

# Vitamin D deficiency may be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity

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**STUDY QUESTION:** Do women with recurrent pregnancy losses (RPL) and low vitamin D have increased prevalence of auto- and cellular immune abnormalities when compared with women with RPL who have normal vitamin D, and does vitamin D have any effect on cellular immunity *in vitro*?

**SUMMARY ANSWER:** A high proportion of women with RPL have vitamin D deficiency and the risk of auto- and cellular immune abnormalities is increased in women with RPL and vitamin D deficiency.

**WHAT IS KNOWN ALREADY:** Vitamin D deficiency in pregnant women is associated with increased risk of obstetrical complications such as pre-eclampsia, bacterial vaginosis associated preterm delivery, gestational diabetes mellitus and small-for-gestational age births.

**STUDY DESIGN, SIZE, DURATION:** A retrospective cross-sectional study of 133 women with RPL who were enrolled in a 2-year period, together with laboratory experiments.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Women with three or more consecutive spontaneous abortions prior to 20 weeks of gestation who were enrolled at the University clinic. Serum vitamin D level, cellular activity and autoimmune parameters *in vivo* and *in vitro* were measured.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Sixty-three out of 133 women (47.4%) had low vitamin D (<30 ng/ml). The prevalence of antiphospholipid antibody (APA) was significantly higher in low vitamin D group (VDlow) (39.7%) than in the normal vitamin D group (VDnl) (22.9%) ( $P < 0.05$ ) and the adjusted odds ratio (OR) for APA in VDlow was 2.22 with the 95% confidence interval (CI) of 1.0–4.7. The prevalence of antinuclear antigen antibody (VDlow versus VDnl; 23.8% versus 10.0%, OR 2.81, 95% CI 1.1–7.4), anti-ssDNA (19.0% versus 5.7%, OR 3.76, 95% CI 1.1–12.4) and thyroperoxidase antibody (33.3% versus 15.7%, OR 2.68, 95% CI 1.2–6.1) was significantly higher in VDlow than those of VDnl ( $P < 0.05$  each). Peripheral blood CD19<sup>+</sup>B and CD56<sup>+</sup>NK cell levels and NK cytotoxicity at effector to target cell (E:T) ratio of 25:1 were significantly higher in VDlow when compared with those of VDnl ( $P < 0.05$  each). Reduction (%) of NK cytotoxicity (at E:T ratio of 50:1 and 25:1) by IgG (12.5 mg/dl) was significantly lower in VDlow than those of VDnl ( $P < 0.05$ ,  $P < 0.01$ , respectively). There were no differences in Th1/Th2 ratios between VDlow and VDnl. When vitamin D<sub>3</sub> was added in NK cytotoxicity assay *in vitro*, NK cytotoxicity at E:T ratio of 50:1 was significantly suppressed with 10 nM/L (nM) ( $11.9 \pm 3.3\%$ ) and 100 nM ( $10.9 \pm 3.7\%$ ) of vitamin D<sub>3</sub> when compared with controls ( $15.3 \pm 4.7\%$ ) ( $P < 0.01$  each). TNF- $\alpha$ /IL-10 expressing CD3<sup>+</sup>/4<sup>+</sup> cell ratios were significantly decreased with 100 nM of vitamin D<sub>3</sub> ( $31.3 \pm 9.4$ ,  $P < 0.05$ ) when compared with controls ( $40.4 \pm 11.3$ ) *in vitro*. Additionally, INF- $\gamma$ /IL-10 expressing CD3<sup>+</sup>/4<sup>+</sup> cell ratio was significantly decreased with 100 nM of vitamin D<sub>3</sub> ( $12.1 \pm 4.0$ ,  $P < 0.05$ ) when compared with controls ( $14.8 \pm 4.6$ ). INF- $\gamma$  and TNF- $\alpha$  secretion from NK cells were significantly decreased ( $P < 0.01$  each), and IL-10, IL-1 $\beta$ , vascular endothelial growth factor and granulocyte colony stimulating factor levels were significantly increased ( $P < 0.01$  each) with vitamin D<sub>3</sub> 100 nM when compared with those of controls.

**LIMITATIONS, REASONS FOR CAUTION:** The prevalence of vitamin D deficiency in women with RPL in this study is open to a possible type I error since women with vitamin D supplementation were excluded from this study.

**WIDER IMPLICATIONS OF THE FINDINGS:** Assessment of vitamin D level is recommended in women with RPL. Vitamin D supplementation should be explored further as a possible therapeutic option for RPL.

**STUDY FUNDING/COMPETING INTEREST(S):** This work was supported by the intramural funding from Department of Microbiology and Immunology, Chicago Medical School at Rosalind Franklin University of Medicine and Science. None of the authors has any conflict of interest to declare.

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**Key words:** vitamin D / recurrent pregnancy loss / natural killer cells / Th1 / autoimmune abnormalities

## Introduction

Vitamin D, a steroid hormone, is well known to be involved in calcium-phosphate homeostasis and bone metabolism (Halloran *et al.*, 1990; Yoshizawa *et al.*, 1997; Panda *et al.*, 2001). The biological activity of vitamin D occurs via two pathways, a slow genomic response and a rapid, non-genomic response (Mizwicki and Norman, 2009). The classical action of vitamin D is a genomic response, and for this action, the vitamin D receptor is localized in the cellular nucleus of various tissues (Rajakumar, 2003; Christakos *et al.*, 2007). In the past decade, non-skeletal effects of vitamin D via non-genomic responses (Bikle, 2009) have been reported in many organs (Reichel *et al.*, 1989; Walters, 1992). The target organs for the non-classical actions of the vitamin D include the adaptive and innate immune systems, pancreatic  $\beta$ -cells, the heart and cardiovascular system, the brain and reproductive tissues. Tissue responses to vitamin D include regulation of hormone secretion, modulation of immune responses, and a control of cellular proliferation and differentiation (Mizwicki and Norman, 2009). Vitamin D was also reported to inhibit proliferation of T helper 1 (Th1) cells and limit their production of cytokines, such as interferon gamma (IFN- $\gamma$ ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF- $\alpha$ ). Conversely, vitamin D induces T helper 2 (Th2) cytokines, such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Lemire *et al.*, 1984; Piccinni *et al.*, 2000; Adams and Hewison, 2008). Furthermore, in many studies vitamin D has been presented as a modifiable environmental factor for Th1-mediated autoimmune disease and appears to be important for susceptibility to and severity of the disease (Lemire *et al.*, 1992; Cantorna *et al.*, 1998; Cantorna, 2000; Huisman *et al.*, 2001; Zella *et al.*, 2003; Kamen *et al.*, 2006; Arnson *et al.*, 2007). Vitamin D also regulates B cell immunity. It down-regulates the proliferation and differentiation of B lymphocytes and inhibits IgG production (Lemire *et al.*, 1984; Rigby *et al.*, 1984; Chen *et al.*, 2007a,b; Adams and Hewison, 2008).

With these immune modulatory effects of vitamin D, it has been speculated that vitamin D could act as an immune regulator during implantation and play an important role in reproductive capacity. In early pregnancy, trophoblasts produce and respond to vitamin D, and some investigators have demonstrated that vitamin D influences local anti-inflammatory responses and induces decidualization for successful pregnancy (Halhali *et al.*, 1991; Stephanou *et al.*, 1994; Du *et al.*, 2005; Barrera *et al.*, 2007, 2008; Diaz *et al.*, 2009). Others find that vitamin D sufficiency is associated with increased IVF pregnancy rates (Ozkan *et al.*, 2010; Rudick *et al.*, 2012). The role of vitamin D in human reproduction has been increasingly recognized as important. However, the effect of vitamin D in recurrent pregnancy losses (RPL) has not been studied yet.

