

# A review of immune cells and molecules in women with recurrent miscarriage

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**Immunological rejection of the fetus due to recognition of paternal antigens by the maternal immune system, resulting in abnormal immune cells and cytokine production, is postulated to be one cause of unexplained pregnancy loss. Although there is evidence for this in rodents, there is less evidence in humans. This article focuses on studies in humans, and reviews the recent literature on the differences in immune cells and molecules in normal fertile women and women with recurrent miscarriage (RM). Although much of the evidence is contradictory, these studies do suggest differences in the expression of some immune cells and molecules in women with RM. Differences in the CD56+ population of cells are seen, and there is some evidence for an alteration in the ratio of Th1 and Th2 cytokines produced by peripheral blood monocytes (PBMCs) and clones of decidual CD4+ cells. There is also some evidence for differences in endometrial cytokine production, and in particular decreased production of pro-inflammatory cytokines such as interleukin-6. Possible reasons for the variations in data are discussed, and the importance of compartment (peripheral blood, endometrium or decidua) in which the cells and molecules are measured and the timing of the sampling, both with respect to the menstrual cycle and pregnancy (at the time or just after miscarriage) is emphasized.**

*Key words:* cytokines/endometrium/immune cells/recurrent miscarriage/uNK cells

## Introduction

Recurrent miscarriage (RM), defined as the loss of three or more consecutive pregnancies in the first trimester of pregnancy, affects approximately 1% of the population (Regan and Rai, 2000). The cause of repeated pregnancy loss is multifactorial, but can be divided into embryologically driven causes (mainly due to an abnormal embryonic karyotype) and maternally driven causes which affect the endometrium and/or placental development (Aplin, 2000; Li *et al.*, 2002). Known causes of maternal defects include coagulation disorders, autoimmune defects, endocrine disorders and endometrial defects (Li, 1998; Regan and Rai, 2000; Li *et al.*, 2002). The aetiology in approximately 50% of cases of RM is unknown, but it has been postulated that a proportion of these repeated pregnancy losses may be due to immune causes. It is likely that there is more than one immune cause of RM, which may include recognition of paternal antigens on the foeto-placental unit by the maternal immune system followed by destruction of the fetus, although actual evidence for this in humans is limited. One of the problems in under-

standing the underlying aetiology of this immune failure is that the mechanisms by which the fetus is protected from the maternal immune system during normal pregnancy are not fully understood. There is considerable evidence for the presence of a specialized immune system within the maternal part of the placenta (the decidua), which is postulated to have a role in this process. This review highlights some of the differences in these specific endometrial and decidual immune cells and molecules that may be involved in immune failure resulting in RM. Although some animal studies are described, the emphasis is on evidence in humans. There is a tendency to extrapolate directly from animal models (particularly those of rodents) to humans, and this has led to assumptions of mechanisms for which the evidence is incomplete. Recurrent miscarriage is normally defined as a loss of three or more miscarriages, but some studies include women with only two miscarriages. In addition, as discussed previously (Li *et al.*, 2002), the timing of the fetal loss may differ between studies and may result in the study of different populations. However, for completeness some of these studies have been

included in this review. The information was obtained from key word and author searches using Medline and Web Science.

