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Short communication

Whole blood flow cytometric analysis of *Ureaplasma*-stimulated monocytes from pregnant women

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ABSTRACT

We hypothesised that circulating monocytes of women with vaginal colonisation with *Ureaplasma* spp., genital microorganisms known to cause inflammation-driven preterm birth, would elicit a tolerised cytokine response to subsequent *in vitro Ureaplasma parvum* serovar 3 (UpSV3) stimulation. Using multi-parameter flow cytometry, we found no differences with regard to maternal colonisation status in the frequency of TNF-α-, IL-6-, IL-8- and IL-1β-expressing monocytes in response to subsequent UpSV3 stimulation ($P > 0.10$ for all cytokines). We conclude that vaginal *Ureaplasma* spp. colonisation does not specifically tolerise monocytes of pregnant women towards decreased responses to subsequent stimulation.

1. Introduction

Nearly half of all cases of spontaneous preterm birth (PTB) can be attributed to infection (Romero et al., 2002), with *Ureaplasma* spp. the most commonly associated microorganisms (DiGiulio et al., 2008). *Ureaplasma* spp. are commensals of the female genital tract of both pregnant and non-pregnant women (Breugelmans et al., 2010). In some pregnant women, ascension of these organisms from the vagina into the uterine cavity leads to colonisation of the amniotic fluid and foetal membranes (Kim et al., 2009), resulting in chorioamnionitis and leading to preterm labour (Namba et al., 2010). However, not all women with intrauterine *Ureaplasma* spp. colonisation will deliver early (Perni et al., 2004).

Increasing evidence supports the role of antimicrobial immune responses in determining pregnancy outcome. Primary exposure to microbial ligands, for example, can induce immune cell reprogramming towards priming for an enhanced response upon restimulation or tolerance, resulting in decreased responsiveness. Peltier et al. (2010) hypothesised that peripheral blood mononuclear cells (PBMCs) isolated from non-pregnant women with a history of PTB would produce greater amounts of TNF-α,

Abbreviations: BFA, Brefeldin A; CCU, colour-changing units; GA, gestational age; IL, interleukin; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PRRs, pattern recognition receptors; PTB, preterm birth; spp., species; TLR, Toll-like receptor; TNF, tumour necrosis factor; *U. parvum*, *Ureaplasma parvum*; UpSV3, *U. parvum* serovar 3.

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suggestive of a primed response when restimulated with bacteria associated with PTB (including *Ureaplasma* spp.). However, no difference was found when compared with women without a history of preterm labour. In contrast, here we hypothesised that vaginal colonisation with *Ureaplasma* spp. would establish monocyte tolerance, such that amniotic ascension of *Ureaplasma* spp. would be asymptomatic in the majority of cases. Therefore, we sought to determine whether blood-derived monocytes from pregnant women colonised with vaginal *Ureaplasma* spp. would elicit a reduced cytokine response compared with non-colonised pregnant women suggestive of immune tolerance to subsequent stimulation with heat-killed *Ureaplasma parvum* serovar 3 (UpSV3).

2. Materials and methods

2.1. Study population

Thirty-two female participants with singleton pregnancies within the second trimester were recruited during their first prenatal visit at King Edward Memorial Hospital (KEMH), Perth, Australia. Exclusion criteria included: a high-risk of PTB (*e.g.* previous history of PTB or preeclampsia), history of recurrent vaginal thrush, current diagnosis of a urinary tract infection and/or the use of antifungals or antibiotics. The study was approved by the Human Research Ethics Committee of the Western Australian Department of Health, Women and Newborn Health Service (2056/EW). All participants provided written informed consent.

2.2. Detection and speciation of Ureaplasma spp.

Flocked vaginal swabs (UTM kit, Copan Diagnostics, Murrieta, CA, USA) were self-collected at recruitment with guidance from a research midwife (Forney et al., 2010). DNA extraction and PCR-HRM analysis of *Ureaplasma* spp. status was carried out as previously described (Payne et al., 2014).