A dominant Th2 immune response is important to maintain maternal-fetal relationship for successful pregnancy (Wegmann *et al.*, 1993). In contrast autoimmunity and dysregulated cellular immune reactions may be responsible for immunological alterations leading to RPL (Souza *et al.*, 2002). On the basis of these immuno-pathological abnormalities of RPL, we aim to investigate the relation of serum vitamin D level and peripheral blood immune parameters, such as Th1 to Th2 cytokine producing CD3<sup>+</sup>/4<sup>+</sup> cell ratio, peripheral blood immunophenotypes, natural killer cell cytotoxicity (NK cytotoxicity) and autoantibodies in women with a history of RPL. In addition, the *in vitro* effect of vitamin D on NK cells, including NK cytotoxicity, perforin expression and cytokine production, and Th1/Th2 cell ratios were investigated.

## Materials and Methods

### Study population

The study was designed as a retrospective cross-sectional study with laboratory experiments. Medical records of 186 women with a history of RPL (defined as three or more consecutive spontaneous abortions prior to 20 weeks of gestation), who registered at the Reproductive Medicine Center, Chicago Medical School at Rosalind Franklin University of Medicine and Science from January 2011 to December 2012, were reviewed consecutively as they registered. Among them, 53 were excluded due to uterine factor (uterine anomaly, uterine synechia, endometrial pathology,  $n = 9$ ), endocrine factor (polycystic ovarian syndrome, diabetes, thyroid disease, abnormal prolactin levels,  $n = 19$ ), genetic factors (parental chromosomal abnormalities,  $n = 1$ ), infectious factors (Chlamydia trachomatis and Ureaplasma urealyticum,  $n = 3$ ), a history of or active autoimmune disease and/or connective tissue disease ( $n = 7$ ), pregnancy ( $n = 5$ ), medical treatment ( $n = 4$ ), and vitamin D supplementation ( $n = 15$ , 10 of them also had endocrine factor). In total 133 women with RPL composed the study group. None had positive findings for uterine, endocrine, genetic, infectious factors, or a history of or active autoimmune disease and connective tissue disease. No one was pregnant or on medical treatment when blood was drawn for laboratory evaluation. Patients with vitamin D supplementation were excluded from the study except patients with prenatal vitamins containing vitamin D 400 units per day. Blood was drawn during early follicular phase and vitamin D levels and immune parameters were tested on the same day.

The sample size calculation was made prior to the study. Type I error rate ( $\alpha$ ) was 0.05 and the power was 0.80 for the two-sided test. To calculate sample size, peripheral blood NK cell levels were utilized as the primary variable since the first goal of the study was to detect the differences in NK cell immunity in vitamin D normal and low groups. Expected sample size was 63 for each group. We collected the sample population consecutively, until we reached the 63 vitamin D deficient population.

**Table 1** Patient characteristics of women with three or more recurrent pregnancy losses (RPL) with normal vitamin D ( $\geq 30$  ng/ml,  $n = 70$ ) and low vitamin D ( $< 30$  ng/ml,  $n = 63$ ).

Characteristics	Vitamin D Normal ( $\geq 30$ ng/ml) ( $n = 70$ )	Vitamin D Low ( $< 30$ ng/ml) ( $n = 63$ )	P-value
Age (years)	37 (33.0–39.5)	35.5 (33.0–39.0)	NS
Gravidity	3 (3–5)	3 (3–5)	NS
Parity	0 (0–1)	0 (0–0.5)	NS
Number of SAB <sup>a</sup>	3 (3–4.5)	3 (3–5)	NS
Primary aborter ( $n$ (%))	49 (70.0)	50 (79.4)	NS <sup>b</sup>
Secondary aborter ( $n$ (%))	21 (30.0)	13 (20.6)	
No. of IVF failure	0 (0–1.5)	0 (0–1)	NS
IVF treatment history ( $n$ (%))	19 (27.1)	15 (23.8)	NS
IVF due to RPL	11 (15.7)	8 (12.7)	NS
IVF due to infertility/subfertility	4 (5.7)	3 (4.8)	
IVF due to advanced age	4 (5.7)	4 (6.3)	
BMI ( $\text{kg}/\text{m}^2$ )	25.7 (23.5–29.5)	27.5 (23.5–29.7)	NS
Vitamin D level (ng/ml)	41 (37–48)	25 (22–28)	$P < 0.0001$
Blood test			
Summer season <sup>c</sup> ( $n$ (%))	36 (51.4)	29 (46.0)	NS <sup>e</sup>
Winter season <sup>d</sup> ( $n$ (%))	15 (21.4)	13 (20.6)	
Other season ( $n$ (%))	19 (27.1)	21 (33.3)	
Prenatal vitamin usage <sup>f</sup> ( $n$ (%))	27 (38.6)	22 (34.9)	NS
Interval from last SAB (months)	9 (6–12)	8 (6–11)	NS
Interval from last IVF cycle (months)	6 (4–9)	6 (5–9.8)	NS
Race			
Caucasian ( $n$ (%))	61 (87.1)	57 (90.5)	NS <sup>g</sup>
Hispanic ( $n$ (%))	6 (8.6)	4 (6.3)	
Asian ( $n$ (%))	3 (4.3)	2 (3.2)	

Data are median (quartiles) unless stated otherwise.

<sup>a</sup>SAB, spontaneous abortion. Primary aborters have never carried a pregnancy with their current partner beyond 20 weeks.

<sup>b</sup>No differences in distribution of primary and secondary aborters in vitamin D normal and low groups.

<sup>c</sup>Summer season; from June to September.

<sup>d</sup>Winter season; from December to February.

<sup>e</sup>No differences in distribution of blood testing seasons in vitamin D normal and low groups, either between summer versus winter, or summer, winter and other season.

<sup>f</sup>Prenatal vitamin containing vitamin D 400 IU per day.

<sup>g</sup>No differences in racial distribution between vitamin D normal and low groups.

Although there is no consensus on optimal serum levels of 25-hydroxy vitamin D, most experts define vitamin D deficiency as a 25-hydroxy vitamin D level of  $< 20$  ng/ml (50 nmol/l) and insufficiency as between 20 and 30 ng/ml (50–75 nmol/l) (Dawson-Hughes et al., 2005; Chen et al., 2007a,b; Holick et al., 2011). Therefore, in this study, study populations were divided into two groups, normal and low vitamin D, depending on their serum vitamin D level (testing was performed in a reference laboratory); normal vitamin D was defined as  $\geq 30$  ng/ml and low vitamin D was  $< 30$  ng/ml. A total of 70 women composed the normal vitamin D group (VDnl) and the rest ( $n = 63$ ) were included in the low vitamin D group (VDlow). There were no differences in age, obstetrical and infertility history, body mass index (BMI), blood testing season, interval from last spontaneous abortion or IVF to blood testing and racial distribution between two groups (Table 1).

## Vitamin D level

Vitamin D levels were measured using the liquid chromatography/tandem mass spectrometry (LC/MS/MS) method at the reference laboratory

(Shah et al., 2012). The average inter-assay coefficient of variation (CV) % across the analytical range of the assay was 7%.

## Analysis of autoantibodies in serum

Antiphospholipid antibodies were tested by enzyme-linked immunosorbent assay (ELISA) as described previously (Gilman-Sachs et al., 1991). High binding polystyrene microtiter 96 wells plates (Thermo Scientific, Rockford, IL, USA) were coated with 20  $\mu\text{l}$  of cardiolipin (45  $\mu\text{g}/\text{ml}$  in ethanol) (Avanti, Alabaster, AL, USA) or one of five other phospholipids (50  $\mu\text{g}/\text{ml}$  in methanol), that is phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid (all from Avanti), or diluents only to determine background values. The plates were allowed to dry overnight at 4°C. After one wash with phosphate buffered saline (PBS, Thermo Scientific), 200  $\mu\text{l}/\text{well}$  of PBS supplemented with 10% bovine serum (Sigma, St Louise, MO, USA) was added to the wells for 2 h at room temperature; then plates were washed once more. Serum from patients and standards (known positive and negative controls) diluted 1:50

in PBS with 10% bovine serum was added in duplicates at 50  $\mu$ l/well. The plates were incubated for 2 h at room temperature, and then washed three times with PBS. Fifty microliters of alkaline phosphatase conjugated antihuman immunoglobulin G (Southern BioTech, Birmingham, AL, USA), IgM and IgA (Invitrogen, Eugene, OR, USA) at saturating concentrations were added to the appropriate wells. After the 1 h incubation at room temperature, the plates were washed three times with PBS and 50  $\mu$ l of 4-nitrophenyl phosphate disodium salt hexahydrate (1 mg/ml, Sigma) was added to each well. After 30 min the reaction was stopped by the addition of 50  $\mu$ l of 3 M sodium hydroxide (Sigma) to each well. The optical density (OD) of each well was determined with a plate reader (Biotek, Winooski, VT, USA) at 405 nm; then the average OD for all duplicate wells was computed and background OD was subtracted. Cut-off values were determined with sera from 60 non-pregnant healthy women between the ages of 20 and 45. An absorbance  $\geq 3$  SD above the mean of the controls for that phospholipid and antibody isotype was considered positive.

