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Transcriptomic analysis of vitamin D responses in uterine and peripheral NK cells

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Abstract

Vitamin D deficiency is prevalent in pregnant women and is associated with adverse pregnancy outcomes, in particular disorders of malplacentation. The active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D3), is a potent regulator of innate and adaptive immunity, but its immune effects during pregnancy remain poorly understood. During early gestation, the predominant immune cells in maternal decidua are uterine natural killer cells (uNK), but the responsivity of these cells to 1,25(OH)$_2$D3 is unknown despite high levels of 1,25(OH)$_2$D3 in decidua. Transcriptomic responses to 1,25(OH)$_2$D3 were characterised in paired donor uNK and peripheral natural killer cells (pNK) following cytokine (CK) stimulation. RNA-seq analyses indicated 911 genes were differentially expressed in CK-stimulated uNK versus CK-stimulated pNK in the absence of 1,25(OH)$_2$D3, with predominant differentially expressed pathways being associated with glycolysis and transforming growth factor β (TGFβ). RNA-seq also showed that the vitamin D receptor (*VDR*) and its heterodimer partner retinoid X receptor were differentially expressed in CK-stimulated uNK vs CK-stimulated pNK. Further analyses confirmed increased expression of *VDR* mRNA and protein, as well as VDR-RXR target in CK-stimulated uNK. RNA-seq analysis showed that in CK-stimulated pNK, 1,25(OH)$_2$D3 induced 38 and suppressed 33 transcripts, whilst in CK-stimulated uNK 1,25(OH)$_2$D3 induced 46 and suppressed 19 genes. However, multiple comparison analysis of transcriptomic data indicated that 1,25(OH)2D3 had no significant overall effect on gene expression in either CK-stimulated pNK or uNK. These data indicate that CK-stimulated uNK are transcriptionally distinct from pNK and, despite expressing abundant VDR, neither pNK nor uNK are sensitive targets for vitamin D.

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Introduction

Human implantation and placental development present a unique challenge for the maternal immune system (Taglauer *et al.* 2010). From embryonic implantation, semi-allogeneic fetal extravillous trophoblast cells invade the maternal decidua and inner myometrium (including remodelling the spiral arteries) facilitating normal haemochorial placentation. Throughout these actions, the materno-fetal immune function remains active, with diverse mechanisms mounted to support fetal development and placentation (Abumaree *et al.* 2012, Erlebacher 2013). From early pregnancy, a high proportion of resident leukocytes, displaying unique phenotypes and functions reside within the uteroplacental interface (Bulmer *et al.* 2010). CD56bright uterine natural killer cells (uNK) (60–70%) are predominant, appearing phenotypically distinct from CD56dim NK cells in peripheral blood (pNK) (<10% total circulating immune cell population). In addition to the classical pro-cytotoxic, anti-tumorigenic and anti-viral effects associated with pNK (Koopman *et al.* 2003), uNK have also been shown to play a pivotal role in embryonic implantation and haemochorial placental development (Lash *et al.* 2010).

Regulation of uNK function involves a complex network of factors (Szekeres-Bartho 2008), and we have postulated that vitamin D plays a role in this process. Vitamin D is a secosteroid that exerts powerful immuno-regulatory responses on cells from both the innate and

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adaptive immune systems following immune activation (Adams & Hewison 2008, Hewison 2011). Early in pregnancy, maternal concentrations of the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3), increase dramatically (Tamblyn *et al.* 2017). Moreover, the enzyme that catalyses synthesis of 1,25$(OH)_2$D3 from precursor 25-hydroxyvitamin D3 (25(OH)D3), 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) (Gray *et al.* 1979, Weisman *et al.* 1979, Zehnder *et al.* 2002), as well as the nuclear vitamin D receptor (VDR) for 1,25$(OH)_2$D3 (Zehnder *et al.* 2002), are abundantly expressed by both maternal decidua (stromal and immune cells) and fetal trophoblast (Evans *et al.* 2004). The precise targets for decidual 1,25$(OH)_2$D3 are yet to be defined, but may include the effects upon VDR-expressing decidual immune cells (Zehnder *et al.* 2002). The aim of the current study was to characterise *ex vivo* the effects of 1,25$(OH)_2$D3 on gene expression in activated uNK from first trimester decidual tissue and to contrast these changes with effects of 1,25$(OH)_2$D3 on paired pNK.

Materials and methods

Ethical approval

Paired decidual and peripheral blood samples were obtained from healthy pregnant women undergoing first trimester elective surgical termination of pregnancy (TOP) (<12 weeks (w) gestational age (GA); range 7–11^{+2} w) following written informed consent (2014–2017; NHS REC approval 06/Q2707/12 and 13/WM/0178). GA was determined by ultrasound measurement of crown rump length and relevant anonymised patient demographics obtained as summarised (Supplementary Table 1, see section on supplementary data given at the end of this article).

Isolation of NK cells from peripheral blood and decidua

CD56+ cell-enriched isolates were prepared by modification of a previously published method (Lash *et al.* 2006). Briefly, whole decidual tissue was minced and DNase/collagenase treated. Lymphocyte purification was then carried out by density gradient centrifugation (Ficoll Plus, GE Healthcare Life Sciences). For initial cell-surface marker analysis, anti-CD56-reactive magnetic bead separation (MidiMACS, Miltenyi Biotec) was performed to obtain CD56+ cells.

Fluorescence activated cell sorting (FACS)

For cell culture and RNA-seq analysis, density-centrifuged cell populations were FACS sorted. Cells were surface stained with primary mAb as summarised and then washed in PBS and re-constituted in 500 µL MACS (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), 2 mM EDTA) for cell sorting. Propidium iodide (PI) was added to assess cell viability at the point of cell sorting, using a BD FACSAria Fusion Flow Cytometer (BD Biosciences, Wokingham, UK). The gating strategy for isolating live CD3-/CD56+ NK from peripheral blood and decidua is shown in Supplementary Fig. 1. Live CD45+ CD3− CD14− CD56+ NKp46+ pNK and uNK were identified (overall purity ≥96%; pNK 99.0–99.2%, uNKs 96.0–98.7%). Monoclonal antibodies (mAbs) used to gate live cells were anti-CD56, anti-NKp46 (Miltenyi Biotec), anti-CD3, anti-CD8, anti-CD14 from BD Biosciences. Isotype-matched IgG and IgM controls conjugated to the appropriate fluorochrome (BD Bioscience and eBioscience) were used for non-specific binding.

