (King et al., 1998, Kopcow et al., 2010). dNK cells are distinct from their peripheral blood (pb) counterpart, however their function in pregnancy is still largely unknown. dNK cells have been implicated in regulating key processes at the maternal-fetal interface, including spiral artery remodelling and extravillous trophoblast (EVT) invasion (Hanna et al., 2006, Wallace et al., 2012).

dNK cells differ from pbNK cells as they exhibit a $CD56^{bright}$ $CD16^{-}CD160^{-}$ phenotype and express a unique repertoire of both activating and inhibitory receptors, with increased expression of CD9, CD69, ILT2, NKp46, NKp44 and NKp30 and the KIR receptors compared to pbNK cells (Searle et al., 1999, Moffett-King, 2002, Koopman et al., 2003, El Costa et al., 2009). dNK cells have a predominantly cytokine secreting role, rather than the traditional cytotoxic function displayed by pbNK cells. dNK cells secrete an array of cytokines, including IL-8 and CXCL10 and angiogenic growth factors, including VEGF and PLGF (Hanna et al., 2006, Wallace et al., 2013c).

During the first trimester of pregnancy, cytotrophoblast cells differentiate into invasive EVT cells. These EVTs migrate from the cell columns of anchoring placental villi and invade the maternal decidua and spiral arteries, reaching the inner third of the myometrium by the second trimester. During this process spiral arteries are remodelled, EVTs replace endothelial cells and vascular smooth muscle cells, modifying the arteries from low-flow, high-resistance into high-flow, low-resistance vessels with an increased diameter to ensure the increasing demands of the fetus are met. Decidual leucocytes, including dNK cells and macrophages, appear in the decidua prior to EVT and are therefore believed to contribute to initiating the remodelling process (Smith et al., 2009).

The main cellular component of the decidua is made up of DSC; epithelial-like cells that are transformed during decidualisation. This process commences after ovulation in the mid-secretory phase of the menstrual cycle, due to rising levels of progesterone. Decidualisation promotes morphological and biochemical changes to the spindle-shaped fibroblast-like endometrial cells converting them into secretory DSC. Two major secretory products of the DSC are IGFBP-1 and prolactin (PRL), classical markers of decidualisation (Gellersen and Brosens, 2003). However DSC also produce a myriad of other factors, including cytokines, chemokines, growth factors, angiogenic factors and hormones (Dimitriadis et al., 2005, Engert et al., 2007). It is these factors that have been implicated in a number of roles, including immune cell recruitment and activation as well as EVT migration and invasion (Pijnenborg, 1998, Kitaya et al., 2000, Verma et al., 2000, Zhu et al., 2009, Godbole and Modi, 2010).

Decidual macrophages make up the second largest population of leukocytes in the decidua. They are characterised by their CD14$^+$ phenotype, however, do not fit in to the conventional M1/M2 macrophage classification. Gene expression profiling suggests that they are skewed towards a M2 alternatively activated phenotype (Gustafsson et al., 2008). Houser and colleagues have demonstrated that decidual macrophages consist of two distinct populations identified by their expression of CD11c, either high (CD11c^{HI}) or intermediate (CDllc^{LO}) expression, which secrete both pro- and anti-inflammatory cytokines (Houser et al., 2011). Decidual macrophages have been implicated in establishing local immune balance, EVT invasion and spiral artery remodelling (Bulmer et al., 1991, Smith et al., 2009, Hazan et al., 2010, Svensson-Arvelund et al., 2015, Lash et al., 2016).

The unique phenotype of dNK cells results from the decidual environment in which they reside, which comprises of maternal DSC and immune cells, and the various factors they secrete. In the present study we have used first trimester DSC and decidual macrophage conditioned media to investigate the effect that secreted factors from these cells have on CD56+ decidual lymphocyte receptor expression and cytokine production. We hypothesise that DSC and decidual macrophage secreted factors will regulate receptor expression and cytokine production. We provide evidence that DSC secreted factors induce the production of the cytokines IL-6 and IL-8 from first trimester CD56+ cells; suggesting that DSC influence the function of dNK cells during early pregnancy.

2. Materials and methods

2.1. DSC isolation

Decidual tissue from 6-13 weeks of gestation was isolated from the products of conception obtained at termination of pregnancy. Decidual tissue was sorted from placental tissue by morphology in a petri dish, minced and digested in serum free M199 media containing 4kU DNase (Sigma Aldrich, UK) and 10kU collagenase (Gibco/ThermoFisher Scientific, Massachusettes, USA) overnight, at room temperature with agitation/rolling. The digested decidua was passed sequentially through 100 and 70 µm filters and layered onto Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) and centrifuged at 710*g* for 20 minutes. The cells in the ‘buffy layer’ were collected and washed in RPMI 1640 with 10% (v/v) fetal calf serum (FCS) supplemented with 2mM L-glutamine (Sigma Aldrich), penicillin (1 unit/ml)/ streptomycin (100µg/ml; Sigma Aldrich) and amphotericin B (2.5µg/ml; Sigma Aldrich) and centrifuged. The supernatant was discarded, and the pellet resuspended in red blood cell lysis buffer (155mM ammonium chloride (VWR, Leicestershire, UK), 9.9mM Trizma base (Sigma Aldrich), pH 7.4), incubated for 5 minutes at room temperature, washed in PBS and centrifuged. The pellet was resuspended in 20ml RPMI media with 10% (v/v) FCS and plated out for stromal cells to adhere to the bottom of the plate/flask. After approximately 15 minutes, non-adherent immune cells were removed. Cells were washed in PBS and cultured in DSC media at 37°C humidified atmosphere, 5% CO_2 in air.