Screening for anti-nuclear antibodies (ANA) was performed by indirect immunofluorescence using a commercially available kit (ImmunoConcepts, Sacramento, CA, USA). Individual autoantibodies to dsDNA, ssDNA, histone, Scl-70, thyroglobulin and thyroperoxidase (TPO) were tested by ELISA using corresponding commercially available kits (Inova Diagnostic, San Diego, CA, USA).

### Immunophenotyping of peripheral blood lymphocytes

For flow cytometry analysis whole blood samples were labeled using fluorochrome-conjugated monoclonal antibodies (mAb) against CD45-PC5, CD3-FITC and CD56-PE, or CD45-PC5 and CD19-FITC (Beckman Coulter, Fullerton, CA, USA). After lyses of red blood cells with Immuno-Lyse according to the manufacturer's instructions and two washes with IsoFlow (Beckman Coulter), samples were analyzed on a FC 500 flow cytometer using CXP software (Beckman Coulter). Lymphocytes were gated based on side scatter characteristic and CD45 expression. Within lymphocytes, T cells were identified as CD3<sup>+</sup> cells, NK cells as CD56<sup>+</sup>/3<sup>-</sup> cells and B cells as CD19<sup>+</sup> cells.

### NK cell cytotoxicity assay

The assay was performed as previously described (Gilman-Sachs *et al.*, 1999). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). The human erythromyelocytic leukemia cell line K562 (ATTC, Manassas, VA, USA) was used as the target cell. K562 cells were pre-stained using PKH67 fluorescent cell linker kit (Sigma). PBMC and K562 cells were mixed in 12  $\times$  75 mm polystyrene test tubes at three different effector to target cell (E:T) ratios (50:1, 25:1 and 12.5:1) in 200  $\mu$ l of RPMI-1640 culture medium (Gibco, Grand Island, NY, USA) with 10% heat inactivated fetal calf serum (FCS, Gemini Bio-Products, Inc., Woodland, CA, USA). Following a 2 h incubation at 37°C and 5% CO<sub>2</sub>, propidium iodide (PI, Sigma) was added to the assay tubes at 10  $\mu$ g/ml and specimens were analyzed on FC500 flow cytometer. K562 cells (green fluorescence) were evaluated for PI incorporation (red) to determine the percent of killed cells. The percent of NK cytotoxicity was calculated after subtracting the background value (percent of PI-positive K562 cells incubated alone which died due to spontaneous cell death) from the percent of target dead cells at each E:T ratio.

The NK cytotoxicity was also evaluated in the presence of IgG (final concentration, 12.5 mg/ml or 6.25 mg/ml, Gammagard liquid immune globulin, Baxter, Weslake Village, CA, USA) following a 2 h incubation time for two different E:T ratios (50:1 and 25:1). To evaluate the effect of vitamin D *in vitro* on NK cytotoxicity, cholecalciferol (vitamin D<sub>3</sub>, Sigma) in 0.1% v/v ethanol at either 10 or 100 nM final concentration (added as 1  $\mu$ l per 1 ml of culture solution), or 0.1% v/v ethanol only (vehicle only control), was

added to the assay tubes containing PBMC and target cells at E:T ratios of 50:1 and 25:1. Each tube was incubated for 2 h at 37°C. Vitamin D<sub>3</sub> was used at 10 and 100 nM in this study since these concentrations have been reported to be effective in other assays using peripheral blood cells (Searing *et al.*, 2010; Shifera *et al.*, 2010), human osteoblasts (Ferlin *et al.*, 2011) and other cell lines (Agmon-Levin *et al.*, 2011; He *et al.*, 2013) and reflect physiologically normal and deficient vitamin D levels.

### Intracellular cytokine analysis for Th1/Th2 cytokine ratios

The assay was performed as previously described (Kwak-Kim *et al.*, 2003). Briefly, freshly isolated PBMCs were resuspended at  $5 \times 10^6$ /ml in RPMI-1640 supplemented with 10% FCS and antibiotic-antimycotic solution. One hundred microliters of the PBMC were incubated for 16 h at 37°C and 5% CO<sub>2</sub> in presence of 25 ng phorbol myristate acetate (PMA)/ml, 1  $\mu$ M ionomycin (both from Sigma) and 0.7  $\mu$ l of GolgiPlug per 1 ml of cell suspension, containing Brefeldin A (BD Biosciences, San Jose, CA, USA). After the incubation, cells were washed and labeled with anti-CD45-PC5, anti-CD-ECD and anti-CD8-FITC to detect CD4 cells by negative selection (CD3<sup>+</sup>CD8<sup>-</sup>) (Beckman Coulter) since PMA and ionomycin down-regulate CD4. Cytofix/Cytoperm and Perm/Wash buffer (BD Pharmingen, Franklin Lakes, NJ, USA) were used according to manufacturer's instructions to fix and permeabilize cells. Next, to detect intracellular cytokines, cells were stained with a PE-conjugated mAbs against cytokines including anti-TNF- $\alpha$ , anti-IFN- $\gamma$  or anti-IL-10 (BD Immunoscience, San Jose, CA, USA). Samples were analyzed using a FC500 flow cytometer (Beckman Coulter). T cells were gated based on CD45 and CD3 expression. Percent of cytokine-positive cells were determined within CD3<sup>+</sup>CD8<sup>-</sup> (CD4) and CD3<sup>+</sup>CD8<sup>+</sup> subpopulations. To calculate Th1/Th2 ratios, the percent of TNF- $\alpha$  or IFN- $\gamma$  expressing CD3<sup>+</sup>CD8<sup>-</sup> T cells was divided by the percent of IL-10 expressing CD3<sup>+</sup>CD8<sup>-</sup> T cells. To evaluate the effect of incubation with vitamin D on cytokine secretion by T cells, cholecalciferol (vitamin D<sub>3</sub>, Sigma) in 0.1% v/v ethanol, either at 10 nM or 100 nM final concentration (1  $\mu$ l per 1 ml of culture solution), or ethanol only for controls was added to PBMC along with PMA and ionomycin and incubated for 16 h.

### Confocal microscopy and analysis of perforin granule polarization

NK cells were isolated by positive selection via magnetically assisted cell sorting (MACS, Miltenyi Biotech, Auburn, CA, USA) according to manufacturer's instruction. Freshly isolated NK cells were incubated with K-562 cells at 1:2 ratio for 24 h at 37°C to form cell conjugates. Vitamin D<sub>3</sub> (10 and 100 nM) was added to the cell suspension to evaluate its effect on effector-target cell conjugation and perforin polarization. After the incubation, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) and stained with FITC-conjugated anti-perforin antibody (BD Biosciences) (Orange *et al.*, 2002). Next, cell suspension was transferred to poly(L-lysine)-coated slides (Sigma) and allowed to adhere. Prolong Gold containing 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used as a nuclear counterstaining dye. Cell conjugates were visualized by using FV10-LD748 (Olympus Corporation, Tokyo, Japan) laser-scanning confocal microscope.

### Cytokines and growth factors by multiplex assays

NK cells were isolated by positive selection via MACS (Miltenyi Biotech). Freshly isolated NK cells were incubated with vitamin D<sub>3</sub> (10 nM and 100 nM) or control solution (0.1% v/v ethanol) in serum-free medium for 24 h at 37°C in 5% CO<sub>2</sub>. After incubation, the supernatants were collected. Pro-, anti-inflammatory cytokines, chemokine and growth factors were



analyzed with Milliplex MAP kit (Millipore Corp, Bethesda, MD, USA) using the MAGPIX instrument (Luminex, Madison, WI, USA) as per manufacturer's instructions. Data were analyzed with software (Milliplex Analyst<sup>®</sup>, Viagene Tech, Carlisle, CA, USA). Analytes were normalized to total protein concentration which was estimated with BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Eleven analytes were determined: IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF).