Cell culture

Paired uNK and pNK were cultured in complete cell culture medium (Penstrep 100 µg/ml, L-glutamine 2 mM, RPMI 1640, fetal calf serum (10%) (Sigma-Aldrich) at 37°C and 5% CO_2 for 24 h, as either unstimulated controls or with cytokine (CK) stimulation using recognised NK cell co-activators: IL-2 (50 IU/mL), IL-12 (10 ng/mL), and IL-15 (10 ng/mL). Unstimulated and CK uNK and pNK were cultured in the presence and absence of 1,25$(OH)_2$D3 (10 nM) (Enzo Lifesciences, Exeter, UK) co-treatment.

Flow cytometric analysis of CD69 and VDR

Expression of VDR (D6 anti-VDR; Santa Cruz Biotechnology) and CD69 (anti-CD69; BD Biosciences), a recognised surface marker of NK cell activation (Clausen *et al.* 2003) was assessed in paired pNK and uNK following 24 h culture in the presence and absence of CK stimulation with or without 1,25$(OH)_2$D3. Briefly, cultured cells were harvested, washed in PBS and incubated with live/dead fixable discrimination dye (Thermo Fisher) on ice for 30 min. Cells were washed with PBS, incubated for 30 min with surface primary mAb at 4°C and then re-washed. For intracellular VDR analysis, a protocol optimised from Bendix *et al*. was used (Bendix *et al.* 2015). Briefly, following surface staining, cells were fixed for 20 min in 100 µL 4% PFA, washed twice in 2 mL 2% FCS-PBS and incubated for 30 min on ice in 75 µL 5% FCS 0.5% Triton X solution (Sigma-Aldrich). Cells were then incubated with anti-VDR mAb for 30 min, washed with 2% FCS-PBS and re-incubated with anti-mouse IgG2a APC secondary mAb under the same conditions. Cells were then re-washed twice in 2% FCS-PBS followed by PBS and stored at 4°C prior to analysis. Flow cytometry data were collected using a multi-channel Dako Cyan flow cytometer (Beckman Coulter). Data were analysed using Flow Jo V10 (Tree star, Inc., Ashland, USA), gating live CD45+ CD56+ NKp46+ cells (Supplementary Fig. 2). The specificity of the D6 anti-VDR mAb in VDR positive control cells (activated T cells) was demonstrated using flow cytometry, immunoprecipitation and chromatin immunoprecipitation (Supplementary Fig. 3).

Confocal imaging of VDR in CD56+ pNKs

CD56+ pNKs were isolated and stimulated overnight with CK in the presence of 1,25$(OH)_2$D3 as described above. Cells were surface-labelled for CD56 using FITC-anti-CD56 (clone REA196, Miltenyi Biotec), then fixed and permeabilised for

VDR labelling with mouse anti-human VDR (D6 clone, Santa Cruz Biotechnology) or mouse IgG2a isotype control according to the protocol described above for flow cytometry but with goat anti-mouse IgG2a-594 and goat-anti-FITC-488 (molecular probes, Fisher Scientific) secondary labelling for 30 min in the presence of Hoechst 33342 (10 µg/mL; Fisher Scientific). After a final wash, cells were re-suspended in ProlongR Gold antifade reagent (Molecular Probes, Cell Signalling Technologies) and placed under coverslip on charged microscope slides. Edges were sealed with nail varnish and 22-slice Z stack images of cells at 63× magnification, resolution 1024×1024 pixels per image were captured using the Zeiss LSM 880, Axio Observer confocal microscope and studied with Zen(2012) software (Supplementary Fig. 4).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted as described previously (Jeffery *et al.* 2012). cDNA was prepared by reverse transcription TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Roche) as per manufacturer's instructions. qRT-PCR was performed on an ABI 7500 qPCR machine using TaqMan assays from Applied Biosystems for VDR (Hs00172113_m1) and multiplexed with VIC labelled 18S rRNA (4319413E) (Applied Biosystems, Roche). Paired uNK and pNK gene expression following 24-h culture in the presence and absence of CK stimulation with and without $1,25(OH)_2D3$ co-treatment was measured. Relative expression compared to paired unstimulated pNK was assessed using fold-change calculated by the $2^{-\Delta\Delta Ct}$ method. All statistical analyses were carried out using Sigmaplot 9.0 software (Systat Inc., San Jose, CA, USA). Experimental means were compared statistically using one-way ANOVA, with the Holm–Sidak method used as a *post hoc* multiple comparison procedure. Statistical analyses for RT-qPCR analyses were carried out using raw ΔCt values.

RNA sequence analysis (RNA-seq)

Total RNA was extracted using the RNeasy Micro Kit and RNeasy MinElute spin columns (Qiagen), as per manufacturer's instructions, eluted in 15 µL RNAse-free water, and immediately stored at −80°C prior to library preparation and sequencing (Source Bioscience Facilities, Nottingham, UK). Following initial QC check using the Agilent BioAnalyzer 2100 (Agilent) cDNA library preparation was performed using Switching Mechanism at the 5′ end of RNA Template (SMART)er Stranded Total RNA-seq Kit – Pico (Clontech, Mountain View) (RNA range = pg). Random primers and locked nucleic acid (LNA) technology were utilised to optimise total coverage. Illumina Adapters and Indexes (barcode markers) (Illumina, Inc.) were added at PCR1 with rRNA cleaved by ZapR in the presence of R-Probes (mammalian specific). The non-cleaved fragments underwent further enrichment in PCR2 cycle. All libraries were QC validated using the Agilent BioAnalyzer 2100 (Santa Clara) to confirm index samples amplified concentration and size distribution. The libraries were pooled and re-validated to assess molarity and size distribution prior to loading (1.8 pM concentration) onto a High Output NextSeq 500 Flow Cell pv2. (Illumina) across four lanes for RNA-seq using 75-base-pair (bp) paired-end reading, generating ~800 million total reads with an average of 25 million per sample. Raw data were returned in the FastQ Phred+33 (Illumina 1.9) format.