2.2. CD56+ cell isolation

CD56+ lymphocytes (including dNK cells) were isolated, as previously described (Wallace et al., 2013c). In brief, non-adhered immune cells, containing the dNK cell fraction, from DSC isolation were purified by use of negative selection with a MagCellect Human NK Cell Isolation Kit (R&D Systems, Abingdon, UK), according to manufacturer's instructions. Purity, as measured by $CD56^+$ cells, was 95.26% ± 0.73% (mean ± SEM, n=19), and viability, immediately upon isolation, was 87.3% ± 1.86% (mean ± SEM, n=19), as assessed by fixable viability dye (eBioscience, Hatfield, UK).

2.3. Decidual macrophage isolation

Decidual tissue was sorted, minced, digested and filtered the same as above. The filtered decidual digest was resuspended in 20 ml 15% (v/v) Percoll® (Sigma Aldrich). Percoll cell suspension was layered on to a Percoll gradient of 5 ml 68% (v/v) and 12.5 ml 45% (v/v) Percoll and centrifuged for 700*g* for 30 minutes at 4°C. The cells between the 15% and 45% Percoll gradient were collected, centrifuged and resuspended in Phenol Red Free RPMI 1640 supplemented with 10% (v/v) FCS, filtered through a 40µm filter, cell number counted, and cells centrifuged at 500*g* for 5 minutes. Cells were resuspended in 80µl MACS buffer (PBS with 0.5% (w/v) BSA (Sigma Aldrich), 2mM EDTA (Sigma Aldrich), adjusted to pH7.2) per 10^7cells and 20µl anti-CD14 antibody coated magnetic beads (Miltenyi Biotec, Surrey, UK) per 10^7 cells, after 15 minutes incubation at 4°C the cells were centrifuged at 300*g* for 10 minutes at 4° to prevent loss of beads. The cell pellet was resuspended in MACS buffer and loaded onto a Large Cell Column with a flow resistor (Miltenyi Biotec) in a magnetic field of a MiniMACS separator (Miltenyi Biotec), cells not bound to the magnetic beads were removed by three washes with 500µl MACS buffer. 2ml of MACs buffer was added to the column, the column was removed from the magnet and the flow resistor removed, then the $CD14^+$ cells were flushed with the plunger. Cells were centrifuged at 500*g* for 5 minutes and resuspended in phenol red free RPMI 1640 supplemented with 10% (v/v) FCS at 1×10^6 cells/ml. Immediately after isolation purity, as measured by $CD14^+$ cells, was 72.26% ± 1.8% (mean ± SEM, n=91) and viability, as assessed by fixable viability dye, was 85.22% ± 1.41% (mean ± SEM, n=69). Conditioned media (CM) was collected after 6 hours of culture and centrifuged at 3000*g* for 5 minutes. The supernatant was stored at -20°c until use.

2.4. Cell culture conditions

Isolated DSC were cultured in DSC medium: RPMI 1640 (Sigma Aldrich) containing 2mM L-glutamine, penicillin (1 unit/ml)/ streptomycin (100µg/ml) and amphotericin B (2.5µg/ml) supplemented with 10% (v/v) FCS. CD56+ cells were cultured in dNK medium: Phenol Red Free RPMI 1640 supplemented with 10% (v/v) FBS, 50ng/ml stem cell factor (SCF; Peprotech, London, UK) and 5ng/ml IL-15 (Peprotech), containing 2mmol/L L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, and 2.5µ*g*/ml amphotericin. Decidual macrophages were cultured in decidual macrophage medium: Phenol Red Free RPMI 1640 supplemented with 10% (v/v) FBS, containing 2mmol/L L-glutamine, 100IU/ml penicillin, 100 µg/ml streptomycin, and 2.5µg/ml amphotericin.

2.5. Re-decidualisation of DSC

DSC were re-decidualised once they had reached confluency, after approximately 2-5 days (James-Allan et al., 2018). The cells were washed with PBS and serum free Hams F10 containing 2mM L-glutamine, penicillin (1 unit/ml)/ streptomycin (100µg/ml) and amphotericin (2.5µg/ml) containing 1µM medroxyprogesterone17-acetate (MPA; Sigma Aldrich) (3.86mg/ml) and 0.5mM 8 Bromo-cyclic AMP (cAMP; BioLog Life Science Institute, Germany) was added. As a vehicle control for MPA, serum free Hams F10 containing 10mM chloroform (BDH (VWR), Pennsylvania, USA) was added to one plate/flask of DSC. The cells were incubated for 3 days, after which CM was collected and centrifuged at 3000*g* for 5 minutes. CM was concentrated 20-fold at 4000*g* prior to storage at -20°c (VivaSpin Columns, 3000 mol wt. cutoff: Sartorius Stedium, Surrey, UK). Decidualisation was confirmed by secretion of decidualisation markers IGFBP1 and PRL, as previously described (James-Allan et al., 2018). These cells are subsequently referred to as re-decidualised DSC (rDSC).