## Statistical analysis

All statistical analyses were performed using SPSS Statistics version 20.0 (2011, SPSS, Inc., Chicago, IL, USA). The statistical comparisons among groups for continuous variables were performed using the Mann–Whitney *U*-test or Kruskal–Wallis one-way analysis of variance. The  $\chi^2$  test or Fisher exact probability test was applied to analyze categorical frequency data. Bonferroni's correction was applied to avoid the errors from multiple comparisons when applicable. A *P*-value of <0.05 was considered significant.

## Results

### Prevalence of low vitamin D in women with RPL

In the 133 women with RPL, 70 (52.6%) had a normal vitamin D level ( $\geq 30$  ng/ml) and 63 (47.4%) had low vitamin D levels (<30 ng/ml), including 41 (30.8%) women with vitamin D insufficiency (20 to <30 ng/ml) and 22 (16.6%) with vitamin D deficiency (<20 ng/ml). The mean

and standard deviation of the serum vitamin D level in VDlow was  $22.5 \pm 5.8$  (ng/ml), whereas  $42.2 \pm 9.8$  (ng/ml) was found for VDnl (Table I).

### Prevalence of autoantibodies

The prevalence of total APA (IgG or IgM autoantibodies to cardiolipin and/or anionic phospholipids) was significantly higher in VDlow than VDnl (39.7% versus 22.9%, *P* < 0.05, OR 2.22, 95% CI 1.0–4.7) (Table II). Nonetheless, there was no difference in prevalence of each autoantibody to cardiolipin or anionic phospholipids (phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol and phosphatidylserine) between VDlow and VDnl.

The prevalence of ANA in VDlow (47.6%) was higher than VDnl (34.3%). However, the difference did not reach a statistically significant level. The prevalence of total anti-nuclear antigen antibody in VDlow (including autoantibodies to dsDNA, ssDNA, histone and Scl70) (23.8 versus 10.0%, *P* < 0.05, OR 2.81, 95% CI, 1.1–7.4) was significantly higher than that of VDnl. Furthermore, the prevalence of anti-ssDNA antibody (19.0 versus 5.7%, *P* < 0.05, OR 3.76, 95% CI 1.1–12.4) in VDlow was significantly higher than that of VDnl. There were no differences in prevalence of anti-dsDNA, anti-histone and anti-Scl70 antibodies between VDlow and VDnl. The prevalence of TPO antibody in VDlow was significantly higher than that of VDnl (33.3 versus 15.7%, *P* < 0.05, OR 2.68, 95% CI 1.2–6.1). However, the prevalence of anti-thyroglobulin antibody was not different between two groups. A comparison of antibody levels in primary and secondary aborters is shown in Supplementary data, Table S1.

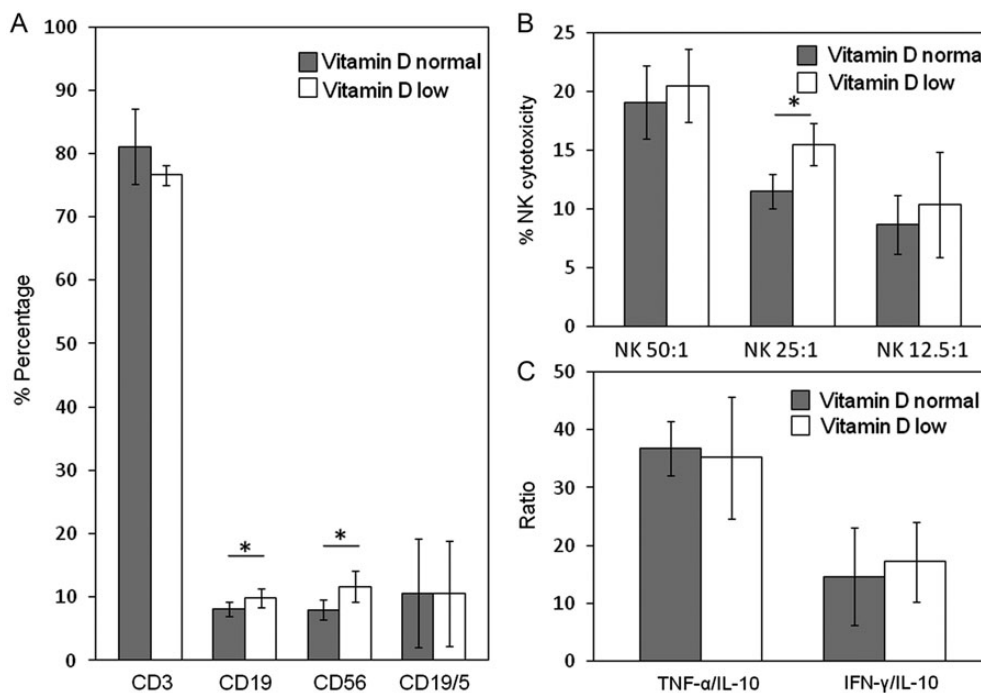
**Table II** Prevalence of antiphospholipid antibody (APA), anti-nuclear antibody (ANA), anti-thyroid antibody and other non-organ-specific autoantibodies in women with three or more recurrent pregnancy losses with normal vitamin D ( $\geq 30$  ng/ml, *n* = 70) and low vitamin D (<30 ng/ml, *n* = 63).

Autoantibodies	Normal vitamin D ( $\geq 30$ ng/ml) ( <i>n</i> = 70) (%)	Low vitamin D (<30 ng/ml) ( <i>n</i> = 63) (%)	Odds ratio (95% CI)	<i>P</i> -value
APA (IgG, IgM) <sup>a</sup>	22.9	39.7	2.22 (1.0–4.7)	<i>P</i> < 0.05
Anti-cardiolipin				
IgG	5.7	11.1	2.06 (0.5–7.4)	NS
IgM	4.3	7.9	1.93 (0.4–8.4)	NS
Anti-anionic phospholipids <sup>b</sup>				
IgG	12.9	20.6	1.76 (0.7–4.5)	NS
IgM	17.1	15.9	0.91 (0.4–2.3)	NS
ANA	34.3	47.6	1.74 (0.9–3.5)	NS
Anti-nuclear antigen <sup>c</sup>	10.0	23.8	2.81 (1.1–7.4)	<i>P</i> < 0.05
Anti-dsDNA	1.4	3.2	2.26 (0.2–25.6)	NS
Anti-ssDNA	5.7	19.0	3.76 (1.1–12.4)	<i>P</i> < 0.05
Anti-histone	1.4	9.5	7.26 (0.8–62.1)	NS
Anti-Scl70	2.9	3.2	1.11 (0.2–8.2)	NS
Anti-thyroid antibody				
Anti-thyroglobulin	8.6	9.5	1.12 (0.3–3.7)	NS
Thyroperoxidase antibody	15.7	33.3	2.68 (1.2–6.1)	<i>P</i> < 0.05

<sup>a</sup>Any IgG or IgM antibodies to phospholipids.

<sup>b</sup>Antibodies to anionic phospholipids including antibodies to phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol and phosphatidylserine.

<sup>c</sup>Any autoantibodies to dsDNA, ssDNA, histone and Scl70.