Bioinformatics and pathway analysis

Bioinformatics analysis of RNA-seq analyses was performed at University of Birmingham, UK using Partek Flow Software (Partek, St Louis, Missouri, USA). Data were trimmed for adapter sequences and pre-alignment QA/QC was performed before being mapped (aligned) using STAR-2.4.1d (Dobin *et al.* 2013). Post-alignment QA/QC was performed with a coverage report generated. Reads were quantified to transcriptome hg38_RefSeq (Genome Reference Consortium GRCh38) and normalised using quantile normalisation. Median total aligned reads were 71567649 (IQR; 64250828–74820738), representing 85.7% (84.5–87.5%) coverage. Differential gene expression was defined by a cut-off fold-change of <-1.5 and >1.5, P value ≤ 0.05 and false discovery rate (FDR)-step up ≤ 0.05. P values were calculated using F-statistics, which calculates variance within samples. Three analyses were assessed: (i) CK uNK versus (vs) CK pNK; (ii) CK uNK vs CK uNK + $1,25(OH)_2D3$; (iii) CK pNK vs CK pNK + $1,25(OH)_2D3$.

Characterisation of common biological processes in the CK-activated uNK and pNK groups was carried out using PathVisio (Version 3.2.4), an open-source pathway analysis and visualisation software tool for biological data analysis, using previously described protocols (Munoz Garcia *et al.* 2018). Briefly, analysis was performed using the 11164 raw gene counts obtained, with corresponding ENTREZ ID and symbol from the HUGO Gene Nomenclature Committee (HGNC) identifiers. A P value <0.05 was applied to determine differentially expressed genes. The Z score, which signifies whether the total number of genes in the pathway are over-represented (Z score >1.96) compared to the complete data set, was then calculated. The curated human pathway collection from the online-pathway repository WikiPathways (Kutmon *et al.* 2016) including the Reactome (Bohler *et al.* 2016) collection (downloaded May 2017) was used.

Results

Comparison of gene expression in cytokine (CK)-stimulated pNK and uNK cells

Principal component analysis (PCA) of unbiased genome-wide RNA-seq analyses revealed wide variance in the transcriptional profiles of CK-stimulated uNK and pNK (Fig. 1A). In both cell types, co-treatment with $1,25(OH)_2D3$ did not result in PCA separation of transcriptional profiles from their respective CK controls. Volcano plots of differentially expressed genes in CK-stimulated uNK relative to CK-stimulated pNK using a cut-off of $p \leq 0.05$ and fold-change $\leq$-1.5 and $\geq$+1.5 showed 1098 upregulated genes in CK-stimulated uNKs (red) and 1188 downregulated genes (green), with those not significantly different in grey ($n = 11163$) (Fig. 1B). Adjustment for FDR identified 911 transcripts to be differentially expressed between CK-stimulated

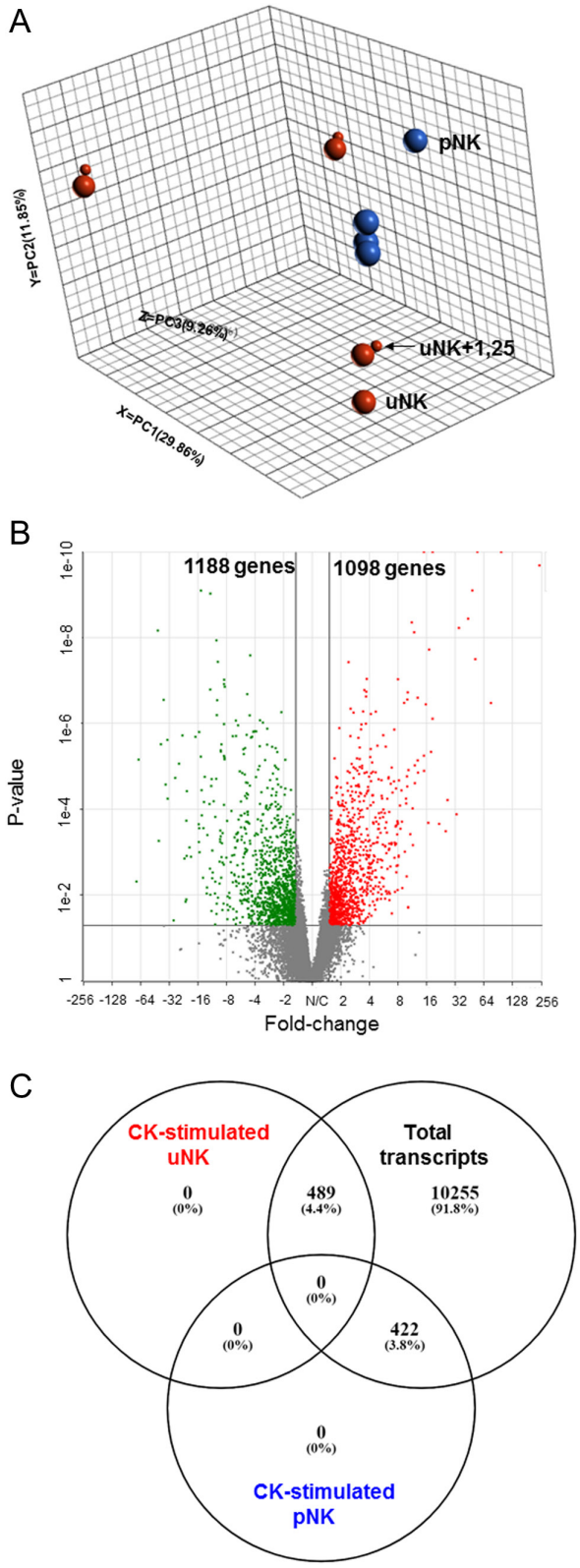

Figure 1 Transcriptomic analysis of cytokine (CK) stimulated pNK and uNK cells. (A) Principal component analysis (PCA) of CK-stimulated uNK and pNKs in the presence and absence of 1,25(OH)$_2$D3. 3-Dimensional dot-plot summarising the main sources of variance across the whole RNA-seq data set by principal components: PC1 29.9%, PC2 11.9%, PC3 9.3%. This includes pNK (red), uNK (blue) in the presence (small dot) and absence (large dot) of 1,25(OH)$_2$D3 co-treatment. (B) Volcano plot summary of differentially expressed genes in CK-stimulated uNK relative to CK-stimulated pNK. Significantly upregulated genes in CK-stimulated uNKs are in red ($n=1098$), downregulated genes in green ($n=1188$), with those not significantly different in grey ($n=11,163$). A cut-off of $P\leq0.05$ and fold-change ≤-1.5 or $\geq+1.5$ was used to determine significance. (C) Venn diagram summarising the distribution of differentially expressed genes in CK-stimulated uNK relative to CK-stimulated pNK compared to total transcripts in CK-stimulated uNK and CK pNK following adjustment for FDR: *P* value ≤0.05; log$_2$ fold-change ≤1.5 or ≥1.5, FDR step-up ≤0.05.

