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Investigation of systemic inflammatory response in first trimester pregnancy failure

J. Calleja-Agius[1,4,*], E. Jauniaux[1], A.R. Pizzey[2], and S. Muttukrishna[3]

[1]Academic Department of Obstetrics and Gynaecology, UCL Institute for Women's Health, University College London, 86-96, Chenies Mews, London WC1E 6HX, UK [2]Research Department of Haematology, UCL Cancer Institute, Paul O'Gorman Building 72, Huntley Street, London WC1E 6BT, UK [3]Anu Research Centre, Department of Obstetrics and Gynaecology, University College Cork, Cork University Maternity Hospital, Wilton, Cork, Republic of Ireland

*Correspondence address. Tel: +356-21693041; Fax: +356-21319527; E-mail: jean.calleja-agius@um.edu.mt

BACKGROUND: The contribution of local and systemic inflammation to the pathophysiology of sporadic first trimester miscarriages remains unclear. The objective of this study was to investigate the inflammatory response in the circulation of women presenting with first trimester miscarriage.

METHODS: Levels of tumour necrosis factor alpha (TNFα), TNF receptors 1 and 2, interferon gamma (IFNγ), interleukin (IL)-6 and IL-10 were assayed using cytometric bead arrays in plasma samples from 29 euploid and 21 aneuploid missed miscarriages, 35 normal pregnant controls and 31 non-pregnant women (NPW). Whole blood flow cytometry was carried out with samples from 17 euploid and 16 aneuploid miscarriages, 18 pregnant controls and 13 NPW.

RESULTS: The plasma of women with euploid miscarriage contained significantly higher circulating levels of TNFα ($P < 0.005$), IFNγ ($P < 0.005$), IL-6 ($P < 0.005$) and IL-10 ($P < 0.01$) than that of pregnant controls, irrespective of gestational age. Significantly ($P < 0.05$) higher TNF-R1 levels at 6–9 weeks, and significantly higher TNFα/IL-6 ($P < 0.001$) and significantly lower TNFα/IL-10 ($P < 0.001$) and IFNγ/IL-10 ($P < 0.001$) ratios at 10–14 weeks, were also found in euploid miscarriage cases compared with pregnant controls. TNFα/IL-10 ratio in plasma was significantly ($P < 0.05$) lower in miscarriages with an abnormal karyotype than those with normal karyotype. Normal pregnant women had a significantly higher plasma level of IFNγ ($P < 0.01$) and IFNγ/IL-10 ratio ($P < 0.005$), a significantly ($P < 0.005$) lower TNF-R1 level, and a significant ($P < 0.05$) increase in stimulated TNFα in monocytes, compared with NPW.

CONCLUSIONS: Our data confirm that there is an inflammatory reaction in normal pregnancy compared with the non-pregnant state, which may be disrupted during miscarriage.

Key words: cytokines / miscarriage / inflammation

Introduction

An inflammatory microenvironment is required for successful implantation and tissue remodeling in the first trimester of pregnancy (Challis *et al.*, 2009). This inflammatory reaction is characterized by an up-regulation of cytokines, chemokines and their receptors. An excessive inflammatory reaction has been associated with recurrent miscarriage or other pregnancy complications such as pre-eclampsia or premature labour (Chaouat *et al.*, 2004).

Miscarriage was initially believed to happen exclusively due to abnormal villous development, as described in anatomical and histological studies in earlier studies (Bouie *et al.*, 1976). However, recent evidence is now proving that early pregnancy failure is a

disorder of the placenta, and that the villous changes described in previous studies are not the cause, but rather the consequence of miscarriage (Jauniaux and Burton, 2005). Defective placentation is mainly characterized by a thinner and fragmented trophoblast shell, reduced cytotrophoblast invasion of the endometrium and incomplete plugging of the lumen at the tips of the spiral arteries (Hustin, 1990). These anatomic changes have been found in about two-thirds of early pregnancy failures (Hustin, 1990; Jauniaux *et al.*, 1994). This leads to the absence of physiological changes in most spiral arteries, with premature onset of the maternal circulation throughout the entire placenta (Jauniaux *et al.*, 2006). *In vivo* and *in vitro* investigations have shown that during the first trimester of normal human pregnancy, the placenta limits, rather than facilitates, oxygen supply to the fetus during

organogenesis (Jauniaux *et al.*, 2003a). There is a very well-controlled phenomenon that has to provide a delicate balance between the fetal metabolic needs and its placenta, and the potential danger of oxidative stress. This physiological hypoxia of the first trimester gestational sac may protect the fetus against the deleterious and teratogenic effects of highly reactive oxygen free radicals (Jauniaux *et al.*, 2006).

Consequently, in early pregnancy failure, the excessive entry of maternal blood into the intervillous space has two main effects on the fetus and the placenta. The first effect is a direct mechanical effect on the villous tissue which becomes enmeshed inside large inter-villous blood thrombi. The second effect is widespread and indirect oxygen-mediated trophoblastic damage and increased apoptosis (Kokawa *et al.*, 1998; Hempstock *et al.*, 2003; Jauniaux *et al.*, 2003b). This mechanism possibly occurs in all miscarriages, with the time at which it occurs in the first trimester depending on the aetiology (Jauniaux *et al.*, 2006). This is in a way analogous to the ischaemia-reperfusion injury that occurs in pre-eclampsia (Chappell *et al.*, 2002).