**Figure 1** Peripheral blood immunophenotype, Natural killer (NK) cell cytotoxicity and T helper (Th) 1/Th2 cell ratios of women with recurrent pregnancy loss comparing groups with low vitamin D (VDlow) ( $n = 63$ ) and normal vitamin D (VDnl) ( $n = 70$ ): **(A)** Proportions (%) of  $CD3^+$  T,  $CD19^+$  B,  $CD3^-/56^+$  NK and  $CD19^+/5^+$  B-I cells in peripheral blood are plotted. VDlow have significantly higher  $CD56^+$  NK cell ( $P < 0.05$ ) and  $CD19^+$  B ( $P < 0.05$ ) cell levels when compared with VDnl; **(B)** NK cytotoxicity at effector-to-target cell ratios (E:T) of 50:1, 25:1 and 12.5:1 is plotted. NK cytotoxicity at E:T of 25:1 of VDlow was significantly higher than VDnl ( $P < 0.05$ ); **(C)**  $TNF-\alpha/IL-10$  and  $IFN-\gamma/IL-10$  expressing  $CD3^+/CD4^+$  T cell ratios are not different between VDnl and VDlow. Values are mean  $\pm$  SD (standard deviation). \* $P < 0.05$ .

### Peripheral blood immunophenotypes and NK cytotoxicity

There were significant differences in the peripheral blood  $CD56^+$  NK cell levels (%) (Mean  $\pm$  SD,  $11.7 \pm 4.5$  versus  $9.0 \pm 4.6$ ,  $P < 0.05$ ) and  $CD19^+$  B cells ( $9.9 \pm 6.5$  versus  $8.1 \pm 4.8$ ,  $P < 0.05$ ) between VDlow and VDnl. No difference was found in the proportion of  $CD3^+$  T cells ( $76.6 \pm 1.6\%$  versus  $81.1 \pm 5.9\%$ ,  $P = NS$ ) or  $CD19^+/5^+$  B-I cell levels ( $10.5 \pm 8.6\%$  versus  $10.6 \pm 8.5\%$ ) between VDlow and VDnl (Fig. 1A). NK cytotoxicity at E:T ratio of 25:1 was significantly different between VDlow and VDnl ( $15.5 \pm 1.5\%$  versus  $11.5 \pm 1.5\%$ ,  $P < 0.05$ ) (Fig. 1B). However, NK cytotoxicity was not significantly different at E:T ratio of 50:1 ( $19.1 \pm 3.1\%$  versus  $20.5 \pm 3.1\%$ ) or 12.5:1 ( $10.4 \pm 4.5\%$  versus  $8.7 \pm 2.5\%$ ) between VDlow and VDnl. To further investigate whether the general trend of vitamin D and NK cytotoxicity of the study groups is continuously present in the smaller groups, NK cytotoxicity of vitamin D normal, insufficient and deficient patients was analyzed (Table III). The vitamin D deficient group had significantly increased NK cell cytotoxicity at embryo transfer ratios of 50:1 ( $22.2 \pm 2.8$  versus  $19.1 \pm 3.1$ ,  $P < 0.05$ ), 25:1 ( $14.3 \pm 2.4$  versus  $11.5 \pm 1.5$ ,  $P < 0.05$ ) and 12.5:1 ( $11.2 \pm 2.2$  versus  $8.7 \pm 2.5$ ,  $P < 0.05$ ) when compared with VDnl group. Vitamin D insufficiency group had significantly increased NK cytotoxicity at E:T ratio of 1:25 only ( $13.8 \pm 2.6$  versus  $11.5 \pm 1.5$ ,  $P < 0.05$ ) when compared with VDnl group.

There was no significant difference in  $TNF-\alpha/IL-10$  (VDlow versus VDnl;  $35.2 \pm 10.6$  versus  $36.8 \pm 4.7$ ) or  $INF-\gamma/IL-10$  ( $17.2 \pm 6.9$

**Table III** The comparisons of NK cytotoxicity in women with recurrent pregnancy losses and vitamin D normal, insufficiency and deficiency levels.

NK cytotoxicity	Vitamin D Normal (>30 ng/ml) $n = 70$	Vitamin D Insufficiency (30–20 ng/ml) $n = 41$	Vitamin D Deficiency (<20 ng/ml) $n = 22$
E:T <sup>a</sup> ratio 50:1	$19.1 \pm 3.1$	$19.9 \pm 3.1$	$22.2 \pm 2.8^b$
E:T ratio 25:1	$11.5 \pm 1.5$	$13.8 \pm 2.6^b$	$14.3 \pm 2.4^b$
E:T ratio 12.5:1	$8.7 \pm 2.5$	$10.1 \pm 4.4$	$11.2 \pm 2.2^b$

Data are expressed as mean and SD of %target cells dead minus background.

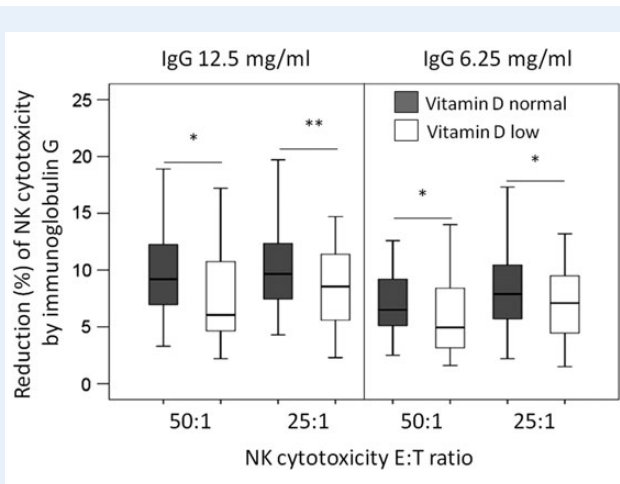
<sup>a</sup>Effector to Target cell ratio.

<sup>b</sup> $P < 0.05$  when compared with vitamin D normal group.

versus  $14.6 \pm 8.4$ ) expressing  $CD3^+/CD4^+$  cell ratios between two groups (Fig. 1C).

### NK cytotoxicity with immunoglobulin G in vitro

The reductions (%) of NK cytotoxicity at E:T ratio of 50:1 ( $9.5 \pm 2.5$ ) and 25:1 ( $9.8 \pm 1.2$ ) by IgG treatment (12.5 mg/dl) were significantly greater in the VDnl group than those in the VDlow group ( $5.8 \pm 1.1$ ,



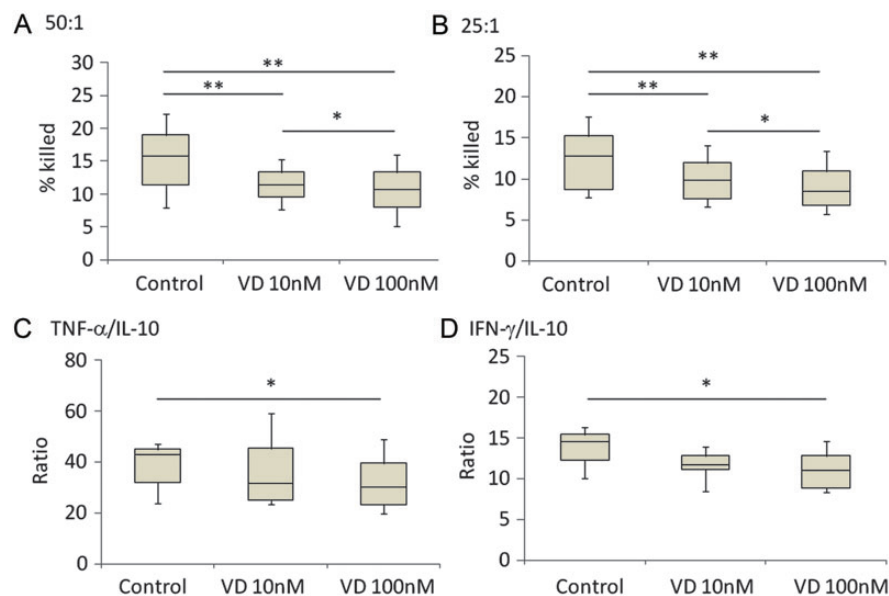
**Figure 2** Reductions (%) of Natural killer (NK) cell cytotoxicity by immunoglobulin G (12.5 mg/dl and 6.25 mg/dl) were significantly lower in low vitamin D (VDlow) when compared with those of normal vitamin D (VDnl) at E:T ratio of 50:1 ( $P < 0.05$  each) and 25:1 ( $P < 0.01$  and  $P < 0.05$  each). Reduction of NK cytotoxicity was calculated as follows:  $[\text{NK cytotoxicity} - \text{NK cytotoxicity with IgG}] / \text{NK cytotoxicity}$ . In the box-and-whisker plots the boxes represent the quartiles, a bar in the box represents the median and whiskers represents the minimum and maximum of the data. \* $P < 0.05$ ; \*\* $P < 0.01$ .