2.6. Effect of rDSC and decidual macrophage CM on CD56+ cell receptor expression and cytokine secretion

In order to determine whether rDSC or decidual macrophages could affect decidual CD56+ cell receptor expression or cytokine production, decidual CD56+ cells were isolated and cultured at 1×10^6 cells/ml in rDSC or decidual macrophage CM in dNK medium for 6 hours. The 20-fold concentrated rDSC CM was diluted to 1-fold with dNK medium. 20-fold concentrated serum free Hams F10 medium containing 1µM MPA and 0.5mM 8 Bromo-cyclic AMP diluted to 1-fold with dNK medium was used as a control. Decidual macrophage control consisted of decidual macrophage medium. Half of the cells were collected for flow cytometry after 6 hours, the other half of the cells were centrifuged and resuspended in dNK medium and incubated for a further 12 hours, after which the CD56+ cell CM was collected and assessed for cytokine secretion.

2.7. Flow cytometry

Decidual CD56+ cells were resuspended in 1ml PBS and stained with fixable viability dye eFluor780 (eBioscience, Hatfield, UK) for 30 minutes at 4°C. Cells were centrifuged at 500*g* for 5 minutes and resuspended in 600µl FACS buffer (PBS with 0.5% (w/v) BSA, 0.05% (w/v) sodium azide) and non-specific binding was blocked using 1µg/ml human IgG and 10µl human FcR binding inhibitor (eBioscience) for 30 minutes at 4°C. After incubation, cells were labelled for 30 minutes at 4°C with primary antibodies as detailed: mouse anti-human CD56-Alexa Fluor 488 (100µg/ml, BD Biosciences, NJ, USA), mouse anti-human CD3-PerCP (25µg/ml, eBioscience), mouse anti-human CD9-PE (6.25µg/ml, BD Biosciences), mouse anti-human CD69-APC (0.75µg/ml, BD Biosciences), mouse anti-human NKp44-PE (6.25µg/ml, BD Biosciences), mouse anti-human NKp46-PE (12.5µg/ml, BD Biosciences), mouse anti-human ILT2-APC (10µg/ml, R&D Sciences), mouse anti-human NK62A-APC (100µg/ml, R&D Sciences), mouse anti-human NKG2C-APC (50µg/ml, R&D Sciences), mouse anti-human NKG2D-APC (50µg/ml, R&D Sciences), KIR2DL1/KIR2DS5-PE (25µg/ml, R&D Sciences) and mouse anti-human KIR2DL2-PE (6.25µg/ml, BD Biosciences). The following isotype controls were used: mouse IgG1 κ-Alexa Fluor 488 (eBioscience), mouse IgG1 PerCP (eBioscience), mouse IgG1 PE (R&D Systems), mouse IgG2a κ-PE (eBioscience), IgG1 κ-APC (eBioscience) and mouse IgG2a κ-APC (eBioscience). Cells were centrifuged at 500*g* for 5 minutes and resuspended in FACS buffer. Flow cytometry was carried out on a LSR II flow cytometer (BD Biosciences). Analysis was carried out by use of FOWJO software (Tree Star Inc, Oregon, USA). Histrograms were gated on viable CD56^{+}cells.

2.8. ELISA

The concentration of IL-6 and IL-8 in decidual CD56+ cell CM was measured using DuoSet ELISA (R&D Systems) according to manufacturer's instructions.

2.9. Statistical analyses

Data was analysed by one-way analysis of variance (ANOVA) with Sidak's multiple comparison test or t-test by use of GraphPad Prism (version 6.0, CA, USA). Significance was accepted at $p<0.05$. ELISA data was transformed by taking the log of the concentration so that variance was not significantly different, and a t-test could be performed on the data.

3. Results

3.1. Receptor repertoire of CD56+ cells is not regulated by DSC or decidual macrophage CM

dNK cells have a unique receptor expression profile and have previously been shown to express the following receptors: CD9, CD69, NKp44, NKp46, ILT2, NKG2A, NKG2C, NKG2D, KIR2DL1 and KIR2DL2 (Wallace et al., 2015). The effect that DSC and decidual macrophage secreted factors had on the expression of CD56+ cell receptors was investigated. Isolated first trimester CD56+ cells were cultured with re-decidualised (rDSC) or decidual macrophage CM for 6 hours after which receptor expression was assessed by flow cytometry (gating strategy, **Figure 1**). Examination of CD56+ cells after 6 hours of culture determined that all receptors were present on the cells. All receptors were expressed in the same proportion in control-treated and rDSC CM-treated cells (**Figure 2**) and in control-treated and decidual macrophage CM-treated cells (**Figure 3**).

3.2. CD56+ decidual cell cytokine secretion is stimulated by DSC

Decidual NK cells have a secretory function; previous studies have shown that first trimester dNK cells secrete the chemokines IL-8 and IL-6 (Wallace et al., 2013b). This study examined if DSC secreted factors have an effect on the secretion of the dNK cytokines IL-8 and IL-6. CD56+ cells were cultured with rDSC CM for 6 hours after which the media was changed and CD56+ cell conditioned media was collected after a further 12 hours of culture. Cells treated with rDSC CM secreted significantly more IL-8 (**Figure 4A**) and IL-6 (**Figure 4B**) than cells treated with control medium.