It has been proposed that there are primary and secondary causes of oxidative stress leading to early pregnancy failure (Hempstock *et al.*, 2003). Primary causes are mainly chromosomal abnormalities which are found in at least 50% of miscarriages and are usually associated with abnormal trophoblast invasion of the uterine deciduas (Hustin, 1990; Greenwold *et al.*, 2003). Secondary causes may involve mater-nal leukocytes and other immune factors including cytokines, such as tumour necrosis factor alpha (TNFα). However, so far, their role in the trophoblast-decidual interaction in normal and abnormal first trimester remains unclear (Jauniaux *et al.*, 2006). Studies have shown that the circulating cytokine levels and the decidual cytokine profile in the decidua are different in women experiencing recurrent miscarriages (Jenkins *et al.*, 2000; Makhseed *et al.*, 2000; von Wolff *et al.*, 2000; Baxter *et al.*, 2001), however little is known about spon-taneous miscarriages. Major fetal abnormalities leading to fetal demise in the first trimester could also lead to secondary placental dysfunction as the placental development becomes increasingly dependent on fetal synthesis towards the end of the first trimester (Shurtz-Swirski *et al.*, 1991).

Previous studies in humans (Raghupathy *et al.*, 2000; Rezaei and Dabbagh, 2002; Raghupathy and Kalinka, 2008) and mice (Doi *et al.*, 1999; Clark and Croitoru, 2001; Joachim *et al.*, 2003) provide evi-dence for an increase in Th-1/Th-2 ratio, with a shift towards Th-1 in recurrent miscarriage. In contrast, peripheral blood mononuclear cells (PBMCs) have been shown to secrete higher levels of Th-1 cyto-kines and lower levels of Th-2 cytokines, in first trimester pregnant women with normal outcome compared with women with spontan-eous miscarriage (Zenclussen *et al.*, 2002) or a history of recurrent miscarriage who subsequently miscarried (Bates *et al.*, 2002). In general, Th-1 cytokines which have been measured include interleukin (IL)-2, TNFα and β, interferon gamma (IFNγ) and Th-2 cytokines include IL-4, IL-5, IL-6 and IL-10. Although similar methods have been used to estimate circulating and intracellular cytokine levels (mainly ELISA and flow cytometry of cultured PBMCs or whole blood) there are conflicting reports in the literature about the state of systemic inflammation in early pregnancy loss, warranting further investigation with karyotyping of the products of conception.

Therefore, the objective of this study is to investigate pro- and anti-inflammatory cytokines and soluble receptor concentrations in maternal plasma and PBMCs in miscarriage patients of normal karyo-type in the first trimester, and to evaluate the systemic inflammatory response in first trimester spontaneous early pregnancy loss compared with normal pregnancy.

Materials and Methods

Plasma and whole blood samples

Pregnant women attending the Early Pregnancy Unit and diagnosed with a missed miscarriage on scan were recruited as cases. A missed miscarriage was diagnosed by ultrasound on the basis of absent fetal heart activity after 5 weeks of gestation or in the presence of an empty gestational sac, before the expulsion of the fetus or placental tissues. Control pregnant women were asymptomatic normal pregnant women with an ongoing pregnancy who came to Antenatal Clinic for booking or to the Early Pregnancy Unit for a reassurance scan, and were followed up prospectively until term delivery. Non-pregnant women (NPW) in the luteal phase of their menstrual cycle were recruited among staff at UCL as a control group for comparison. All blood samples were collected as part of an ongoing prospective study, for which the participants gave informed consent. In the case of women with missed miscarriage, the blood was sampled im-mediately upon diagnosis. The study was approved by the UCLH Research Ethics Committee.

All pregnant cases were <14 weeks' gestation as calculated from the first day of the last menstrual period and confirmed by ultrasound meas-urement of crown-rump length. Inclusion criteria were healthy non-smoking women with a spontaneous singleton pregnancy, and not on any medication until the time of blood sampling. In the case of missed miscarriages, there was an ultrasound confirmation of an absent fetal heartbeat. Exclusion criteria were patients with a history of recurrent miscarriage, the presence of a hydatiform mole or a multiple pregnancy, any congenital uterine anomaly, cervical incompetence, large uterine fibroids, a known thrombophilia or any other medical condition needing chronic drug treatment.

The products of conception obtained from patients with missed miscarriage who underwent elective removal of products of conception, were karyotyped at a commercial laboratory (TDL, London) using standard culturing, suspension harvest and G-band analysis methodology as previously described (Greenwold and Jauniaux, 2002).

Venous blood (10 ml) was collected by sterile venepuncture and 1.5 ml was aspirated aseptically for whole blood cell culture and analysis, while the rest was centrifuged within 2 h of collection, and the plasma supernatant was stored at −20°C until assayed.

Study groups

In total, there were 106 successfully karyotyped placentae. There were 50 placentae with male karyotype, of which 29 were euploid and 21 were tri-somies (majority Trisomies 16 and 22, but also Trisomies 7, 8, 13, 20 and 21 were found in individual cases). There were 51 placentae with female karyotype, of which 25 were euploid and 26 were aneuploid (7 monoso-mies XO and 19 trisomies). There were also five triploidies.