$P < 0.05$ ;  $7.5 \pm 0.8$ ,  $P < 0.01$ , respectively) (Fig. 2). When 6.25 mg/dl of IgG was added, the reductions (%) of NK cytotoxicity at E:T ratio of 50:1 ( $6.3 \pm 1.9$ ) and 25:1 ( $7.5 \pm 1.1$ ) were significantly greater in the VDnl group when compared with those in the VDlow group ( $4.9 \pm 1.1$ ,  $P < 0.05$ ;  $6.4 \pm 0.6$ ,  $P < 0.05$ , respectively).

### ***In vitro* effect of vitamin D on NK cytotoxicity and Th1/Th2 cytokine expression**

PBMC containing NK effector cells and K562 targets were incubated for 2 h with vitamin D at two final concentrations, 10 or 100 nM, and controls before testing for NK cytotoxicity *in vitro* ( $n = 18$  each). The NK cytotoxicity at E:T ratio of 50:1 was significantly suppressed with vitamin D at both 10 nM ( $11.9 \pm 3.3\%$ ,  $P < 0.01$ ) and 100 nM ( $10.9 \pm 3.7\%$ ,  $P < 0.01$ ) when compared with controls ( $15.3 \pm 0.7\%$ ). Again, vitamin D suppressed NK cytotoxicity in a dose-dependent manner (vitamin D 10 nM versus 100 nM,  $P < 0.05$ ) (Fig. 3A). NK cytotoxicity at E:T ratio of 25:1 was also suppressed significantly by 10 nM vitamin D ( $10.1 \pm 3.9\%$ ,  $P < 0.01$ ) and 100 nM of vitamin D ( $8.6 \pm 4.4\%$ ,  $P < 0.01$ ) when compared with controls ( $12.1 \pm 4.8\%$ ) in a dose-dependent manner (vitamin D 10 nM versus 100 nM,  $P < 0.05$ ) (Fig. 3B).

When 100 nM vitamin D was added to T cell cultures for 16 h, there was a significant reduction in TNF- $\alpha$ /IL-10 expressing CD3<sup>+</sup>/CD4<sup>+</sup> cell ratios ( $31.3 \pm 9.4$ ) when compared with controls ( $40.4 \pm 11.3$ ,  $P < 0.05$ ). On the other hand, no statistically significant difference was

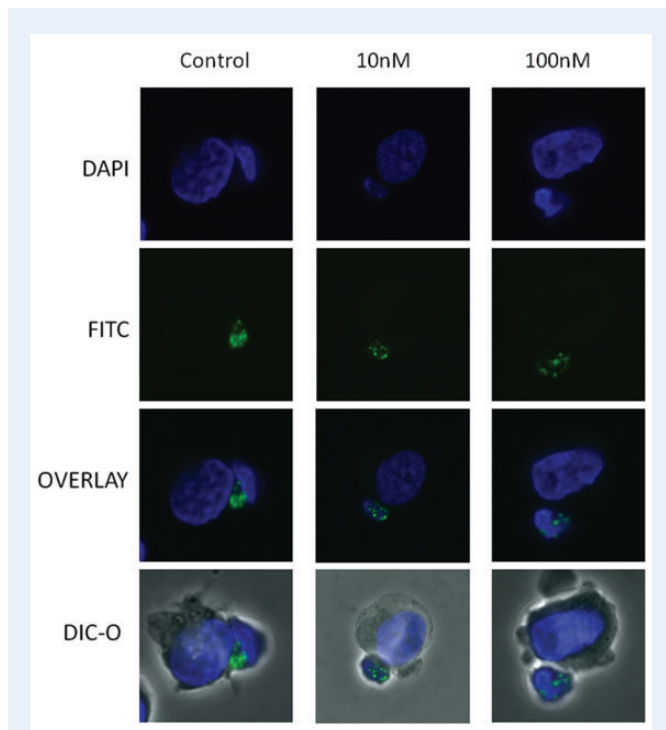


**Figure 3** The effect of 10 and 100 nM vitamin D on Natural killer (NK) cell cytotoxicity *in vitro* ( $n = 18$  each): (A) NK cytotoxicity at E:T ratio of 50:1 was significantly suppressed by vitamin D<sub>3</sub> 10 nM ( $11.9 \pm 3.3\%$ ) and 100 nM ( $10.9 \pm 3.7\%$ ) when compared with controls ( $15.3 \pm 4.7\%$ ) ( $P < 0.01$  each). In addition there was a significant difference between NK cytotoxicity with vitamin D<sub>3</sub> 10 nM or 100 nM ( $P < 0.05$ ); (B) NK cytotoxicity at E:T ratio of 25:1 was significantly suppressed by 10 nM ( $10.1 \pm 3.9\%$ ) and 100 nM ( $8.6 \pm 4.4\%$ ) ( $P < 0.01$  each) of vitamin D<sub>3</sub> when compared with controls ( $12.1 \pm 4.8\%$ ). In addition there was a significant difference between NK cytotoxicity with vitamin D<sub>3</sub> 10 nM and that with vitamin D<sub>3</sub> 100 nM ( $P < 0.05$ ); (C) TNF- $\alpha$ /IL-10 expressing CD3<sup>+</sup>/4<sup>+</sup> cell ratios were significantly decreased with 100 nM of vitamin D<sub>3</sub> ( $31.3 \pm 9.4$ ,  $P < 0.05$ ) when compared with controls ( $40.4 \pm 11.3$ ) but not changed with 10 nM of vitamin D<sub>3</sub> ( $37.2 \pm 12.2$ ,  $P = \text{NS}$ ); (D) INF- $\gamma$ /IL-10 expressing CD3<sup>+</sup>/4<sup>+</sup> cell ratio was significantly decreased with 100 nM of vitamin D<sub>3</sub> ( $12.1 \pm 4.0$ ,  $P < 0.05$ ) when compared with controls ( $14.8 \pm 4.6$ ) but not changed with 10 nM of vitamin D<sub>3</sub> ( $12.9 \pm 4.4$ ,  $P = \text{NS}$ ). In the box-and-whisker plots the boxes represent the quartiles, a bar in the box represents the median and whiskers represents the minimum and maximum of the data. \* $P < 0.05$ ; \*\* $P < 0.01$ .

found when T cells were treated with 10 nM vitamin D ( $37.2 \pm 12.2$ ) when compared with controls (Fig. 3C). When vitamin D 100 nM was added, the INF- $\gamma$ /IL-10 expressing CD3<sup>+</sup>/CD4<sup>+</sup> cell ratio ( $12.1 \pm 4.0$ ,  $P < 0.05$ ) was also significantly reduced when compared with controls ( $14.8 \pm 4.6$ ) (Fig. 3D). However, there was no significant difference with 10 nM vitamin D ( $12.9 \pm 4.4$ ) when compared with controls.

### In vitro vitamin D effect on perforin granules polarization in NK cells

Confocal images of NK cell cytotoxicity against K562 cells revealed that NK cells conjugated with K562 cells in media exhibit polarized expression of perforin in the cytoplasm of NK cells towards the direction of target K562 cells (Fig. 4). NK cells conjugated with K562 cells in 10 nM or 100 nM vitamin D<sub>3</sub> demonstrate depolarized perforin expression in the cytoplasm. Depolarization and restricted perforin expression was more exaggerated with vitamin D<sub>3</sub> 100 nM than vitamin D<sub>3</sub> 10 nM. These findings suggest that vitamin D inhibits perforin release and disturbs the polarization usually found before killing occurs.



**Figure 4** Confocal images of Natural killer (NK) cell cytotoxicity against K562 cells. NK and K562 cells were cultured with control media containing EtOH (Control), 10 or 100 nM vitamin D. NK cells were fixed, permeabilized and stained with anti-perforin mAbs. The first row shows nuclear staining with DAPI. The second row demonstrates confocal images of perforin with FITC staining. The third row is overlay of DAPI and FITC staining. The last row shows a differential interference contrast-overlay (DIC-O) of all images. NK cells attached with K562 cells in control solution have polarized perforin expression in the cytoplasm of NK cells and to the direction of K562 cells. NK cells attached with K562 cells in vitamin D<sub>3</sub> 10 and 100 nM demonstrate depolarized perforin expression mainly in the nucleus of NK cells.