3.3. CD56+ decidual cell cytokine secretion is not stimulated by decidual macrophages

To determine if decidual macrophage secreted factors have an effect on the secretion of cytokines IL-8 and IL-6. CD56+ cells were cultured with decidual macrophage CM for 6 hours after which the media was changed and dNK conditioned media was collected after a further 12 hours of culture. IL-8 (**Figure 5A**) or IL-6 (**Figure 5B**) secretion showed no significant changes when treated with decidual macrophage CM compared to control.

4. Discussion

During the first trimester of pregnancy, dNK cells are the predominant leukocyte within the decidua and therefore come in to contact with other decidual cells, such as DSC and macrophages. dNK cells differ from pbNK cells due to their unique receptor repertoire and cytokine-secreting phenotype, which dictate their role during early pregnancy, including spiral artery remodeling and interaction with EVT (Moffett-King, 2002, Smith et al., 2009). In this study we have demonstrated that secretion of the cytokines IL-8 and IL-6 from first trimester CD56+ cells is stimulated by first trimester DSC secreted factors, but not secreted factors from decidual macrophages. This suggests that DSC interact with dNK, influencing their function and possibly their role during the first trimester of pregnancy.

In the decidua NK cells are known to be in close contact with trophoblast cells. A crucial step in placentation during early pregnancy is the remodeling of spiral arteries leading to high volume, low resistance vessels that transport maternal blood to the placenta. Smith and colleagues demonstrated that this is a multi-step process, which begins with a trophoblast-independent stage in which disruption of vascular smooth muscle cells and the endothelial cell layer occur in the absence of invading trophoblasts but in the presence of dNK and macrophage cells (Smith et al., 2009). The ability of dNK cells to secrete soluble factors has been shown to induce VSMC disruption prior to EVT interaction with spiral arteries inferring that the factors that dNK secrete play an important role in spiral artery remodeling. It is also known that factors, such as IL-8 and CXCL10, secreted by dNK promote invasion and chemotaxis of EVT and therefore also contribute to the trophoblast-dependent stage of spiral artery remodeling (Hanna et al., 2006, Wallace et al., 2013a).

Results from this study demonstrate that conditioned media from rDSC stimulate CD56+ decidual cells to secrete increased amounts of the cytokines IL-8 and IL-6. dNK cells have previously been shown to secrete an array of factors, including these cytokines (Hanna et al., 2006). It is known that trophoblasts, including EVTs, express the respective receptors CXCR1 and IL-6Rα, suggesting that these dNK-secreted cytokines may act upon trophoblast cells during the first trimester of pregnancy (Jovanovic and Vicovac, 2009, Jovanovic et al., 2010, Hanna et al., 2006). IL-8 and IL-6 are chemokines that have been implicated in the invasion of trophoblast cells in the first trimester of pregnancy, including stimulating migration and chemotaxis of trophoblast cells (Jovanovic and Vicovac, 2009, Jovanovic et al., 2010). Consequently, as dNK cells come in to contact with DSC as their number increases during decidualisation and the first trimester of pregnancy, it is possible that crosstalk between DSC and dNK stimulate dNK to produce factors that could assist in both trophoblast-independent and trophoblast-dependent spiral artery remodelling.

It has been shown that alongside dNK cells there are other innate lymphoid cell (ILC) populations found in the decidua during the early stages of pregnancy. Three populations of ILCs have been identified, including an ILC1 subset which produce IFNγ and two ILC3 populations, lymphoid tissue inducer (LTi)-like cells and NCR^+ILC3 which release IL-17/TNF and IL22/IL-8, respectively (Vacca et al., 2015). NCR^+ILC3 cells contribute to IL-8 production in the decidua. Although subpopulations of ILCs was not investigated in the CD56+ cells isolated in this study it would be interesting to investigate if DSC CM is stimulating specific cells, such as NCR^{+}ILC3, which is leading to the increase in IL-8 secretion observed.

It is known that there is a large infiltration of NK cells into the decidua during the first trimester of pregnancy, however the mechanism as to how these cells move into the decidua is still largely unknown. Possible hypotheses include that they are recruited from the pbNK population to the decidua where they undergo differentiation (Keskin et al., 2007), or that $CD56^{dim}CD16^{-}$ pbNK cells migrate to the decidua during pregnancy due to the interaction of cytokines on their surface and the corresponding receptors expressed on trophoblasts or decidual stromal cells (Hannan et al., 2006). Others have suggested that dNK cells may differentiate from endometrial NK (eNK) cells which become differentiated due to local mediators in the decidua when pregnancy occurs. However, results from this study suggest that DSC secreted factors could be stimulating dNK cells to secrete chemokines that attract additional NK cells to the decidua. This supports the idea that there is a feedback mechanism in the decidua in which maternal DSC and immune cells attract each other to populate the decidua in the first trimester of pregnancy.