For the purpose of this study, in order to avoid the risk of maternal con-tamination, the study group included 29 patients with missed miscarriage of normal male karyotype (14 at 6–9 and 15 at 10–14 weeks), 21 patients with missed miscarriage of aneuploid (trisomic) male karyotype (9 at 6–9 and 12 at 10–14 weeks) and the control groups included 35 pregnant women (16 at 6–9 and 19 at 10–14 weeks) and 31 NPW. All patients were matched for age, parity, ethnicity and BMI.

Multiplex bead arrays

TNF receptors 1 and 2, TNFα, IFNγ, IL-6 and IL-10 were assayed in maternal plasma using the BD™ Cytometric Bead Array Human Soluble Protein Flex Sets (BD Biosciences, San Jose, Canada). Cytometric bead array permitted multiplexed analysis of different cytokines and receptors on smaller quantities of plasma, compared with conventional ELISA systems. The experiment was set up according to the manufacturer's instructions and as previously published (Chen *et al.*, 1999). Acquisition of the sample data was performed using the BD FACSArray™ bioanalyzer flow cytometer (BD Biosciences, CA, USA) and data were presented in graphical and tabular formats using the FCAP Array™ Software. The limits of detection for each assay were 0.7 pg/ml for TNFα, 1.8 pg/ml for IFNγ, 1.6 pg/ml for IL-6, 0.13 pg/ml for IL-10, 5.2 pg/ml for TNF-R1 and 1.4 pg/ml for TNF-R2. The intra-assay coefficient of variation was 10.2% for TNFα, 10.8% for IFNγ, 6.4% for IL-10, 9.4% for IL-6, 2.6% for TNF-R1 and 7.1% for TNF-R2. The inter-assay coefficient of variation was 5% for TNFα, 5% for IFNγ, 8% for IL-6, 11% for IL-10, 10.1% for TNF-R1 and 5.6% for TNF-R2.

Whole blood flow cytometry for cellular expression and inflammatory response

Whole blood flow cytometry performed in a subgroup of patients with miscarriage with normal male karyotype ($n = 17$: $n = 7$ at 6–9 weeks and $n = 10$ at 10–14 weeks), miscarriages with aneuploid male karyotype ($n = 16$: $n = 6$ at 6–9 weeks and $n = 10$ at 10–14 weeks), pregnant controls ($n = 18$: $n = 8$ at 6–9 weeks and $n = 10$ at 10–14 weeks) and NPW ($n = 13$) of the same cohort of patients. The patients in the whole blood flow cytometry subgroup were matched for age, parity, ethnicity and BMI.

Optimal experimental conditions were selected based on initial validation experiments with a range of doses of (0.1–40 ng/ml) of lipopolysaccharide (LPS) and incubation times (2–20 h). All experiments were carried out as previously described by our group (Calleja-Agius *et al.*, 2011) using 40 ng/ml of LPS as an inflammatory stimulus with an incubation period of 12 h. In brief, dual antibody labelling was carried out with monocyte (CD 14) specific and cytokine or receptor-specific mouse anti-human antibodies (AbD Serotec, Oxford, UK). Using aseptic techniques, 1.5 ml of whole blood from each individual patient was mixed with 1.5 ml of Dulbecco phosphate-buffered saline without Ca^{++}, Mg^{++} and the final solution divided into aliquots. Ten microlitres of monensin sodium solution (a Golgi apparatus inhibitor used to stop cytokine release by the cell; Sigma-Aldrich, St. Louis, USA) and 10 µl of LPS 40 ng/ml solution were added to one aliquot (stimulated), while only 10 µl of monensin sodium solution (Sigma-Aldrich) without LPS was added to the other aliquot. Both aliquots were placed in a water bath at 37°C, shaking at 20 revs/min for 12 h incubation. Each aliquot was further divided depending on the antibody combination, and Fix and Perm® Cell Permeabilization Reagents (Caltag™ Invitrogen, UK) were added following the manufacturer's instructions. Each sample was read by the flowcytometer Dako CyAn Advanced Digital Processing, operated through the Summit 4.31 Software. The results were then saved in fetal calf serum 2.0 file format for post-acquisition spectral compensation and data analysis. The cell populations were classified as monocytes, granulocytes and lymphocytes by their granularity and size, and further gating was done on CD-14 positive cells, which represent monocytes.

Statistical analysis

Data were log transformed to approximate normal distribution, and two-way analysis of variance was carried out using category (pregnant controls versus miscarriage of normal karyotype) and gestational age subgroups (6–9 and 10–14 weeks). All patients were matched for age, BMI, parity and ethnicity. Median and interquartile ranges were calculated for each patient category within each gestational group. Comparison of cytokine levels in plasma between patient categories in each gestational group were tested for significance using an unpaired *t*-test of the log-transformed data. This analysis was repeated for intracellular cytokine levels in monocytes, both at baseline and at stimulated levels using 40 ng/ml LPS, including the % rise for each cytokine upon stimulation. In this case, significance testing was carried out using Mann–Whitney *U*-test, since the number of samples was limited. Descriptive statistics and significance testing was carried out using SPSS v.17 for Windows (SPSS Inc., Chicago, IL, USA). A $P \leq 0.05$ was considered statistically significant.