uNK and pNK, with 422 down-, and 489 upregulated in CK-stimulated uNK relative to CK-stimulated pNK (*P* value ≤0.05; log$_2$ fold-change ≤1.5 or ≥1.5, FDR step-up ≤0.05) (Fig. 1C). Significantly regulated genes in CK-stimulated uNK relative to CK-stimulated pNK included upregulation of *NCAM1* (CD56) (fold-change 5.29) and downregulation of *FCG3RA* (CD16A) (fold-change -14.57), both of which are well-recognised differentially expressed markers for CD56bright NK versus CD56dim pNK subsets (Koopman *et al.* 2003, Poli *et al.* 2009*b*). Interestingly, granzyme (*GZMK*) (fold-change (7.83)) and *CD96* (2.53), which permits adhesive interactions and modulates responses between activated T and NK cells (Georgiev *et al.* 2018), were both upregulated in CK-stimulated uNK. *CD276* (7.71), a regulator of T cell cytotoxicity (Picarda *et al.* 2016), was also upregulated in CK uNK.

To better delineate the differences in transcript expression between CK-stimulated uNK and pNK, complementary pathway analysis was performed on FDR-adjusted differentially expressed data using WikiPathways (WP) workflows. A broad spectrum of enriched canonical pathways was identified, with significantly enriched pathways ranked from a high to low Z score (Supplementary dataset 1). Pathways identified as being significantly enriched based on a Z score ≥1.96 are shown in Table 1. Overall, 29 WP pathways were significantly enriched in CK-stimulated uNK relative to CK-stimulated pNK, including pathways related to hypoxia-inducible factor (HIF)1α and peroxisomal proliferator-activated receptor (PPAR)γ regulation of glycolysis, and transforming growth factor (TGF)α signalling (Supplementary Fig. 5A, B, C, D, E, F, G, H, I, J K, L, M, N, O, P, Q, R and S).

VDR expression in uNK is upregulated following cytokine stimulation

RNA-seq analysis revealed that *VDR* mRNA expression is significantly upregulated in CK-stimulated uNK (fold-change 8.56) relative to CK-stimulated pNK. Analysis of differentially expressed pathways in CK-stimulated uNK versus CK-stimulated pNK in Supplementary

Table 1 Pathway analysis of differential gene expression in CK uNK versus CK pNK.

Pathway	Positive	Measured	Total	% Positive	Z score	*P* Value
HIF1A and PPARG regulation of glycolysis	6	6	19	100.00	3.64	0
TGF-beta signalling pathway	51	111	133	45.95	3.38	0.003
Cori cycle	8	10	53	80.00	3.33	0
Gastric cancer network 1	14	22	30	63.64	3.29	0.001
Retinoblastoma gene in cancer	38	80	98	47.50	3.16	0.001
Extracellular vesicle-mediated signalling in recipient cells	12	19	31	63.16	3.01	0
Regulation of sister chromatid separation at metaphase-anaphase transition	10	15	16	66.67	2.96	0.004
Calcium regulation in the cardiac cell	31	65	164	47.69	2.88	0.003
Primary focal segmental glomerulosclerosis FSGS	20	38	78	52.63	2.86	0.003
Fluoropyrimidine activity	13	22	58	59.09	2.82	0.001
Integrin-mediated cell adhesion	30	64	102	46.88	2.72	0.004
Focal adhesion	46	108	201	42.59	2.57	0.014
Chemokine signalling pathway	42	99	172	42.42	2.42	0.024
Ebola virus pathway on host	39	91	141	42.86	2.41	0.018
B cell receptor signalling pathway	36	83	99	43.37	2.4	0.009
Nanoparticle-mediated activation of receptor signalling	12	22	36	54.55	2.36	0.016
Lamin A-processing pathway	4	5	8	80.00	2.35	0.015
Signal transduction of S1P receptor	11	20	26	55.00	2.3	0.022
MFAP5-mediated ovarian cancer cell motility and invasiveness	6	9	18	66.67	2.29	0.026
Microglia pathogen phagocytosis pathway	15	30	44	50.00	2.22	0.027
Histone modifications	27	61	68	44.26	2.21	0.024
Association between physico-chemical features and toxicity associated pathways	22	48	78	45.83	2.19	0.026
Human thyroid-stimulating hormone (TSH) signalling pathway	24	54	67	44.44	2.1	0.032
Mesodermal commitment pathway	35	84	154	41.67	2.08	0.024
Apoptosis-related network due to altered Notch3 in ovarian cancer	20	44	54	45.45	2.04	0.047
Regulation of Wnt/B-catenin signalling by small molecule compounds	7	12	33	58.33	2.03	0.036
T Cell antigen receptor (TCR) signalling pathway	32	77	93	41.56	1.97	0.047
Purine metabolism	6	10	75	60.00	1.96	0.044
G protein signalling pathways	25	58	97	43.10	1.96	0.047

Summary of WikiPathways data-base analysis for CK uNK versus CK pNK. Frequency of significant differentially expressed genes (*P* value <0.05, Z score >1.96), total genes measured as a proportion of the total frequency of pathway enriched genes were ranked based on Z score.

dataset 1 also showed enhanced expression of pathways associated with VDR and its heterodimer partner retinoid X receptor (RXR) (Z score 1.83, $p=0.07$) (Fig. 2), as well as non-genomic actions of $1,25(OH)_2D3$ (Z score 1.88, $P=0.062$) (Supplementary Fig. 5A). This suggests that capacity for $1,25(OH)_2D3$-VDR responses is elevated in CK-stimulated uNK relative to CK-stimulated pNK. Studies were therefore carried out to further characterise VDR expression in uNK and pNK. Flow cytometry showed that pNK and uNK expressed similar levels of the NK marker NKp46. Analysis of CD56 expression showed that uNK cells were characterised by more distinct populations of CD56bright and CD56dim cells than pNK (Fig. 3A). Both CD56 and NKp46 showed no change in response to either CK stimulation or $1,25(OH)_2D3$ treatment in either uNK or pNK cells for median fluorescence intensity (MFI) (Fig. 3A) or frequency of expression (data not shown). As shown in Fig. 3B and C, CK stimulation significantly upregulated expression of the activation marker CD69 in both uNK (median 47.9% IQR 31.5–56.6 to 68.1%; 48.9–76.4) and pNK (17.7%; 11.5–20.3 to 70.5%; 57.4–72.8), but treatment with $1,25(OH)_2D3$ had no significant effect on CD69 expression in either the unstimulated or CK-stimulated uNK or pNK populations (Fig. 3C).