### Models used to study RM

In RM, pregnancy loss very often occurs during the first trimester, prior to week 13 of pregnancy. This is the period of pregnancy during which placental formation takes place, and pregnancy loss at this stage may occur as a consequence of abnormal development of the fetoplacental unit. (Fetal abnormality may be another explanation.) Obtaining placental tissue at this time in on-going human pregnancies is clearly not possible, and therefore the mechanisms of abnormal development postulated to occur in pregnancies destined to miscarry are difficult to study. Various alternative approaches have been adopted to study the role of immune cells and molecules in the aetiology of recurrent miscarriage. These include the analysis of immune cell populations and cytokines in: (i) the peripheral blood of women who suffer RM and normal fertile women either before pregnancy or at the time of miscarriage (Hill *et al.*, 1995; Raghupathy *et al.*, 2000; Makhseed *et al.*, 2001); (ii) endometrial tissue obtained from women with RM and normal fertile women in the peri-implantation period in the non-pregnant state (Quenby *et al.*, 1999; Lim *et al.*, 2000; von Wolff *et al.*, 2000); and (iii) placental tissue obtained at the time of miscarriage from women with a history of RM, from women with a spontaneous, non-RM and from women requesting terminations of normal pregnancy (Lea *et al.*, 1995; Piccinni *et al.*, 1998; Vassiliadou and Bulmer, 1998a). While the study of placental tissue might appear to be the best approach, there are difficulties, particularly with respect to components of the immune system, in determining whether observed differences are due to pro-inflammatory events as a consequence of the miscarriage. More recently, comparisons have been made between placental tissue from women with unexplained RM with chromosomally normal and abnormal fetuses (Quack *et al.*, 2001; Yamada *et al.*, 2001); as both these groups of women have undergone similar miscarriage events, differences observed in the women with normal fetuses may be more use in gaining an understanding of the mechanism of RM. However, even in this case the differences seen may be due to different mechanisms of miscarriage resulting from embryonic-driven and maternally driven causes and a molecule or cell which is important in both causes would be missed. It has recently been suggested that the importance of chromosomal abnormalities in RM has been vastly underestimated (Quenby *et al.*, 2002). Determination of fetal karyotype in future studies will be needed to either support or refute this hypothesis.

### Immune cells

#### *Populations of immune cells in the endometrium and materno-fetal interface*

The population of leukocytes in human decidua and endometrium has been studied extensively (reviewed in Johnson *et al.*, 1999; Bulmer, 1995; 1996) and is distinctly different to that of peripheral blood. In particular there are essentially no B cells (Bulmer *et al.*, 1991; Johnson *et al.*, 1999) and very few neutrophils (Salamonsen and Lathbury, 2000). The population

consists mainly three cell types, namely T cells, macrophages and uterine natural killer (uNK) cells (also called large granular lymphocytes) (Bulmer, 1995; 1996; Johnson *et al.*, 1999). These uNK cells differ from the majority of NK cells found in the peripheral blood; the majority express CD56 and CD38, but not the classical T-cell or NK-cell markers CD3, CD4, CD8, CD16 and CD57 (Bulmer *et al.*, 1991). A small proportion of uNK cells are similar to the peripheral NK cells and show minimum expression of CD56. These are sometimes referred to as CD56<sup>dim</sup> cells, while the major population of uNK cells are called CD56<sup>bright</sup> cells. The numbers and proportions of each cell type vary through the menstrual cycle and in early pregnancy. T cells make up approximately 45% of leukocytes in the proliferative endometrium, and although their absolute numbers remain constant throughout the cycle and in early pregnancy their relative numbers decrease as the proportion of uNK cells increase (Bulmer *et al.*, 1991). Macrophages make up approximately 15–20% of endometrial leukocytes, their numbers increase slightly during the secretory phase of the cycle and early pregnancy so that they comprise 20% of leukocytes in the placental bed in early pregnancy (Bulmer, 1995). The CD56+ uNK cell numbers show the most dramatic menstrual cycle-dependent changes. During the proliferative phase of the cycle their numbers are approximately equal to those of T cells, but by the mid-secretory phase of the cycle they comprise 70% of the endometrial leukocytes and their numbers increase further during early pregnancy (King *et al.*, 1989; Bulmer *et al.*, 1991). The exact role of the uNK cells is not known. Initially, they were regarded as a potential threat to the fetus since early studies in mice showed increased numbers in abortion-prone animals (Baines and De Fougères, 1988). More recent studies however have shown that the placentae of NK-deficient mice are hypertrophic and result in fetal death (Guimond *et al.*, 1997), suggesting a positive role. In humans, CD56+ cells undergo apoptosis a few days prior to menstruation, but are maintained if pregnancy occurs and when rescued by hCG (King *et al.*, 1989; Koh *et al.*, 1995; Lea *et al.*, 1997), suggesting that they are important in menstruation and early pregnancy.