Results

Demographics

There were no statistical differences between the groups regarding maternal age, parity, BMI and ethnicity. There were no significant differences between the mean gestation durations in each patient subgroup within both studies.

Miscarriages of normal karyotype compared with pregnant controls at 6–9 weeks' gestation

Differences in cytokine levels and ratios were standardized to the comparator group, and expressed as percentages of the levels or ratios in the comparator group.

Maternal plasma concentrations

Patients with miscarriage of normal karyotype had significantly higher circulating levels of TNFα (337.1%, $P = 0.005$), TNF-R1 (34.1%, $P < 0.05$), IFNγ (299.5%, $P < 0.005$), IL-6 (195%, $P < 0.005$) and IL-10 (2584.6%, $P = 0.01$) in the 6–9 gestational group compared with gestation-matched normal pregnant women (Fig. 1). There was no statistical difference in the cytokine ratios between the two patient categories at this gestational stage.

Cellular expression

At 6–9 weeks, there was a significant (24.6%, $P < 0.05$) increase in unstimulated IL-6 levels in monocytes in miscarriages of normal karyotype compared with pregnant controls. Otherwise, there was no significant difference in any of the baseline or stimulated cytokine levels or receptor levels.

Miscarriages of normal karyotype compared with pregnant controls at 10–14 weeks' gestation

Maternal plasma concentrations

Patients with miscarriage of normal karyotype had significantly higher circulating levels of TNFα (615.7%, $P < 0.001$), IFNγ (375.6%, $P < 0.001$), IL-6 (115%, $P < 0.001$) and IL-10 (3330.8%, $P < 0.001$) in the 10–14 weeks gestational groups compared with gestation-matched normal pregnant women (Fig. 1). In this gestational group, there was also a significantly higher ratio of TNFα/IL-6 (197.7%,
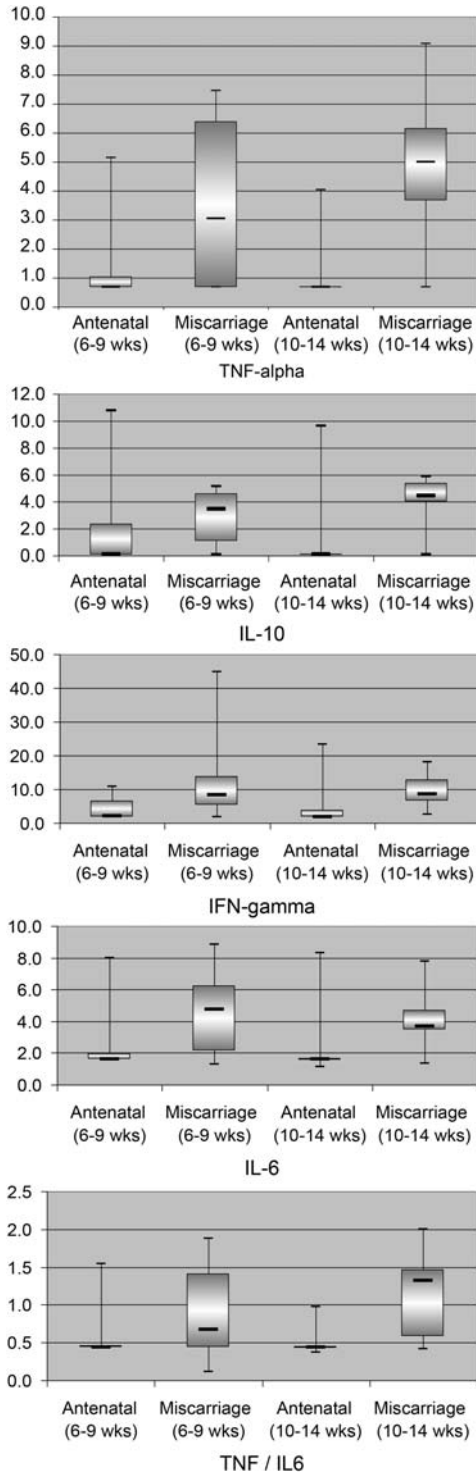
Figure 1 Median and interquartile range for all cytokines and receptors (pg/ml) measured in plasma in both gestational subgroups, in pregnant controls and miscarriages of normal karyotype. Significance testing was carried out using unpaired *T*-test to compare pregnant controls and euploid miscarriages. At 6–9-week gestation: $n = 16$ pregnant controls, $n = 14$ euploid miscarriages; and at 10–14-week gestation: $n = 19$ pregnant controls, $n = 15$ euploid miscarriages. *Y*-axis is cytokine/receptor (pg/ml).
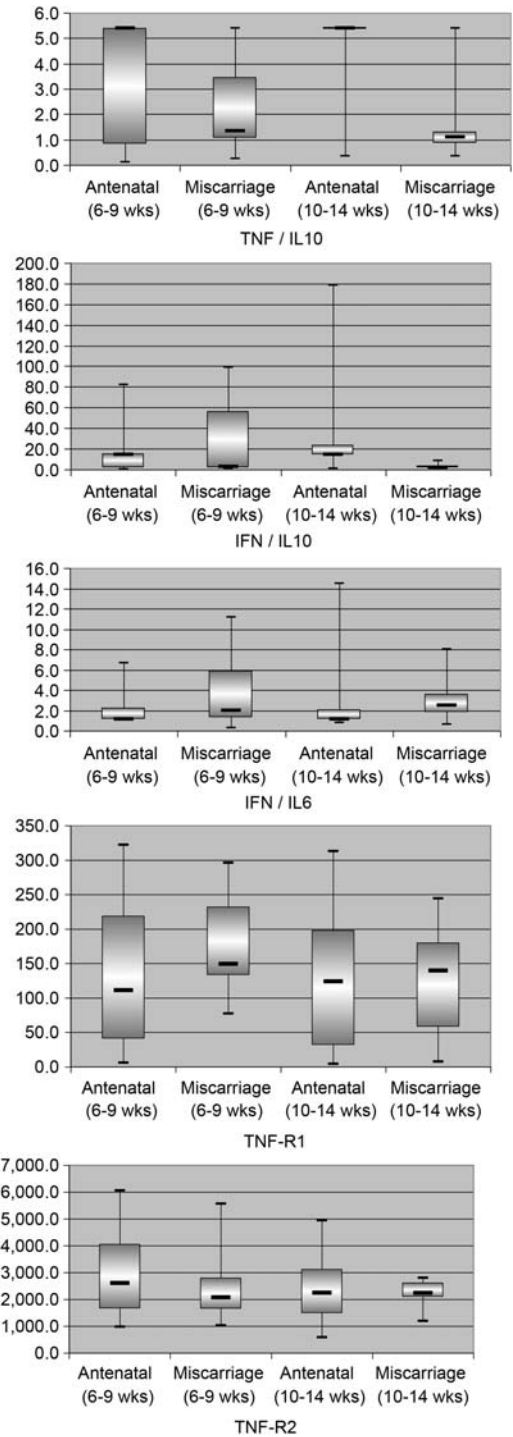
Figure 1 Continued