DSC are known to secrete an array of factors including RANTES, IL-8, CXCL10 and IL-15 (Sharma et al., 2016). These factors have a range of functions, including the recruitment, migration and proliferation of dNK cells. Evidence has shown that DSC express ligands that act on dNK activating receptors, leading to cytokine, chemokine and angiogenic factor production (Hanna et al., 2006). RANTES, IL-8 and CXCL10 are chemokines found in the endometrium, with levels highest during the late secretory phase of the menstrual cycle, suggesting that they may regulate the recruitment of immune cells to the decidua (Arici et al., 1998, Hornung et al., 1997, Kitaya et al., 2004). IL-15, which is secreted by DSC (Kitaya et al., 2000, Okada et al., 2000), is known to have activating and proliferative effects on NK cells (Carson et al., 1994) and has been shown to induce uterine NK cells to secrete an array of factors (Cooper et al., 2001, Eriksson et al., 2004) and stimulate their proliferation (Verma et al., 2000).

Macrophages are the second most abundant leukocyte type in the decidua. Macrophages are typically classified in to either M1, representing a classical pro-inflammatory, anti-microbial activation, or M2 an anti-inflammatory phenotype. However, it is thought that decidual macrophages have a unique phenotype that differs from peripheral blood monocytes as they do not fit in to the conventional M1/M2 classification, due to having aspects of both an anti- and a pro-inflammatory phenotype (Houser et al., 2011, Svensson et al., 2011). It is known that decidual macrophages secrete an array of factors, including the pro-inflammatory cytokines TNFα and IL-1β and the anti-inflammatory cytokines IL-10 and TGFβ (Houser et al., 2011).

dNK cells and macrophages are in close contact within the decidua, therefore it is likely that crosstalk between these cells occurs. However, little is known regarding the interaction between immune cells during pregnancy. There is evidence to suggest that secreted factors could influence dNK cells, for example TGFβ has been shown to suppress the activation of dNK subpopulations (Zhang et al 2019). Additionally, some functional regulation has been shown with macrophages inhibiting NK cell killing of invasive trophoblast cells (Co et al 2013). Therefore, we hypothesised that factors secreted from macrophage cells during the first trimester of pregnancy will influence the phenotype of dNK cells, including their cytokine secretion and receptor expression. In contradiction to our hypothesis, and the results from DSC secreted factors, decidual macrophage secreted factors did not influence dNK IL-6 or IL-8 secretion during the first trimester of pregnancy in this study.

dNK cells express a distinctive panel of activating and inhibitory receptors that differs from pbNK cells. These receptors are crucial to their interaction with EVT as EVT express a unique combination of MHC molecules, including HLA-C, HLA-E and HLA-G which interact with receptors expressed by dNK cells. The expression of these receptors is induced in the mid-secretory phase of the menstrual cycle, in conjunction with decidualisation, suggesting that the production of cytokines in the endometrium activates the receptor expression on NK cells converting them to dNK cells.

We found that the receptors studied were expressed by CD56+ cells isolated from first trimester pregnancies, however *in vitro* culture with rDSC or decidual macrophage conditioned media did not alter their expression. This suggests that DSC or decidual macrophage secreted factors from early pregnancy do not affect the expression of CD56+ cell receptors investigated in this study despite both secreting a wide range of factors. However, the cells used in this study were isolated from the decidua and therefore will have already been in contact with decidual factors *in vivo* and it may not be possible to modify these further. It would be interesting to determine whether NK cells isolated from the endometrium that have not previously been exposed to the decidual environment would have a different response.

We observed some variation in receptor expression, which could be due to patient variability, or due to potential subpopulations of CD56+ cells, including ILCs. We have previously shown that the expression of these receptors on dNK cells does not differ in the first trimester when pre- and post-10 weeks gestation were compared (Wallace et al 2015), therefore this variation is unlikely to be due to the gestational range of the samples. Zhang et al (2017) suggest that that interactions between trophoblast and dNK cells during the first trimester of pregnancy supress dNK cell functions by inhibiting the expression of their activating receptor NKG2D (Zhang et al., 2017). However, this report only observed changes in NKGD2 receptor expression with direct co-culture of cells, but not with trophoblast conditioned media, which matches the results in this study, suggesting receptor-ligand interactions rather than soluble factors having a role in this process.

Decidualisation transforms endometrial stromal cells in to secretory decidual stromal cells and we have shown that the secretory products from these cells can influence the phenotype of NK cells within the decidua in the first trimester of pregnancy. Interestingly decidual macrophage secretory factors did not have the same effect as DSC cells, suggesting that they may not interact with NK cells during the early stages of pregnancy and therefore have an alternate function within the decidua. dNK have been shown to have abnormalities in pregnancies with impaired spiral artery remodelling, and therefore an increased risk of developing complications such as pre-eclampsia and intrauterine growth restriction (Fraser et al., 2012, Wallace et al., 2013c, Wallace et al., 2014, Wallace et al., 2015). Therefore, the interaction with DSC within the decidua in the first trimester of pregnancy is likely to be crucial to induce the unique dNK phenotype so that normal placentation occurs.

5. Conclusion

In summary, we have found that secreted factors from DSC interact with CD56+ cells simulating cytokine secretion in the first trimester of pregnancy. These findings suggest that DSC, but not decidual macrophages, influence the phenotype of NK cells in the decidua in the early stages of pregnancy.

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Figure 1. Representative gating strategy of flow cytometry data of isolated decidual lymphocytes. Freshly isolated cells, at 0hr, were gated on forward (FSC)/side-scatter (SSC) (A), this population was then gated on viability as assessed by negativity for eFluordye (B), the population of alive cells was gated for CD56 and CD56^{bright} positivity (C). Data are of a sample, gestational age 10 + 0 weeks. Grey line indicates IgG control, and darker line indicates antibody to CD56.