### Cytokines and growth factor profiles derived from NK cells treated with vitamin D

The concentrations of IFN- $\gamma$  (vitamin D<sub>3</sub> 10 nM,  $P < 0.05$ ; 100 nM,  $P < 0.01$ ) and TNF- $\alpha$  (vitamin D<sub>3</sub> 10 nM,  $P < 0.05$ ; 100 nM,  $P < 0.01$ ) in the NK cell culture supernatant incubated with vitamin D<sub>3</sub> were significantly decreased in a dose-dependent manner when compared with those of controls ( $n = 18$  each) (Figs. 3B and 5A). In contrast, the concentrations of IL-10, VEGF, G-CSF (Vitamin D<sub>3</sub> 10 nM,  $P < 0.05$ ; 100 nM,  $P < 0.01$ , respectively) and IL-1 $\beta$  (vitamin D<sub>3</sub> 10 nM,  $P < 0.01$ ; 100 nM,  $P < 0.01$ ) were significantly increased when compared with controls in a dose-dependent manner (Fig. 5C–F). IL-2, IL-4, IL-6, IL-8 and GM-CSF were not detected in supernatant (below the sensitivity level of the assay).

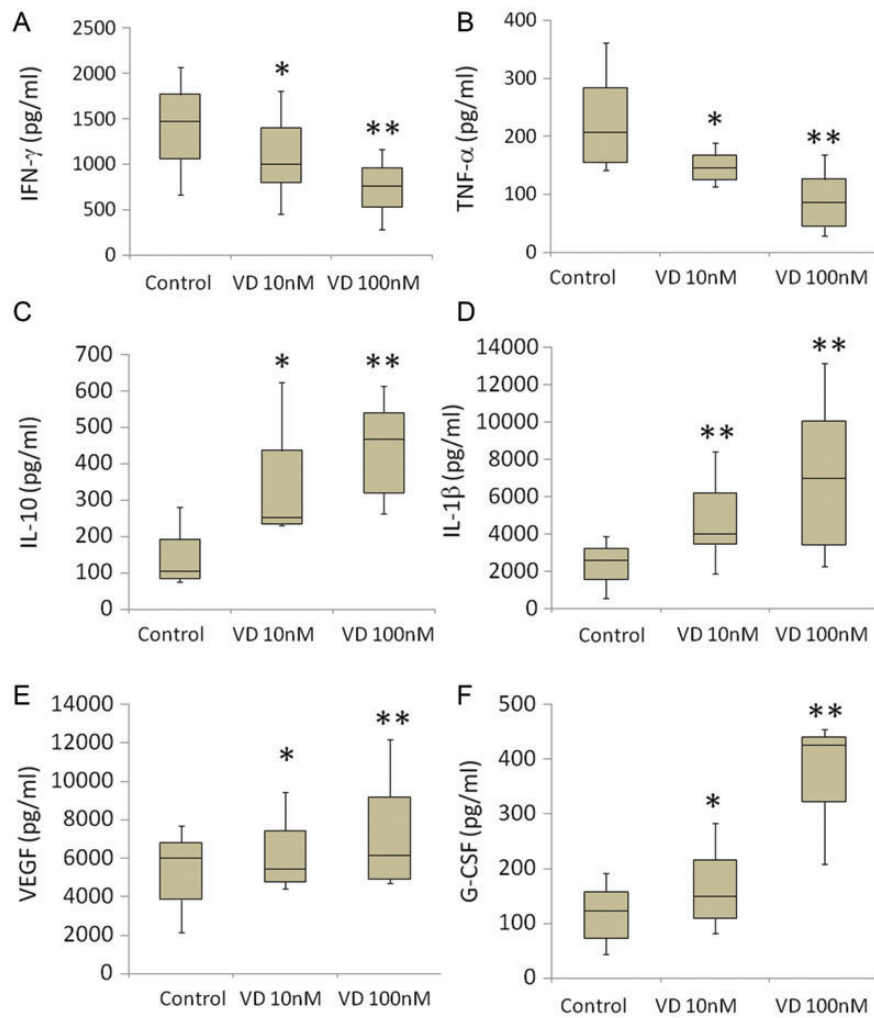
### Discussion

It is well known that vitamin D deficiency is prevalent among pregnant women (Dent and Gupta, 1975). Vitamin D deficiency in pregnant women is associated with increased risk of obstetrical complications such as pre-eclampsia (Bodnar *et al.*, 2007), bacterial vaginosis associated preterm delivery (Bodnar *et al.*, 2009), gestational diabetes mellitus (Maghbooli *et al.*, 2008) and small-for-gestational age births (Bodnar and Simhan, 2010). We report a high proportion of women with RPL have vitamin D deficiency, which is associated with increased cellular and autoimmunity. Women with RPL have increased prevalence of various autoantibodies, such as APA, ANA and TPO antibody (Roussev *et al.*, 1996). In this study, a low vitamin D level was associated with increased odds of positive APA (OR 2.22), anti-nuclear antigen antibody (OR 2.81), anti-ssDNA (OR 3.76) and TPO antibody (OR 2.68) in women with RPL. It has been reported that patients with antiphospholipid antibody syndrome have increased prevalence of vitamin D deficiency ( $< 30$  ng/ml) when compared with healthy controls (Orbach *et al.*, 2007; Agmon-Levin *et al.*, 2011), which is in agreement with the results of this study.

Hashimoto's disease is the most common autoimmune thyroid disease in women with RPL. We demonstrated that the prevalence of TPO antibody in VDlow was significantly higher than that of VDnl. This was consistent with previous study of Tamer *et al.* (2011). Vitamin D levels have been reported to be inversely correlated with TPO antibody titer (Bozkurt *et al.*, 2012; Camurdan *et al.*, 2012). In animal models, vitamin D was shown to prevent autoimmune thyroiditis by inhibiting lymphocyte proliferation and secretion of inflammatory cytokines (Li *et al.*, 2007). Therefore, we speculate that vitamin D deficiency might be associated with the pathogenesis of Hashimoto's disease. Although prevalence of antibodies to nuclear antigens and anti-ssDNA antibody were significantly higher in VDlow when compared with VDnl, there was no difference in the prevalence of ANA between VDlow and VDnl group.

Low vitamin D appears to be important for autoimmune disease susceptibility and severity (Armsen *et al.*, 2007) and vitamin D deficiency was associated with an increased presence of autoantibodies via B cell hyperactivation and autoantibody production (Ritterhouse *et al.*, 2011). In our study, peripheral blood CD19<sup>+</sup> B cell levels were significantly higher in RPL women with VDlow than those with VDnl. Elevated 19<sup>+</sup> B cell levels in VDlow may reflect autoimmune activation since vitamin D plays a regulatory role in autoantibody production by inhibiting the





**Figure 5** Concentrations of pro-, anti-inflammatory cytokines, and growth factor in culture supernatants from freshly isolated Natural killer (NK) cells treated with vitamin D (10 nM and 100 nM) or control solution for 48 h ( $n = 18$ , each). (A) IFN- $\gamma$ ; (B) TNF- $\alpha$ ; (C) IL-10; (D) IL-1 $\beta$ ; (E) VEGF and (F) G-CSF. In the box-and-whisker plots the boxes represent the quartiles, a bar in the box represents the median and whiskers represents the minimum and maximum of the data. \* $P < 0.05$ ; \*\* $P < 0.01$ .

ongoing proliferation of activated B cells and inducing their apoptosis (Chen et al., 2007a,b). From these findings, it can be inferred that vitamin D plays a role in regulating B cell proliferation and function during successful pregnancy.