$P < 0.001$), and significantly lower TNFα/IL-10 (-79.6%, $P = 0.001$) and IFNγ/IL-10 (-86.1%, $P < 0.001$) ratios, in miscarriages compared with normal pregnancy (Table I).

Cellular expression

At 10–14 weeks, there was a significant decrease (-56.4%, $P < 0.05$) in the level of stimulated IFNγ in miscarriages of normal karyotype

Table I Median and interquartile range for all cytokine ratios measured in plasma in the 10–14 week gestational subgroup, in pregnant controls (n = 19) and miscarriages (n = 15).

Cytokine, ratio	Pregnant controls, 10–14 weeks	Miscarriage, 10–14 weeks	P-value (unpaired T-test), comparison between controls versus miscarriages at 10–14 weeks
TNFα/IL-6	0.44 (0.44, 0.54)	1.31 (0.58, 1.46)	<0.001
TNFα/IL-10	5.39 (5.39, 5.64)	1.10 (0.85, 1.31)	0.001
IFNγ/IL-6	1.13 (1.13, 2.08)	2.48 (1.79, 3.56)	0.08
IFNγ/IL-10	13.85 (13.85, 23.5)	1.92 (1.41, 3.54)	<0.001

There were no significant differences for the cytokine ratios in the 6–9 week gestational subgroup.

compared with pregnant controls. There was no significant difference in any of the other baseline or stimulated cytokine levels or receptor levels.

Comparison of euploid with aneuploid missed miscarriages

Maternal plasma concentrations

The levels of TNFα, TNF-R1, TNF-R2, IFNγ, IL-6 and IL-10 were not significantly different between miscarriages with normal and abnormal karyotype, irrespective of gestational stage. TNFα/IL-10 ratio in the plasma was significantly (P < 0.05) lower in miscarriages with an abnormal karyotype compared with those with normal karyotype in both gestational groups (Fig. 2).

Cellular expression

There was no significant difference between intracellular levels of TNFα, TNF-R1, TNF-R2, IFNγ, IL-6 and IL-10 in the monocytes of both groups.

Comparison of normal pregnant state with luteal phase of the non-pregnant state

Maternal plasma concentrations

Normal pregnant women in the first trimester (6–14 weeks) had significantly higher (163%, P = 0.005) plasma levels of IFNγ, and significantly lower (−50.5%, P = 0.001) TNF-R1 than NPW. There was also a significantly higher (117%, P = 0.001) IFNγ/IL-10 ratio in normal pregnant controls than NPW. There was no significant difference in TNFα, TNF-R2, IL-6 or IL-10 levels, or in ratios of TNFα/IL-10, TNFα/IL-6 or IFNγ/IL-6 (Fig. 3 and Table II).