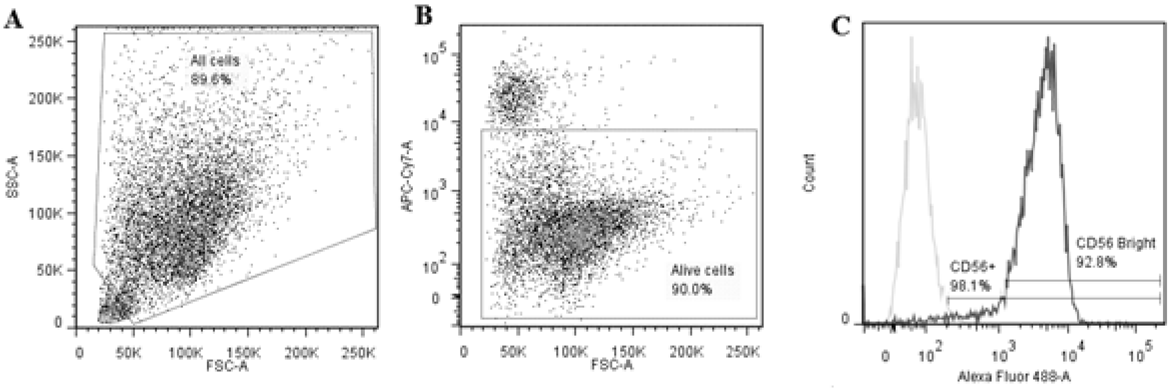
Figure 2. Percentage of CD56+ cells positive for receptors after stimulation with rDSC CM. Isolated cells were cultured with rDSC CM for 6 hours and receptor expression subsequently assessed by flow cytometry. Data shown as mean ± SEM, paired t-test: not significant, n=6.

Figure 3. Percentage of CD56+ cells positive for receptors after stimulation with decidual macrophage CM. Isolated cells were cultured with decidual macrophage (dMØ) CM for 6 hours and receptor expression subsequently assessed by flow cytometry. Data shown as mean ± SEM, paired t-test: not significant, n=10.

Figure 4. Secretion of IL-6 and IL-8 from CD56+ cells treated with rDSC CM. Isolated cells were cultured with rDSC CM for 6 hours. Secretion of IL-8 and IL-6 was measured by ELISA in media collected after a further 12 hours of culture with dNK medium. **A**: Secretion of IL-8 between control-treated and rDSC-treated cells (n=18). **B**: Secretion of IL-6 between control-treated and rDSC-treated cells (n=21). Data was log transformed to ensure equal variance. Paired t-test, data shown as mean ± SEM, **** p<0.0001, *** p<0.001.

Figure 5. Secretion of IL-6 and IL-8 from CD56+ cells treated with decidual macrophage CM. Isolated cells were cultured with decidual macrophage (dMØ) CM for 6 hours. Secretion of IL-8 and IL-6 was measured by ELISA in media collected after a further 12 hours of culture with dNK medium. **A:** Secretion of IL-8 between control-treated and decidual macrophage-treated cells (n=7). **B:** Secretion of IL-6 between control-treated and decidual macrophage-

treated cells (n=10). Data was log transformed to ensure equal variance. Paired t-test, data shown as mean ± SEM, ns denotes not significant.