Without stimulation the frequency of NK cells that expressed VDR was low for both uNK (36.2%; 33.1–36.5) and pNK (15.8%; 5.81–61.6). CK stimulation significantly increased VDR frequency in uNK (74.2%; 65.6–81.3), although the absolute level of VDR expression by each expressing cell (MFI) was not significantly increased (Fig. 3D). $1,25(OH)_2D3$ had no significant effect on VDR protein expression in either uNK or pNK in the presence or absence of CK stimulation (Fig. 3C). Expression of *VDR* mRNA was also significantly induced by CK stimulation in uNK ($P=0.0001$) and pNK ($P=0.017$). Interestingly, this effect was suppressed in both cell types by co-treatment with $1,25(OH)_2D3$ (Fig. 3D). Confocal imaging of CK-stimulated pNK cells (Supplementary Fig. 4) showed co-localisation of VDR protein with both DNA (Hoescht staining) and the plasma membrane (CD56), consistent with both membrane and nuclear localisation of VDR.

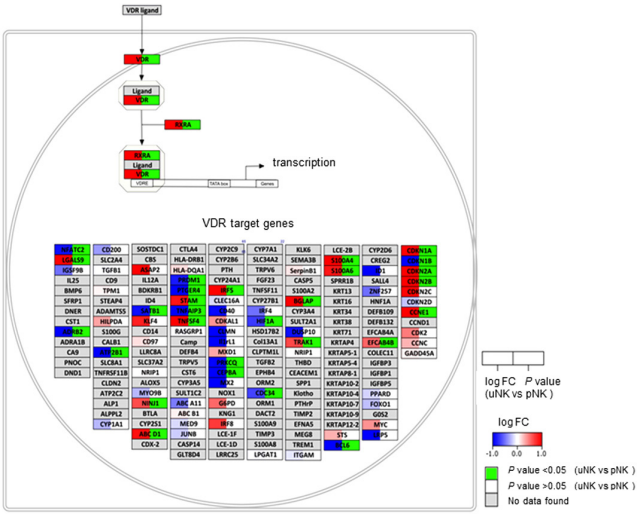

Figure 2 Pathway analysis of vitamin D receptor (VDR) signalling-related genes in CK-stimulated uNK versus CK-stimulated pNK. Visualisation of genes with enhanced (red) or suppressed (blue) expression in CK-stimulated uNK versus CK-stimulated pNK. Individual genes are shown in boxes and box colour split into two parts, (1) the $\log_2$ fold-change in the left part of the box (blue downregulated, white not changed, red upregulated) and (2) *P* value for statistical significance is shown in the right part of the box (green when significant). Pathway elements not assessed in the dataset are shown in grey.

Effects of 1,25(OH)$_2$D3 on gene expression in CK-stimulated pNK and uNK cells

When compared to CK-stimulated pNK, CK-stimulated pNK treated with 1,25(OH)$_2$D3, showed only 71 genes differentially expressed genes (fold-change ±1.5; $P \leq 0.05$). Of these, 33 (46.5%) were downregulated and 38 (53.5%) upregulated by 1,25(OH)$_2$D3. In CK-stimulated uNK treated with 1,25(OH)$_2$D3 66 genes were differentially expressed relative to CK stimulation of uNK alone. Of these, 46 (69.7%) were down- and 19 (30.3%) upregulated by 1,25(OH)$_2$D3. In contrast to pNK, 1,25(OH)$_2$D3 primarily targeted uNK genes implicated in cell processing, specifically cell adhesion, apoptosis, migration and angiogenesis ($n=27$; 41.5%) (Table 2). Due to the relatively small number of genes differentially expressed in response to 1,25(OH)$_2$D3 in either CK-stimulated pNK or uNK, adjustment of data to incorporate fold-change ±1.5, *P* value ≤0.05, FDR step up <0.05 resulted in no significant effect of 1,25(OH)$_2$D3 for either CK-stimulated uNK or CK-stimulated pNK. Furthermore, pathway analysis using non-adjusted differentially expressed gene data for either CK-stimulated uNK or CK-stimulated pNK cell treated with 1,25(OH)$_2$D3 showed no significant enrichment of any specific pathways by 1,25(OH)$_2$D3 (data not shown), and no commonality with any of the pathways that are known to be regulated by 1,25(OH)$_2$D3 in other immune cells (Fig. 4).

Discussion

Previous studies using DNA microarrays have described transcriptomic variations in CD56bright pNK and CD56dim pNK cells, as well as CD56 bright uNK (Koopman *et al.* 2003). In the current study, pNK or uNK were not sub-categorised according to CD56 brightness, because CD56dim pNK predominate in peripheral blood (approximately 95% of total pNK cell population), whilst CD56bright uNK are the prevalent NK type in decidua (>95% of total uNK population). These differences imply that uNKs are constitutively active. In agreement with this, we observed that uNK were larger with abundant perforin and granzyme. Importantly, no change in CD56 or NKp46 activation receptor expression was measured in uNK or pNK in response to CK stimulation or treatment with the active form of vitamin D, 1,25(OH)$_2$D3. CD56bright NKs are considered efficient immunomodulatory cytokine producers, and only become cytotoxic following appropriate activation. However, in contrast to NK from most other sites, uNKs do not express the activating receptor CD16 which mediates antibody-dependent cellular cytotoxicity (Poli *et al.* 2009*a*). It appears that whilst uNKs maintain an intrinsic capacity to exert cytotoxic functions when specifically challenged, this function is regulated in normal early pregnancy (Moffett & Colucci 2014). Further undefined local mechanisms, such as cytokine or hormone secretion, or cross-talk with other immune cell types within the decidual microenvironment are also thought to suppress the potential lytic effects of uNKs (Hanna *et al.* 2006). In the current study, pro-inflammatory IL-2, IL-12 and IL-15 were utilised for CK challenge, as these are recognised NK cell activators, with both IL-12 and IL-15 constitutively expressed within the decidua from initial implantation (Kovacs *et al.* 1992, Zourbas *et al.* 2001). Although IL-2 concentrations appear low within normal decidua (Jokhi *et al.* 1994), significantly elevated IL-2 has been reported in the setting of malplacentation (Garzia *et al.* 2013), where vitamin D deficiency is also more common (Robinson *et al.* 2011).