Several studies have reported a link between RPL and altered cytotoxicity and level of peripheral NK cells (Kwak-Kim et al., 2005). Preconception evaluation of NK cell activity in women with RPL has been reported to predict pregnancy outcome of the subsequent pregnancy (Aoki et al., 1995; Kwak et al., 1995). Furthermore, elevated peripheral NK cells in pregnant women predict spontaneous abortions with normal karyotype in index pregnancy (Yamada et al., 2001; Kwak-Kim et al., 2010). Recently, increasing evidence supports a novel immune-regulatory role of vitamin D (Erkkola et al., 2009; Sandhu and Casale, 2010). However, whether vitamin D has any regulatory role on NK cell has not been investigated well. In this study, we demonstrated that the proportion of NK cells in RPL women with VDlow was significantly higher

than that of RPL women with VDnl. Additionally, NK cytotoxicity at E:T ratio of 25:1 was significantly higher in VDlow when compared with that of VDnl.

It is possible that vitamin D levels are simply associated with general Th1-biased autoimmune conditions and the low vitamin D levels are not the major cause of elevated NK cytotoxicity, since immune mediated RPL is associated with Th1 autoimmune condition. To elucidate this possibility, we further analyzed NK cytotoxicities (*in vivo*) in three subgroups, vitamin D normal, insufficiency and deficiency. Women with vitamin D deficiency ( $<20$  ng/ml) had significantly increased NK cytotoxicity at all E:T ratios when compared with those of VDnl and step-wise increases in NK cytotoxicities were present among women with VDnl, vitamin D insufficiency and deficiency. Therefore, vitamin D and NK cytotoxicity seem to have a direct inverse relationship. Some might argue that the increase in NK cytotoxicity observed in VDlow group might be actually an indirect effect of increased systematic Th1 immunity, not of vitamin D's

direct effect on perforin action in NK cells. With the confocal microscope study, we found that vitamin D suppresses perforin secretion from the cytoplasm and consequently affects the killing of the target cell. Hence, vitamin D may be involved in regulation of both NK cell population and cytotoxicity. As increased NK cell proportion and/or cytotoxicity are known to be risk factors for reproductive failures, low vitamin D level may have further implication in reproductive failure. IgG has been reported to suppress NK cytotoxicity *in vivo* and *in vitro* (Kwak *et al.*, 1996; Ruiz *et al.*, 1996). Additionally, we demonstrated that NK cell response to IgG in VDnl was significantly greater than that of VDlow. NK cells in decreased vitamin D micro milieu might have decreased response to IgG possibly via either altered perforin degranulation process or signal transduction pathways. This finding may explain the differences in success rate of intravenous immunoglobulin G treatment in women with RPL (Carp, 2007).

When vitamin D was added to *in vitro* assays, NK cytotoxicity was significantly reduced in a dose-dependent manner in this study. We also found that vitamin D suppressed secretion of type I cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , by NK cells and increased type II cytokine such as IL-10. Hence, vitamin D induces a NK type 2 (NK2) shift. NK1 shift over NK2 has been reported in preeclamptic women when compared with normal pregnancy and non-pregnant healthy women (Borzychowski *et al.*, 2005). In addition, women with RPL also had NK1 shift when compared with normal controls (Fukui *et al.*, 2008). Consequently, low vitamin D level may contribute to the NK1 shift in RPL women. In this study, we used vitamin D3 as an *in vitro* treatment and not 25(OH)D or 1,25(OH) $_2$ D. Thus, we demonstrated that PBMCs have the ability to activate vitamin D through its entire metabolic pathway, utilizing both a vitamin D-25-hydroxylase and the 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase that function in an autocrine fashion to affect the desired target cell. Vitamin D has been reported to inhibit proliferation of Th1 cells and limit their cytokine production, such as IFN- $\gamma$ , IL-2 and TNF- $\alpha$ . Conversely, vitamin D induces T helper 2 (Th2) cytokines, such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 from T cells (Adams and Hewison, 2008). From our study and others, vitamin D induces a type I shift in both NK and T cells. Taken together, an adequate level of vitamin D or supplementation may be important to maintain proper innate and acquired immunity for successful pregnancy.

In this study, we demonstrated that vitamin D induced VEGF and G-CSF production in peripheral NK cells. Lower expression of VEGF has been reported in women with pre-eclampsia or intrauterine growth restriction (Dunk and Ahmed, 2001; Kalkunte *et al.*, 2009). Molvarec *et al.* (2010) reported that the percentage of VEGF-producing peripheral blood NK cells was significantly lower in women with pre-eclampsia compared with that of healthy pregnant women. Consequently, dysregulation of VEGF production by NK cells at the feto-maternal interface may cause poor angiogenesis and pregnancy complications such as RPL or pre-eclampsia. G-CSF is produced by human decidual stromal cells and endometrial glandular cells, and its receptor, c-fms, is present on the trophoblast cells. It plays a role in autocrine and paracrine manners in the decidua and placenta, and in a human trial, pregnancy loss rate was significantly reduced by G-CSF (Scarpellini and Sbracia, 2009). Therefore, vitamin D can be a potential therapeutic option to regulate angiogenesis and prevent recurrent pregnancy losses.

It has been reported that dominant pro-inflammatory Th1 immune response is associated with RPL or multiple implantation failures

(Raghupathy *et al.*, 2000; Kwak-Kim *et al.*, 2003). Vitamin D has been reported to block the induction of Th1 cytokines in T cells, while promoting Th2 responses (van Etten and Mathieu, 2005). In this study, the Th1 / Th2 cytokine-expressing CD3 $^+$ /CD4 $^+$  Th cell ratio was not significantly different between RPL women with VDlow and VDnl. However, in this study we demonstrate that vitamin D has a dose-dependent effect on an induction of a polarized Th2 shift *in vitro*. This result is consistent with previous studies which demonstrate that vitamin D skews the T cell compartment from a Th1 towards a Th2 type (Smolders *et al.*, 2008; Correale *et al.*, 2009). It is possible that the mean difference in vitamin D levels between VDlow and VDnl in this study ( $\sim 20$  ng/ml or 50 nM) may not be sufficient to shift the Th1/Th2 ratio *in vivo*, since an *in vitro* effect of vitamin D on Th1/Th2 shift was only demonstrated at 100 nM of vitamin D $_3$  concentration. Most of the differences in immunological biomarkers between VDnl and VDlow in this study are relatively small and may not be clinically relevant in terms of vitamin D levels. Indeed, vitamin D effect on Th1 to Th2 shift was induced when relatively higher level of vitamin D $_3$  was added. A prospective study evaluating subsequent pregnancy outcome in the normal and low vitamin D groups is needed to clarify the clinical relevance of low vitamin D levels in women with RPL.

In summary, a high proportion of women with RPL have vitamin D deficiency and the vitamin D deficiency has immunological implications in RPL. Vitamin D is associated with B and NK cell immunity and Th1 / Th2 balance, and women with low vitamin D have a tendency to develop APA and other autoantibodies, which are related to autoimmune disease and poor reproductive outcome. Therefore, assessment of vitamin D seems to be a high yield practice in women with RPL and autoimmune or cellular immune abnormalities. Conversely, women with low vitamin D level have significantly increased odds for autoimmune abnormalities, which is a risk factor for reproductive failure. NK cell cytotoxicity and Th1 polarization were significantly reduced *in vitro* by vitamin D, and vitamin D reduced perforin secretion and polarization in NK cells. In addition, vitamin D suppressed type I cytokine production and increased type 2 and growth factors from NK cells. Therefore, these results raise the possibility that vitamin D could be available as a new therapeutic option for reproductive failure. Further study is needed to elucidate immune-regulatory role of vitamin D, and whether supplemental vitamin D administration can prevent the onset of pregnancy loss.

## Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

## Authors' roles

J.K.-K. initiated and designed the study, and is responsible for the facility in which the study is conducted. K.O. wrote the first draft of the manuscript and was responsible for the analysis and interpretation of the data. A.H. and S.D. assisted for the analysis and interpretation of the data. J.K.-K., A.G.-S. and K.B. helped in acquisition analysis and interpretation of data. All authors have read the manuscript, critically revised it for important intellectual content, agreed that the work is ready for submission and eventually accepted the responsibility for the manuscript's content.

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## Conflict of interest

None declared.

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