Cellular expression

In monocytes, there was a significant increase (61%; P < 0.05) in stimulated TNFα in pregnant women compared with NPW. Upon
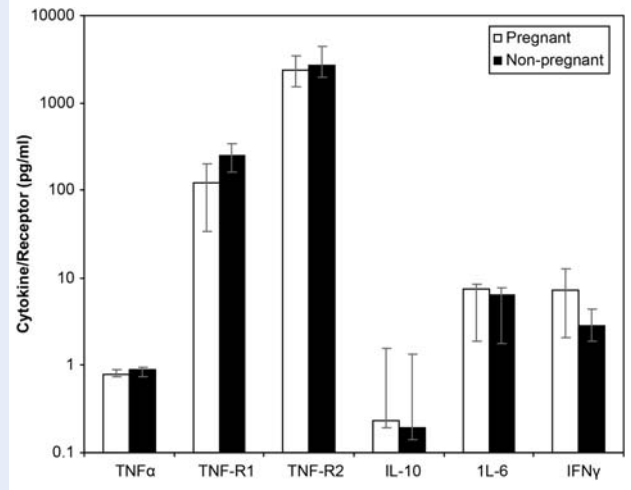

Figure 2 Median and interquartile range for TNFα/IL-10 ratio measured in plasma in both gestational subgroups, in euploid (n = 29) and aneuploid (n = 21) male miscarriages. Significance testing was carried out using Mann–Whitney. There was a significantly (P < 0.05) lower TNFα/IL-10 ratio in the plasma of miscarriages of abnormal karyotype compared with those with normal karyotype.
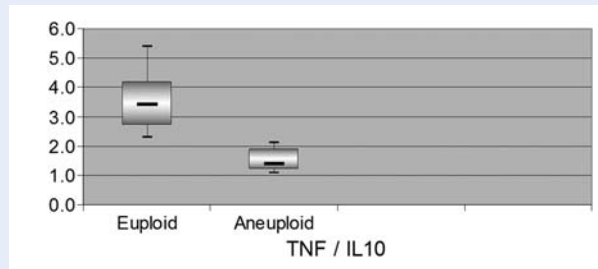

Figure 3 Median and interquartile range for all cytokines and receptors (pg/ml) measured in plasma in pregnant controls between 6–14-week gestation (n = 35) and NPW (n = 31). Significance testing was carried out using unpaired t-test. *P < 0.05, **P < 0.01.

Table II Median and interquartile range for all cytokine ratios measured in plasma in pregnant controls between 6–14-week gestation (n = 35) and NPW (n = 31).

Cytokine ratio	Pregnant controls (6–14 weeks) Median (interquartile range)	Non-pregnant women Median (interquartile range)	P-value
TNFα/IL10	3.48 (1.36, 5.61)	4.74 (3.04, 5.38)	0.09
TNFα/IL6	0.11 (0.10, 0.44)	0.14 (0.11, 0.44)	0.8
IFNγ/IL10	32.00 (13.85, 98.46)	14.74 (2.24, 18.92)	0.001
IFNγ/IL6	0.97 (1.06, 8.00)	0.44 (1.13, 2.05)	0.19

Significance testing was carried out using unpaired t-test.
NPW, non-pregnant women.

Table III Median and interquartile range for all cytokines and receptors measured in monocytes, in pg/ml, in pregnant controls (n = 18) and NPW (n = 13).

Cytokine/receptor	Pregnant controls (6–14 weeks) Median (interquartile range)	Non-pregnant women Median (interquartile range)	P-value
TNFR-1 median	2.53 (1.63, 3.81)	1.29 (1.00, 3.67)	0.29
TNFR-2 median	15.57 (12.33, 20.04)	9.73 (9.39, 12.99)	0.12
TNFα (0 LPS)	1.53 (0.65, 5.73)	1.92 (1.20, 2.29)	0.69
TNFα (40 LPS)	8.50 (7.14, 20.48)	5.27 (3.85, 9.19)	0.03
% rise in TNFα	537.91 (207.82, 1209.81)	235.36 (109.65, 491.71)	0.09
IFNγ (0 LPS)	80.29 (69.15, 95.47)	88.21 (85.91, 94.89)	0.07
IFNγ (40 LPS)	81.00 (72.62, 93.11)	64.13 (53.28, 89.26)	0.12
% rise in IFNγ	−0.29 (−1.44, 18.54)	−26.69 (−37.07, −1.91)	0.32
IL-10 (0 LPS)	1.57 (1.34, 3.01)	2.13 (1.54, 3.77)	0.60
IL-10 (40 LPS)	1.79 (0.97, 4.41)	3.35 (1.77, 8.77)	0.34
% rise in IL10	5.45 (−30.49, 93.64)	97.71 (−4.51, 258.61)	0.25
IL-6 (0 LPS)	1.94 (1.65, 3.65)	2.30 (1.60, 3.62)	0.68
IL-6 (40 LPS)	6.37 (3.64, 9.37)	5.10 (3.51, 6.06)	0.54
% rise in IL-6	97.58 (37.41, 224.85)	74.42 (−20.67, 135.00)	0.94

Significance testing was carried out using unpaired t-test. Cytokines were measured at basal level and upon stimulation with 40 ng/ml LPS. The % rise from basal to stimulated was calculated as [(stimulated-basal)/basal] multiplied by 100. TNF-R1 and TNF-R2 were measured at basal level only.

stimulation with 40 ng/ml LPS, there was a median rise of 537.9% from baseline in TNFα in pregnant controls, when compared with a median rise of 235.3% in NPW. Other differences were not significant although IFNγ levels decreased less in pregnant controls when compared with NPW, upon LPS stimulation (0.3 versus 26.7%), and there was only a median rise of 5.5% in IL-10 upon LPS stimulation in samples from pregnant women, when compared with a median of 97.7% in NPW (Table III).