The first aim of the current study was to compare the transcriptomic profiles of uNK versus pNK following immune activation by CK stimulation. The second objective of the study was to determine if CK-stimulated uNK and pNK were responsive to 1,25(OH)$_2$D3 in a similar manner to that reported for other components of the immune system such as macrophages and T cells (Adams & Hewison 2008). Since the effects of 1,25(OH)$_2$D3 on NK cell function in decidua and maternal blood are still unclear, NK cell subsets were not further sub-classified.

In the current study, approximately 900 genes were differentially expressed in CK-stimulated uNK versus CK-stimulated pNK, similar to previous DNA array analyses for unstimulated CD56dim pNK versus

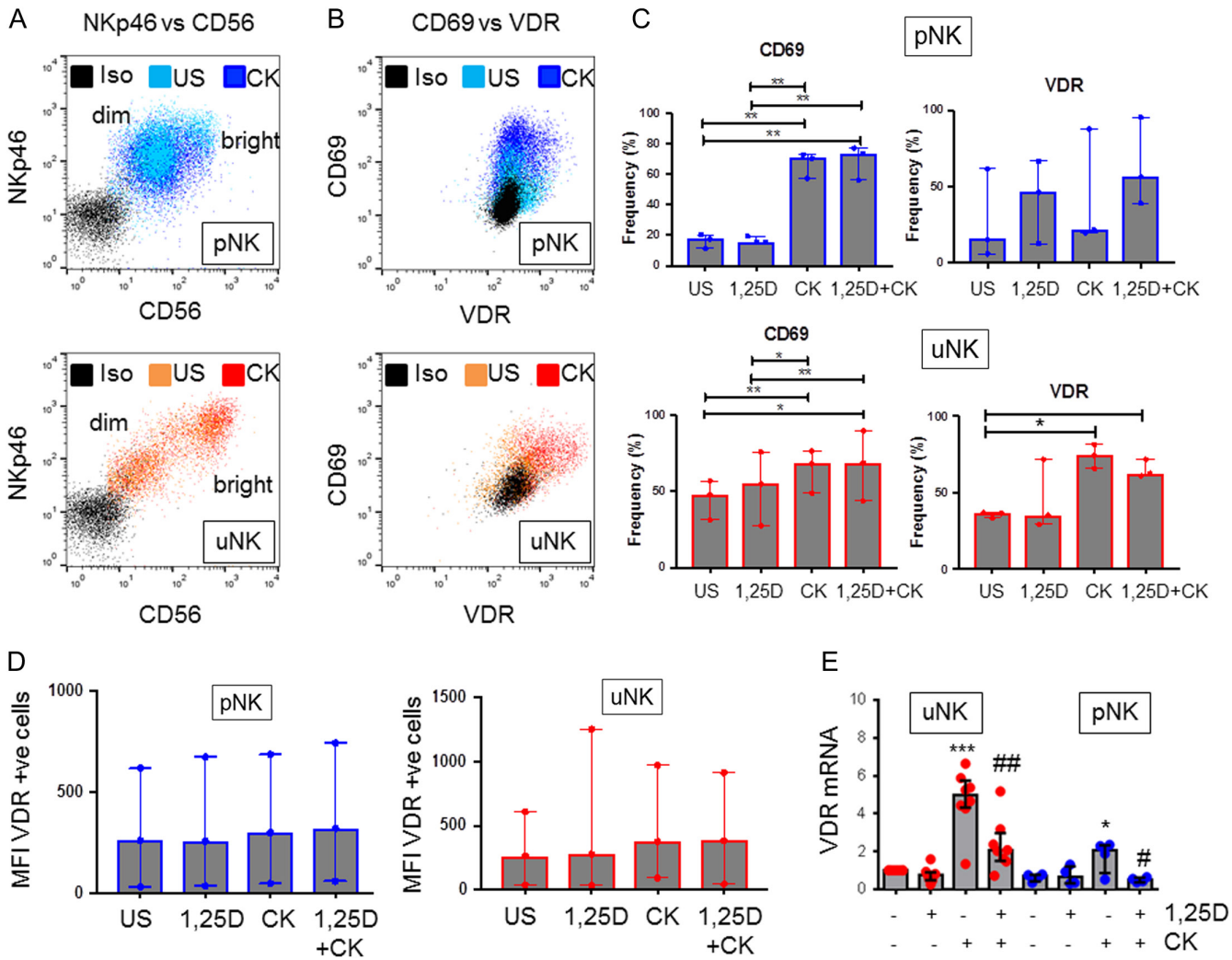

Figure 3 Expression of VDR in paired peripheral blood and uterine natural killer cells. (A) Representative flow cytometry plots for expression of NKp46 and CD56 in isolated unstimulated (US) and cytokine (CK)-stimulated peripheral blood natural killer cells (pNK) and uterine natural killer cells (uNK) relative to isotype control (Iso). (B) Representative flow cytometry plots for expression of VDR and CD69 in US and CK-stimulated CD3-CD56+ uNK and pNK cells relative to Iso control. (C) Summary of VDR and CD69 surface protein expression in US- and CK uNK and -pNK cells in the presence or absence of 1,25$(OH)_2$D3 (1,25D). (D) Median fluorescence intensity (MFI) for VDR expression in pNK and uNK cells. (E) mRNA expression for *VDR* in isolated US and CK uNK and pNK cells in the presence and absence of 1,25$(OH)_2$D3. Relative expression compared to US uNK cells; median value with bars denoting inter-quartile range. Effect of CK stimulation and 1,25$(OH)_2$D3 was assessed by two-way ANOVA. Stars indicate level of significant difference between groups for which multiple comparisons analysis indicated significance *<0.05, **<0.01.

unstimulated CD56bright uNK (Koopman *et al.* 2003). In CK-stimulated uNK a comparable number of up- (429) and downregulated (422) genes were measured when adjusted for FDR. By contrast, in the absence of CK stimulation, it was reported that uNK almost exclusively exhibited enhanced transcription relative to pNK (Koopman *et al.* 2003). uNK responses to CK stimulation appear highly distinct from paired pNK. In the circulation, pNK function as an early cytolytic response to tumours or viral infection (Vivier *et al.* 2011, Chiossone *et al.* 2018). By contrast, consistent with previous reports, we observed that uNK are poorly cytolytic and express low levels of pNK cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (Kopcow *et al.* 2005, Vacca *et al.* 2006) and appear less reactive with low CD107 (degranulation marker) expression relative to pNKs in response to CK challenge (data not published). The unique phenotype of uNK may reflect their adaptation within this unique maternal tissue microenvironment to aid decidualisation, embryonic implantation and subsequent fetal development (Pollheimer *et al.* 2018).