#### *Differences in immune cell population in RM patients*

##### *CD56+ cells*

Several studies have shown increased numbers of CD56+ NK cells in the peripheral blood of women with RM either prior to or during pregnancy compared with healthy fertile non-pregnant or pregnant controls (Aoki *et al.*, 1995; Kwak *et al.*, 1995). Studies have also shown that levels of peripheral blood CD56+ cells both prior to and during pregnancy can predict pregnancy outcome in women with RM (Coulam *et al.*, 1995; Emmer *et al.*, 2000). In contrast to normal fertile women, where peripheral CD56+ NK cell activity decreases during the first trimester of pregnancy, CD56+ NK cell activity remains high in women with RM (Higuchi *et al.*, 1995; Kwak *et al.*, 1995). Other studies have shown that the high CD56+ NK cell number and activity is only seen in pregnant RM women with chromosomally normal fetuses, thus suggesting that the presence of high CD56+ levels are a cause rather than an effect of RM (Coulam *et al.*, 1995; Yamada *et al.*, 2001).

However, another study has shown no differences in the levels of peripheral blood CD56+ cells in women undergoing missed miscarriage with chromosomally normal and abnormal fetuses (Yamamoto *et al.*, 1999a).

In contrast to the increased numbers of CD56+ cells in peripheral blood, a decreased number of decidual CD56+ NK cells are reported in the placental tissue from spontaneous miscarriages in RM women compared with tissue from spontaneous miscarriages in women without RM and women requesting termination (Yamamoto *et al.*, 1999b; Quack *et al.*, 2001). A decreased cytotoxic capability of decidual CD56+ NK cells in placental tissue from spontaneous aborters has also been shown (Vassiliadou and Bulmer, 1998a), though women with RM were not included in this study.

Two separate immunohistochemical studies have shown increased numbers of CD56+ cells in the non-pregnant endometrium of women with RM (Clifford *et al.*, 1999; Quenby *et al.*, 1999), and lower numbers were seen in women with RM who subsequently had a live birth compared with those who miscarried (Quenby *et al.*, 1999). This is in contrast to a flow cytometric study which showed similar numbers of CD56+ cells in the endometrium of women with RM and control subjects, although the women with RM did have increased numbers of endometrial CD56<sup>dim</sup> CD16+ cells compared with control subjects (Lachapelle *et al.*, 1996). However, the latter report suggested that CD3+ cells were the major leukocyte within the endometrial leukocyte population, both in women with RM and fertile controls. This is in disagreement with numerous in-vivo immunocytochemical studies (Bulmer 1995; 1996) and suggests that flow cytometry may not be the best means of studying these endometrial leukocyte populations.

Taken together, these results suggest that there are alterations in the CD56+ population of leukocytes in women with RM. However, whether these are increased or decreased depends on whether peripheral blood, first-trimester decidua or peri-implantation endometrium is analysed. CD56+ cells comprise <10% of peripheral blood leukocytes, and therefore these changes may not be significant to total peripheral blood cellular activity. The fact that there appears to be decreased numbers of CD56+ cells in the decidua and increased numbers in the endometrium is more difficult to explain, but could be due to the presence of two different populations of CD56+ cells, either CD16+ or CD16-. The increased number in the endometrium could be due to CD56+, CD16+ cells as suggested previously (Lachapelle *et al.*, 1996), while the decreased number reported in decidua could be the CD56+, CD16- population. The recent study showing increased numbers of CD16+ cells in early pregnancy decidua of women with RM (Emmer *et al.*, 2002) would support this.

#### CD3+ T cells

The second most abundant population of leukocytes within the endometrium and decidua are the CD3+ T cells. Studies have shown no differences in numbers of CD3+ T cells in the peripheral blood of RM and normal fertile women prior to pregnancy (Kwak *et al.*, 1995; Yahata *et al.*, 1998).