Discussion

This study demonstrates that there is an inflammatory reaction in normal pregnancy compared with the non-pregnant state, which may be altered in spontaneous miscarriage. Our findings also show that there are higher plasma and intracellular levels of some pro-inflammatory cytokines in pregnant compared with NPW, confirming that normal pregnancy is an inflammatory state as described previously (Redman and Sargent, 2010). In cases of spontaneous miscarriage with normal karyotype, plasma cytokine levels were generally higher than in pregnant controls.

However, a limitation of this study concerns reliability of the statistical significance of the results due to multiple testing in the flow cytometry study, which is exacerbated by the relatively small sample sizes. The low sample sizes are because of the difficulty in getting chromosomally normal miscarriage samples.

In normal pregnancy, there is an increase in systemic inflammation (Challis et al., 2009), which explains the higher plasma levels of IFNγ and IFNγ/IL-10 in normal pregnant women compared with NPW in circulation, and the higher rise in TNFα (and possibly lower IL-10) upon stimulation of monocytes. This is in contrast with the findings of another study carried out on cultured PBMCs (Bates et al., 2002), where the levels of the pro-inflammatory cytokine,

IFNγ, were decreased while IL-10 levels were higher in pregnant controls compared with NPW. However, in our study, all NPW had their blood samples taken in the luteal phase, unlike in the latter study. Moreover, we have compared cytokine and soluble receptor levels both in plasma and intracellularly in monocytes. In contrast to previous investigations where experiments were done on cultured PBMCs (Bates et al., 2002; Sacks et al., 2003), in this study we used a whole-blood cell culture system which has been successfully used by other groups (Colin et al., 2004; Zahran et al., 2006; van Nieuwenhoven et al., 2008), together with a Golgi apparatus inhibitor used to stop cytokine release by the cell, in order to closely reflect the in vivo conditions. Whole blood analysis is more physiological, when compared with studies involving cultured PBMCs, because in the latter, the purification step itself can alter cytokines and other markers of inflammation. The TNFα/IL-10 ratio was not significantly different between the pregnant and NPW, however this may be attributed to the low detectable levels of TNFα.

Both pro-inflammatory (TNFα, IFNγ) and anti-inflammatory (IL-6, IL-10) cytokines were significantly elevated in the plasma of patients presenting with miscarriages of normal karyotype when compared with pregnant controls, irrespective of gestation. Since the Th-1/Th-2 ratio gives a better picture of the overall immune response, rather than the individual cytokine levels, the cytokine ratios were calculated. The significantly higher TNFα/IL-6 ratio in miscarriages of normal karyotype compared with normal pregnancy is consistent with a shift towards Th-1 type of immune response with an increase in pro-inflammatory cytokines, which has already been shown previously by others investigating miscarriages sampled after diagnosis (Makhseed et al., 1999, 2000; Raghupathy et al., 2000). However, in the late first trimester (10–14 weeks), the TNFα/IL-10 and IFNγ/IL-10 ratios were lower in euploid miscarriages compared with normal pregnancy. This contradicts the hypothesis of a shift in the

Th-1/Th-2 ratios. We hypothesize that there could be changes in oxidative stress in the feto–placental unit happening at around 9 weeks gestation (Jauniaux et al., 2000; Jauniaux et al., 2001), which could modulate the cytokine balance both in normal pregnancies and in miscarriages. This can explain our findings that in the early first trimester (6–9 weeks), there were no changes in the Th-1/Th-2 ratios, even though there was a rise in the plasma levels of both pro- and anti-inflammatory cytokines in women with euploid miscarriages compared with normal pregnancy. With the premature entry of maternal oxygenated blood in late first trimester miscarriages leading to an increase in free radicals in the placenta (Jauniaux et al., 2000), there may be a compensatory rise in the anti-inflammatory cytokine IL-10 in the maternal circulation, possibly to dampen the rise in pro-inflammatory cytokines in the placenta. Our preliminary results of measuring the placental cytokine levels in normal first trimester and miscarriages show increased TNFα and decreased receptors in villous tissue of miscarriages.

Our study contributes to this literature by confirming that the miscarriages studied were of normal karyotype. Other studies have recruited women with recurrent miscarriage, but did not specify the karyotype of the products of conception of the index miscarriage. Karyotype of the fetus which is eventually miscarried has been shown to have an effect on the cytokine levels present in the maternal circulation of recurrent miscarriages (Yamada et al., 2004). However, in this study, we investigated this difference in spontaneous miscarriages. A comparison was done between male euploid and aneuploid miscarriages. In this case, the TNFα/IL-10 ratio in plasma was significantly lower in miscarriages with an abnormal karyotype compared with those with normal karyotype. However since the TNFα/IL-10 ratio was already lower in euploid miscarriages compared with normal pregnancy, it would appear that the difference in immune function in miscarriage is actually exaggerated in aneuploid miscarriages.