Detailed pathway analysis verified that CK-stimulated uNK are phenotypically distinct from CK-stimulated pNK, with cell metabolism, signalling and processing, and cancer pathways consistently over-expressed in

Table 2 Effect of 1,25$(OH)_2$D3 on gene expression in CK pNK and uNK.

	Genes expressed in pNK			Genes expressed in uNK		
Functional classification	Total transcripts	Upregulated by 1,25$(OH)_2$D3	Downregulated by 1,25$(OH)_2$D3	Total transcripts	Upregulated by 1,25$(OH)_2$D3	Downregulated by 1,25$(OH)_2$D3
Cell structure	3	*TTLL1, FAM161A*	*CMYA5*	2		*CAMSAP2, SOBP*
Cell survival, proliferation, invasion, adhesion, angiogenesis, trafficking	15	*TMEM144A, MMP14, PIK3R6, ENG, RABL2A, DEPDC1B, TRIM35, CAB39L, AP5S1*	*TSPAN4, SERPINI1, NRP2, RAPGEFL1, FAM195A, AP4M1*	27	*TSPAN2, ARAP3, ITGAM, RARRES3, ADGRE5, FGL2, DYSF, NINJ1, ZFP91-CNTF, DENND6B, BGLAP, DOCK3, RNF165, TAX1BP3, TRIM35, TAGLN2, RGS3*	*ADGRG1, TMPRSS6, CYGB, C8orf44-SGK3, LZTS3, MARCKSL1, NACAD, TCF7L2, PHOSPHO2-KLH23, P2RY11*
Immune function	3	*LXN, MTCP1, RSAD2*		4	*SERPINB1, IGALS9, RELT*	*RSAD2*
Metabolism and lipogenesis	17	*ACOT1, ACSF2, CCBL1, MTHFD2L, PPP1R3F, AMPD3, GRHPR*	*PM20D2, TKFC, KDELC1, BDH2, GMPR, BCKDHB, NBR2, NPR2, ENPP1, NR2F6*	7	*TMEM56-RWDD3, SARDH, CYP1A1, CDPCP1, ACSL1, GDE1, ADA*	
Genetic	10	*CBX8, DNAJC30, RPA3, EXOSC7, PPFIBP1*	*TUT1, GTPBP3, MIR600HG, TCEANC, TYRO3P*	9	*TFEC, WWC2, ZBTB7A, HIC1, EFL1, HNRNPLL, TRAK1, CREG1*	*BHLHB9*
Ion transport	1	*SLC35E3*		2	*SLC31A1*	*SLC22A1*
Unknown function	10	*LOC101929767, TTC32, LOC100287042, PROB1*	*ARMCX4, LOC441666, LOC100506804, PRRC4, CBWD6, THAP8*	8	*ZSWIM5, LOC101928464, TNRC6C-AS1, POMGNT2, PLEKho2, NARS2, INTS6L*	*C1QTNF3-AMACR*
Anti-sense, non-coding, snoRNAs	11	*BRWD1-IT2, SNORA67, SNORA17B, LOC100129083, ADNP-AS1, RPL13P5*	*C22orf34, SNHG25, TRIM47, MRPL42P5, GCSHP3*	6	*SNORA17A, SIGLEC17P, NPPA-As1*	*CCDC102A, ALMS1-IT1, LOC100505771*

Summary of total genes differentially induced or suppressed by 1,25$(OH)_2$D3 in CK-stimulated pNK and CK-stimulated uNK (fold-change <−1.5 or >+1.5, $P \leq 0.05$), with sub-categorisation according to transcript function.

CK-stimulated uNK relative to CK-stimulated pNK. These observations are consistent with the known roles of tissue-resident uNK in placental development and fetal implantation. The significant inter-pathway transcript variance between CK-stimulated uNK and pNK underlines the complex diversity of uNK cell function. HIF1A and PPARγ regulation of glycolysis (Z score 3.64) was the most significantly differentially expressed pathway in CK-stimulated uNK (Supplementary Fig. 5B). The Cori cycle, a lactate–glucose carbon recycling system between muscle and liver that decreases circulating lactate was also enriched in CK-stimulated uNK (Z score 3.33) (Supplementary Fig. 5D). Genes associated with glycolysis and gluconeogenesis were also enhanced in CK-activated uNK (Z score 1.83, 14/30 genes, $P=0.067$), further underlining the active metabolic remodelling in uNK relative to pNK. During early pregnancy decidualisation is associated with enhanced glucose influx (Frolova & Moley 2011, Kommagani *et al.* 2013). Recent studies have shown that the decidua acts in a manner similar to that reported for tumours by generating energy via a high rate of glycolysis, even under aerobic conditions rather, than conventional oxidative phosphorylation (Zuo *et al.* 2015). This is referred to as a decidual Warburg-like glycolysis similar to that reported in tumours and was dependent on expression of HIF1α and the lactate transporter protein (MCT4) (Zuo *et al.* 2015). Collectively, these data suggest that a key feature of activated uNK is to regulate glucose metabolism and lactate transport within the decidua. Dysregulation of glucose metabolism in the human uterus is associated with subfertility and aberrant implantation (Kommagani *et al.* 2013). Glycolytic flux targets in uNK may therefore represent novel translational strategies to precisely time and/or therapeutically extend the window of receptivity in women at high risk for early pregnancy loss. Consistent with previous microarray analysis of first trimester uNK and adult non-paired pNK (Wang *et al.* 2014), the current study showed that CK-stimulated uNK are enriched for pathways associated with transcriptional regulation, which may serve to differentially regulate NK development and function in the decidual microenvironment. Whether pNK undergo significant transcriptional alterations following recruitment to the decidua or, alternatively, uNK represent an entirely distinct subset of NK cells remains unclear (Hanna *et al.* 2003, Vacca *et al.* 2011).