A
All cells
89.6%
SSC-A
FSC-A
B
APC-Cy7-A
Alive cells
90.0%
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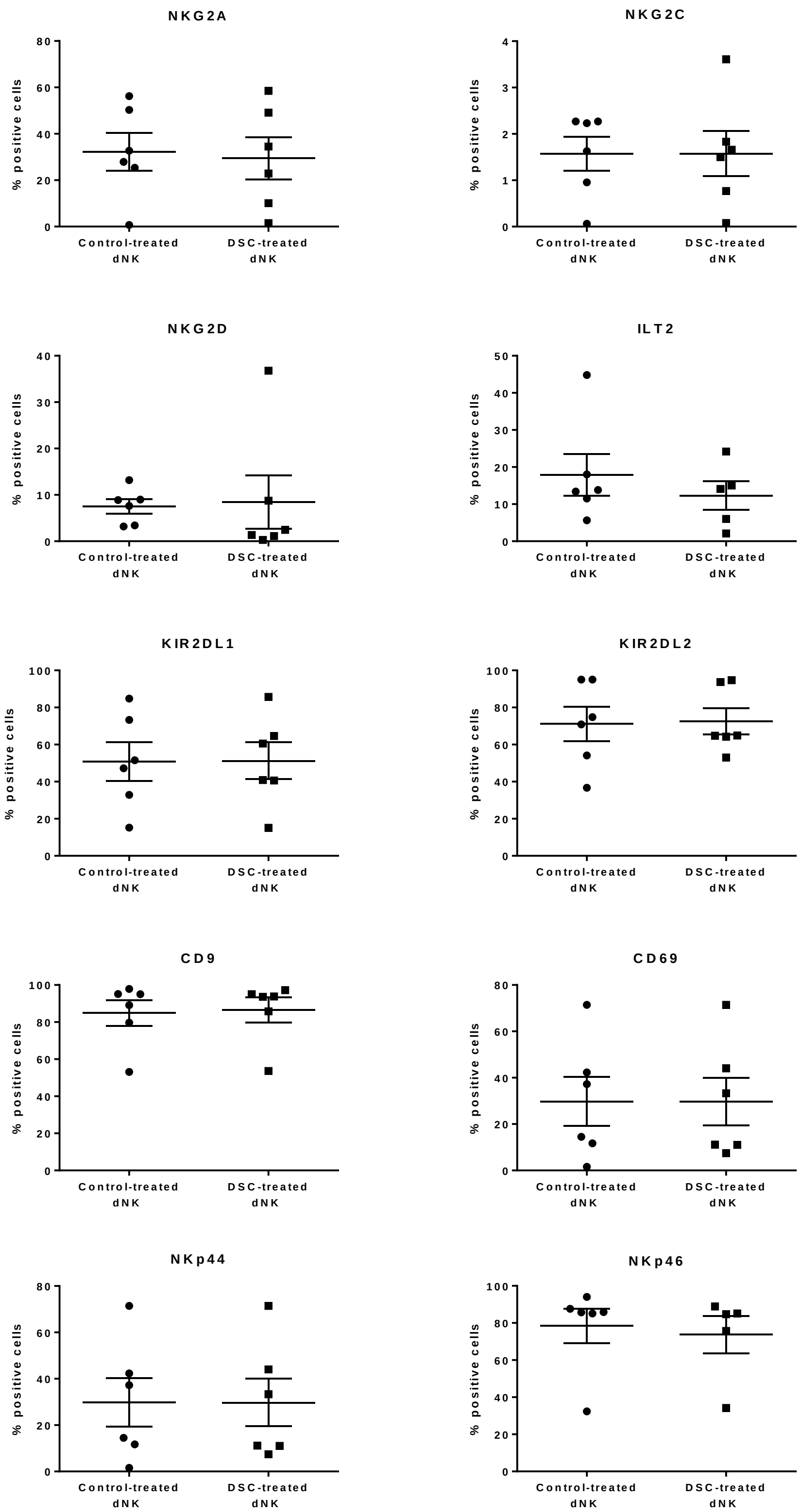
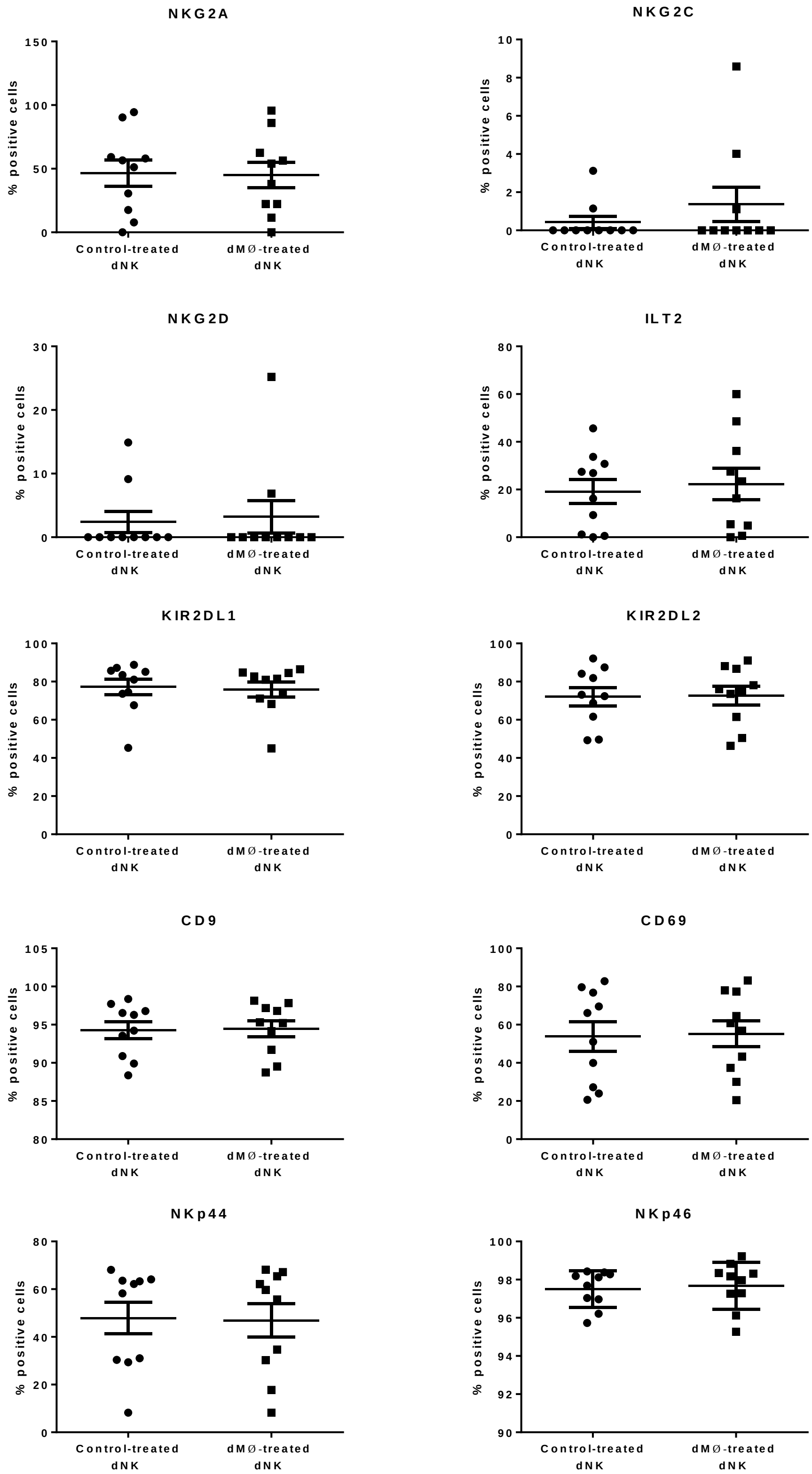
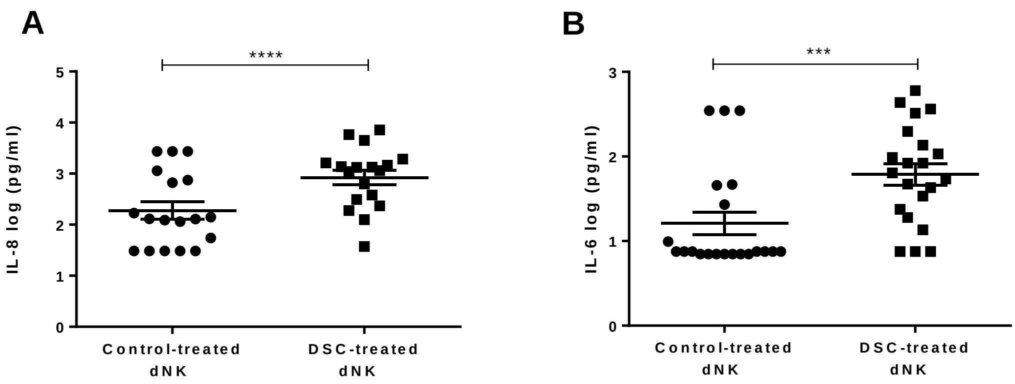
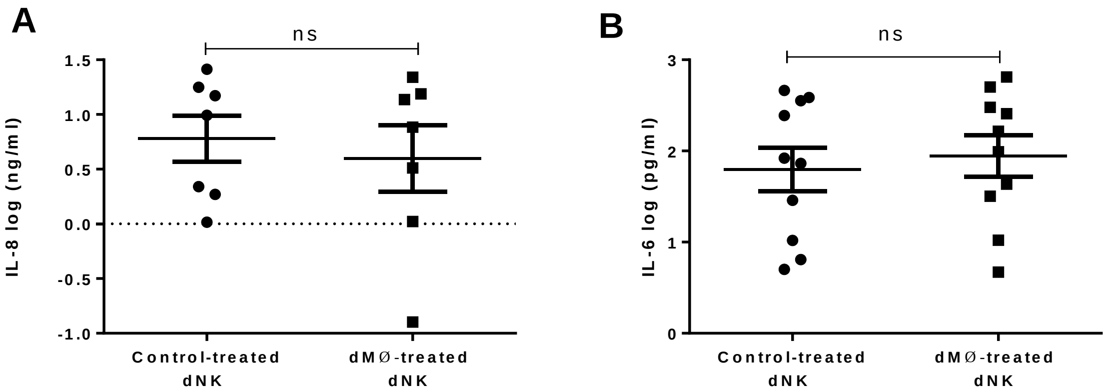
C
Count
CD56+
98.1%
CD56 Bright
92.8%
Alexa Fluor 488-A