In order to avoid the risk of maternal contamination, only male euploid miscarried placentae were included in this study. However, one limitation of including only miscarriages of normal male karyotype is the introduction of a bias whereby the maternal immune response is altered by the H-Y antigen present in male fetuses. It has been shown that in patients with recurrent miscarriages, male fetuses are at an increased risk of being miscarried due to the increased maternal immune response against H-Y antigens. Anti-H-Y immunity may partly explain the increased inflammatory response found during miscarriage of male fetuses of normal karyotype (Christiansen et al., 2010; Nielsen et al., 2010a; Nielsen et al., 2010b). In view of this, a subanalysis was carried out to compare cytokine levels between women miscarrying a euploid male fetus, and those controls who eventually delivered a healthy male baby at term. There was a trend for higher circulating levels for the pro-inflammatory cytokines (TNFα and IFNγ) in the miscarriage group, but it did not achieve statistical significance, probably due to the small sample size.

In contrast to our findings in the maternal plasma, the cytokine levels in monocytes of euploid miscarriages showed significantly lower levels of the pro-inflammatory cytokine, IFNγ upon LPS stimulation, and higher basal IL-6 levels, when compared with pregnant controls. In a smaller study with $n = 5$, other investigators found no shift in Th-1/Th-2 cytokine ratio in PBMCs of pregnant women prior to ending up having a spontaneous miscarriage (Marzi et al., 1996). However, similar to our findings, others have shown that there is a rise in anti-inflammatory, rather than pro-inflammatory cytokines in PBMCs of pregnant women who subsequently miscarry (Bates et al., 2002). Although, in contrast to our study the patients recruited in the latter study were recurrent and not spontaneous miscarriagers, and the karyotype of the products of conception was also unknown, the trend in the cytokine shift intracellularly in monocytes is similar. This is inspite of the fact that in our study, the blood was sampled upon confirmation of the missed miscarriage, while in the latter study, the blood was sampled prior to the actual miscarriage. This may suggest that the changes in cytokine levels may be a cause rather than a consequence of the actual miscarriage.

Cytokines such as TNFα do not exist in a stored form, but are synthesized upon activation of cells derived from the monocyte/macrophage lineage (Aggarwal et al., 1985b). LPS stimulation could lead up to a 100-fold increase in the level of cytokine secretion (Beutler et al., 1986). The production of IFNγ in decidual mononuclear cells has recently been shown to be up-regulated by IL-2 and IL-12 by increasing the susceptibility of these monocytes to LPS, with TNFα being independent of such a mechanism (Negishi et al., 2011). In our study, in pregnant women, the rise in pro-inflammatory TNFα levels upon LPS stimulation was higher compared with non-pregnant controls, while the increase in stimulated IFNγ was higher than euploid miscarriages. IFNγ may play a beneficial role in early pregnancy. Uterine natural killer cells have been shown to depend on IFNγ during normal trophoblast invasion in mice (Ashkar and Croy, 2001), and this cytokine has been shown to be essential in endometrial angiogenesis in pigs (Tayade et al., 2007).

TNFα receptors, TNF-R1 and TNF-R2, are shed from the cell surface and exist as soluble receptors in circulation neutralizing the effect of TNFα (Aggarwal et al., 1985a). These receptor levels have been shown to be decreased in recurrent miscarriage (Chernyshov et al., 2005). Early in the first trimester, TNF-R1 levels were higher in the plasma of miscarriages with normal karyotype compared with pregnant controls, who in turn had lower levels than NPW. The high TNF-R1 levels may explain the overall low levels of TNFα present in the plasma in our study since the assay measures free TNFα.

The classification of IL-6 into Th-1 or Th-2 cytokine remains controversial (Chaouat et al., 2007). In the present study, we have analyzed our data classifying IL-6 as a Th-2 cytokine, as there is literature showing that IL-6 has anti-inflammatory properties in pregnancy as explained above. We found that TNFα/IL-6 ratios were significantly higher in miscarriages than in pregnant controls, supporting IL-6 as a Th2 cytokine. However, in studies on premature labour (Genc and Ford, 2010; Wei et al., 2010) and pre-eclampsia (Vural et al., 2010), IL-6 has been classified as a pro-inflammatory cytokine. IL-6 has been detected in coelomic and amniotic fluid of early human pregnancy (Jauniaux et al., 1996) and has been shown to be involved in the promotion of Th-2 differentiation and inhibition of Th-1 polarization (Dubinsky et al., 2008). During the first trimester, IL-6 could be involved in the generation of new vessels in the placental villous tissue, and tissue remodeling associated with placentation, thus making it a Th-2 cytokine at this gestation (Jauniaux et al., 1996).

Our group has further shown, using the same methodology as in this study, that women with threatened miscarriage who end up miscarrying have a shift towards a Th-1 type of immune response in their circulation, when compared with women with threatened miscarriage

who have a live birth with no adverse pregnancy outcome (Calleja-Agius *et al.*, 2011). This further confirms that the shift towards a Th-1 type of immune response in plasma of miscarriages of normal karyotype can contribute to prediction of early fetal demise.

This current study sheds new light on the role of cytokines in human pregnancy and pregnancy failure.

Authors' roles

J.C.A. wrote the first draft of the paper and carried out sample collection and the actual experiments, together with the statistical analysis. E.R.J. contributed to the study design, interpretation of the data, and to the writing of the manuscript. A.R.P. contributed to the experimental design and data analysis. S.M. contributed to the experimental design, data analysis, presentation and interpretation of data.

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Conflict of interest

All authors declare no conflict of interest.

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