In contrast, one study has shown a significantly decreased number of CD3+ T cells in the peripheral blood during pregnancy in women with RM who subsequently miscarried compared with those who had a live birth and normal pregnant controls (Kwak

*et al.*, 1995). Two recent studies have investigated a subpopulation of CD3+ T cells that also express the CD56+ uNK cell marker. These studies have shown a decrease in the number of CD56+CD3+ cells in the peripheral blood prior to pregnancy (Yahata *et al.*, 1998; Yamamoto *et al.*, 1999b). No differences in the numbers of CD3+ T cells in endometrium from RM and control women have been reported (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999); neither were there any differences in numbers of CD3+ cells in early pregnancy decidua from normal fertile women and women with RM (Yamamoto *et al.*, 1999b; Quack *et al.*, 2001) and in decidua from normal pregnancies and after spontaneous abortion (Vassiliadou and Bulmer, 1998a). However, a decreased number of CD56+CD3+ cells in the decidua of women with RM compared with control women has been reported (Yahata *et al.*, 1998; Yamamoto *et al.*, 1999b).

T cells can also be classified according to protein components of their T-cell receptor (TCR) which are found in close association with the CD3 complex. The TCR consists of two polypeptide chains (either  $\alpha$  and  $\beta$  or  $\gamma$  and  $\delta$ ) which are held together by disulphide bonds. The majority of peripheral blood T cells express  $\alpha\beta$ , but  $\gamma\delta$  T cells are found in epithelial cell layers where they are thought to play a role in preventing the invasion of infectious agents. Extensive studies by Clark and colleagues have suggested that these different populations of T cells play important roles in successful pregnancy outcome in mice, with  $\alpha\beta$  cells being important immediately after implantation, and decidual  $\gamma\delta$  T cells being important in preventing recurrent abortions occurring after day 8.5 (Clark *et al.*, 2002). In abortion-prone mice the production of interleukin (IL)-10 and transforming growth factor  $\beta$ 2 (TGF $\beta$ 2) by V $\gamma$ 1.1 $\delta$ 6.3 T cells, which infiltrate into the decidua on day 8.5, has been shown to be important in preventing miscarriage (Clark *et al.*, 1997; Arck *et al.*, 1999). There is less evidence available to date for the importance of this mechanism in humans. The ratio of specific subpopulations of peripheral blood  $\gamma\delta$  T cells (V $\gamma$ 1, V $\delta$ 1 to V $\gamma$ 9V $\delta$ 2) is reported to be different in pregnant women with a history of RM compared with controls (Barakonyi *et al.*, 1999). Although several reports have suggested the presence and importance of  $\gamma\delta$  T cells in the human decidua (Szekeres-Bartho *et al.*, 2001), other investigations have shown that most human decidual T cells are  $\alpha\beta$  positive, with only 5–10% of T cells expressing  $\gamma\delta$  (Vassiliadou and Bulmer, 1998a).

The balance between the CD4+ and CD8+ T-cell subsets has also been investigated, and studies have shown a shift towards a higher CD4+/CD8+ ratio in endometrial biopsies from women with RM (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999).

Thus, although there appear to be no differences in the total T-cell numbers in women with RM, there may be differences in subpopulations of T cells which may be important.

#### Macrophages

No significant differences have been found in the number of macrophages in first-trimester decidua from women with RM either with chromosomally normal and abnormal fetuses, or in first-trimester decidua from spontaneous abortions and controls (Vassiliadou and Bulmer, 1996; Quack *et al.*, 2001). However, an increase in the number of macrophages in the non-pregnant endometrium of women with RM, together with an increased number of endometrial macrophages in RM women who

subsequently miscarried compared with those who had a live birth has been reported (Quenby *et al.*, 1999).

#### Cell activation markers

Differences in the absolute numbers of leukocytes may not reflect differences in the activity—and therefore function—of the cells. Perhaps a better understanding of the role of these cells in RM would be obtained from the measurement of their activities. The activation status of both T cells and CD56+ has been investigated by measurement of expression of CD25 (IL-2 R $\alpha$ ) (T-cell activation marker) and CD69 (NK-cell activation marker). Increased expression of CD69 on peripheral blood CD56+ (both CD56<sup>bright</sup> and CD56<sup>dim</sup>) of women with unexplained RM compared with normal controls has been reported (Ntrivalas *et al.*, 2001). These peripheral blood CD56+ cells from women with RM also expressed significantly greater CD69 when cells were co-cultured with trophoblast cell lines compared with CD56+ blood cells from controls. In a study which included a small number of women, levels of IL-2R $\alpha$  in peripheral blood obtained from women with RM in the first trimester of pregnancy were higher than those of healthy pregnant and healthy non-pregnant women (MacLean *et al.*, 2002). An increased number of CD25+ cells have been shown in the first-trimester decidua of women with RM with chromosomally normal fetuses compared with decidua from elective terminations and women with RM with chromosomally abnormal fetuses (Quack *et al.*, 2001). An increased number of CD25+ cells has also been shown in the first-trimester decidua of women undergoing spontaneous abortion compared with decidua from elective terminations (Vassiliadou *et al.*, 1999). Further studies are required on the activity of these cells in women with RM, not only with respect to the activation markers, but also as an investigation into functional parameters such as cytotoxicity and secretory activity.