2.3. Whole blood in vitro stimulation with heat-killed UpSV3

A clinical isolate of UpSV3 was chosen for stimulation (Payne et al., 2014). Exponential phase cultures were quantified, as previously described (Beeton et al., 2012), and heat-killed by incubation of 2 mL cultures at 60 °C for 4 h. Cells collected by centrifugation at 15,000 × *g*, 4 °C for 20 min were resuspended to 10^7 CCU/mL in sterile 1× PBS (Sigma, St. Louis, MO, USA). 10B broth (Media Preparation Unit, Melbourne University, Melbourne, Australia) was used as a specificity control; an equivalent volume was centrifuged and the 'pellet' resuspended in an equivalent volume of PBS. 300 μL aliquots of fresh, whole heparinised blood collected at recruitment were stimulated with heat-killed UpSV3 (10^6 CCU/mL), 10B (equivalent volume to UpSV3), LPS (0.5 μg/mL; Sigma) or left unstimulated (baseline) for 4 h at 37 °C in the presence of Brefeldin A (BFA; 0.5 mg/mL; Sigma). Unstimulated blood without BFA treatment was included as a control for non-specific antibody binding.

2.4. Intracellular staining and flow cytometry of stimulated monocytes

Stimulated whole blood was stained using a Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies), followed by cell surface staining for anti-CD14 APC-Cy7 (M5E2) at 2 μg/mL and anti-CD33 PerCP-Cy5.5 (WM53) at 1 μg/mL (both Biolegend, San Diego, CA, USA). Cells were washed using a 1X PBS wash buffer containing 0.5% BSA ("BovoStar", Bovogen Biologicals, East Keilor, VIC, Australia) and 0.1% NaN_3. Red cells were lysed in FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA) and remaining cells permeabilised with FACS™ Permeabilising Solution 2 (BD Biosciences). Intracellular cytokines were stained using anti-IL-1β AF647 (JK1B-1; 0.5 μg/mL), anti-IL-6 PE (MQ2-13A5; 1.25 μg/mL), anti-IL-8 FITC (E8N1; 1.25 μg/mL) and anti-TNF-α BV421 (MAb11; 5 μg/mL) (all Biolegend). Washed cells were resuspended in 200 μL Stabilising Fixative (BD Biosciences) and intracellular flow cytometry performed within 24–48 h on a FACSCanto II flow cytometer (BD Biosciences) and analysed using FlowJo software (Treestar, Ashland, OR, USA). Compensation was performed at each acquisition using compensation beads (anti-mouse and anti-rat Ig, κ/Negative Control Compensation Particles Sets, BD Biosciences). Cytokine expression by $CD14^+CD33^+$ monocytes was determined using gating shown in Supplementary Figure 1, with a minimum of 2000 live $CD14^+CD33^+$ monocytes acquired.

2.5. Statistical analysis

Comparisons of demographic and birth characteristics between *Ureaplasma* spp. non-colonised and colonised women were made using Mann–Whitney tests for continuous data and Fisher's exact tests for categorical data. Frequencies of cytokine-expressing cells were transformed to the natural logarithm to correct data normality and assessed for association with *Ureaplasma* spp. colonisation using generalised linear regression analysis with a general estimating equation approach. Models were adjusted for maternal age, gestational age at swab, and baseline frequencies of cytokine-expressing cells, and assessed for an interaction between *Ureaplasma* spp. colonisation status and stimulant. Differences in response to stimulation were evaluated after fitting the final model using post-estimation Wald tests. Stata 12 statistical software (StataCorp, College Station, TX, USA) was used for statistical analysis. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

Of the 32 participants included in this study, 41% ($n = 13$) were vaginally colonised with *Ureaplasma* spp., of which 62% ($n = 8$) were colonised with *U. parvum* only, 15% ($n = 2$) with both *U. parvum* and *U. urealyticum*, and 23% ($n = 3$) with *U. urealyticum* only (Supplementary Table 1). All participants were enrolled at their first prenatal clinical visit.