Of the total transcripts measured in CK-activated uNK and pNK, only 66 (0.59%) and 71 (0.64%) respectively were significantly regulated by 1,25$(OH)_2$D3 ($P \leq 0.05$, fold-change ±1.5). Although transcriptomic analysis indicated that CK uNK and CK pNK respond differentially to 1,25$(OH)_2$D3, this effect was statistically insignificant as the current analysis was not significantly powered to account for multiple comparisons (FDR step up <0.05). Sample size and participant heterogeneity are potential reasons for this. Intra-participant variability for pNK was

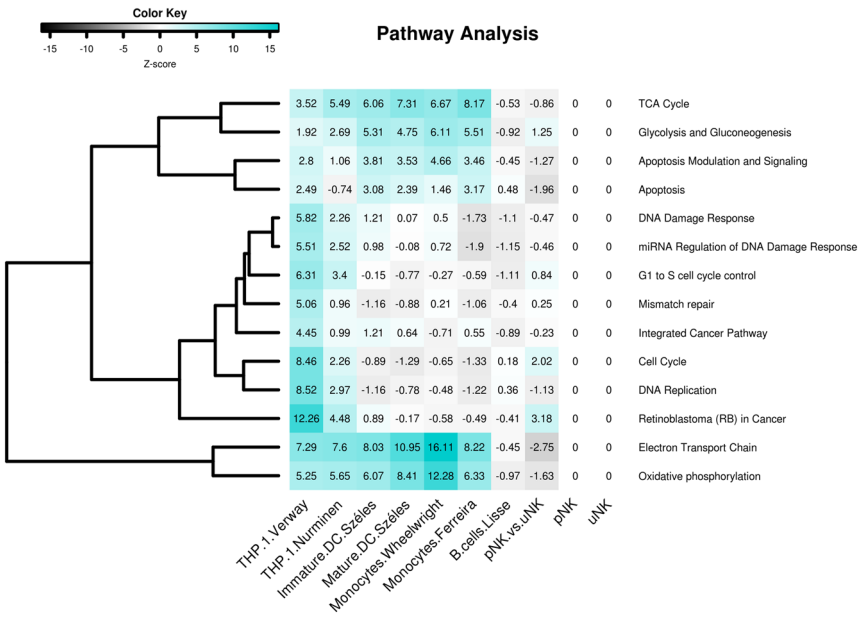

Figure 4 Pathway analysis of transcriptomic responses to 1,25(OH)$_2$D3-treated in THP-1 cells, dendritic cells, monocytes and B cells compared to effects in pNK and uNK. Heat map representing Z-scores for pathways significantly altered by 1,25(OH)$_2$D3 comparing pathway analysis on datasets from THP-1 cells, dendritic cells (DC), monocytes and B cells with effects of 1,25(OH)$_2$D3 on CK-stimulated pNK and uNK. Z-scores >1.96 indicate that more genes are significantly altered in this pathway compared to the complete dataset. Genes differentially expressed between CK-stimulated uNK and pNK in the absence of 1,25(OH)$_2$D3 (pNK vs uNK) showed minimal overlap with the effect of 1,25(OH)$_2$D3 on immune cells. The effects of 1,25(OH)$_2$D3 on CK-stimulated pNK or uNK showed no commonality with the other immune cells.

high, whereas the uNK demonstrated comparatively low variability. The heterogeneity in pNK gene expression may be in part attributable to differences in gestational age, maternal age, ethnicity and smoking status (Supplementary Table 1), albeit n numbers are small. Cellular heterogeneity may also be a contributing factor to sample variability as recent single-cell RNA sequencing data indicates three highly distinct decidual NK subsets to be resident within first trimester decidua (Vento-Tormo *et al.* 2018).

Analysis of commonly regulated biological pathways, as detailed previously (Munoz Garcia *et al.* 2018), demonstrates that responses to 1,25(OH)$_2$D3 in CK-stimulated uNK and pNK diverge significantly from other immune subsets including monocytes, dendritic cells and B cells (Fig. 4). This may in part reflect different treatment regimens. Here a physiological treatment dose (10 nM) of 1,25(OH)$_2$D3 was utilised, whereas for monocytic THP-1 cells a 10-fold higher treatment dose (100 nM) was applied (Seuter *et al.* 2016). Temporal variations may also be a contributory factor. For THP-1 cells, 46 genes were induced/ suppressed after 2.5 h, 288 at 4 h and 1204 at 24 h. In the current study we assessed mRNA expression in NK cells after 24 h to maximise potential variations in gene expression; however, both pNK and uNK cells may show different patterns of gene regulation by 1,25(OH)$_2$D3 at earlier or later time points. The distinct transcriptional responses to 1,25(OH)$_2$D3 by NK cells may also reflect differential distribution of VDR.

Data reported here provide further evidence for the distinct phenotype of uNK cells relative to circulating pNK. By comparing the gene expression profile of these cells under conditions of immune activation, we confirmed enrichment of distinct functional pathways in uNK such as TGF-β signalling (Supplementary Fig. 5C), which has previously been shown to promote uNK development (Keskin *et al.* 2007). Our data have also revealed novel features of CK-stimulated uNK, notably enrichment of pathways associated with metabolism, suggesting a role for uNK cells in immunometabolic activity within the decidua. The lack of 1,25(OH)$_2$D3 response by uNK and pNK contrasts the expression of VDR by both cell types and the enhancement of this receptor in CK-stimulated uNK vs pNK. Given the abundant levels of 1,25(OH)$_2$D3 within decidual tissue (Tamblyn *et al.* 2017), and the potent immunomodulatory actions of 1,25(OH)$_2$D3 (Chun *et al.* 2014), the lack of response by NK cells is intriguing. Further studies are required to better define the role of the VDR in NK cells. Data in the current study suggest possible cell membrane expression of VDR and this may support non-genomic responses to 1,25(OH)$_2$D3 (Hii & Ferrante 2016). In the case of uNK, it is also possible that 1,25(OH)$_2$D3 is able to influence these cells indirectly via other immune cells such as macrophages that have an established transcriptional and functional response to 1,25(OH)$_2$D3. It is now clear that uNK play a key role in mediating the cross-talk between immune cells within the decidua (Vento-Tormo *et al.* 2018), and vitamin D may therefore play a role in this process. Finally, the data presented in the current study have focused on transcriptional activity but it is also possible that 1,25(OH)$_2$D3 exerts epigenetic effects on NK cells independent of transcription. In recent studies of uNK cells, it has been shown that repeated pregnancies are associated with uNK cells that have a unique transcriptomic and epigenetic signature consistent with trained immune responses (Gamliel *et al.* 2018). Based on its established epigenetic activity (Carlberg 2019), it is tempting to speculate that vitamin D may contribute to this facet of uNK function.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0509.

Declaration of interest

Vitabiotics Pregnacare generously supported this work through the charity Wellbeing of Women, which independently selected the research through its AMRC-accredited peer review process led by its Research Advisory Committee. Dr Tamblyn and her work were not impacted or influenced in any way by its sources of funding. The other authors have nothing to disclose.

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