NKG2A
NKG2C
NKG2D
ILT2
KIR2DL1
KIR2DL2
CD9
CD69
NKp44
NKp46
% positive cells
Control-treated dNK
DSC-treated dNK


NKG2A
150
100
50
0
% positive cells
Control-treated dNK
dMØ-treated dNK
NKG2C
10
8
6
4
2
0
% positive cells
Control-treated dNK
dMØ-treated dNK
NKG2D
30
20
10
0
% positive cells
Control-treated dNK
dMØ-treated dNK
ILT2
80
60
40
20
0
% positive cells
Control-treated dNK
dMØ-treated dNK
KIR2DL1
100
80
60
40
20
0
% positive cells
Control-treated dNK
dMØ-treated dNK
KIR2DL2
100
80
60
40
20
0
% positive cells
Control-treated dNK
dMØ-treated dNK
CD9
105
100
95
90
85
80
% positive cells
Control-treated dNK
dMØ-treated dNK
CD69
100
80
60
40
20
0
% positive cells
Control-treated dNK
dMØ-treated dNK
NKp44
80
60
40
20
0
% positive cells
Control-treated dNK
dMØ-treated dNK
NKp46
100
98
96
94
92
90
% positive cells
Control-treated dNK
dMØ-treated dNK


A
****
IL-8 log (pg/ml)
0
1
2
3
4
5
Control-treated dNK
DSC-treated dNK
B
***
IL-6 log (pg/ml)
0
1
2
3
Control-treated dNK
DSC-treated dNK


A
ns
IL-8 log (ng/ml)
-1.0
-0.5
0.0
0.5
1.0
1.5
Control-treated dNK
dMØ-treated dNK
B
ns
IL-6 log (pg/ml)
0
1
2
3
Control-treated dNK
dMØ-treated dNK