#### Cytokines

Cytokines have traditionally been divided into families dependent upon the immune cell of origin and the immunological effects that they bring about. CD4+ T-helper cells are the major immune cells involved in cytokine production, and these can be divided into three functional subsets based on their cytokine production. Th1 cells produce interferon gamma (IFN $\gamma$ ), IL-2 and tumour necrosis factor beta (TNF $\beta$ ), and these are the main effectors of cell-mediated immune responses. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which are the main effectors of antibody-mediated humoral responses. The third T-helper cell population is that of the Th0 cells; these are precursor cells which can be converted to either Th1 or Th2 type cells and can produce both Th1 and Th2 cytokines as well as TNF $\alpha$  and granulocyte-macrophage colony-stimulating factor (GM-CSF). A further family of cytokines are the pro-inflammatory cytokines, such as IL-1, TNF $\alpha$ , IL-6 and leukaemia inhibitory factor (LIF); these are produced by macrophages and are involved in the inflammatory events associated with tissue damage and repair. It is now known that all these cytokines are also produced by cells other than immune cells, including the epithelial and stromal cells of the endometrium and the decidual and cytotrophoblast cells of the placenta. This is particularly pertinent to their potential role in reproductive failure, as T-helper cells are only a minor population of cells

within the secretory endometrium and first-trimester placental tissue and therefore may not be the main source of cytokines present in the fetoplacental unit. Cytokines act locally, and therefore measurements of amounts present in the fetoplacental unit post-implantation are of greater significance than measurements in the peripheral blood, or measurements prior to implantation. In addition, there is a great deal of interaction between different cytokines, and therefore differences in levels of single cytokines may be misleading. This is particularly true for the Th1 and Th2 cytokines where the relative amounts of each type of cytokine are important in determining the final response.

Studies in rodents, carried out by Thomas Wegmann and colleagues during the early 1990s, have provided strong evidence that successful pregnancy is associated with a predominant Th2 cytokine profile, and that Th1 cytokines are detrimental to pregnancy (Wegmann *et al.*, 1993). Many of the investigations in mice have been carried out with matings of CBA/J female mice with either DBA/2 or BALB/C males. CBA/J $\times$ DBA/2 matings result in a high loss of fetuses, while CBA/J $\times$ BALB/C result in normal pregnancy outcomes. Placental production of IFN $\gamma$ , TNF $\alpha$  and IL-2 is greater in the CBA/J $\times$ DBA/2 than the CBA/J $\times$ BALB/C cross (Tangri and Raghupathy, 1993), and peripheral blood lymphocytes from the abortion-prone mice produce more IFN $\gamma$ , TNF $\alpha$  and IL-2 when challenged than do similarly challenged cells from the non-abortion-prone mice (Tangri *et al.*, 1994). Injection of TNF $\alpha$ , IFN $\gamma$  or IL-2 into non-abortion-prone mice results in increased abortion rates (Chaouat *et al.*, 1990), while injection of IL-10 into abortion-prone mice decreases abortion rates (Chaouat *et al.*, 1995). It is postulated that the different cytokine profiles are due to the response of the female reproductive tract to the different paternal antigens presented by the DBA/2 and BALB/C mice (Raghupathy, 1997). More recently, it has been suggested that even in mice the Th1/Th2 hypothesis represents an oversimplification of the situation, and the importance of other cytokines has been acknowledged (Chaouat *et al.*, 2002). Nevertheless, these studies have provoked numerous studies to be conducted in humans.

#### Th1 and Th2 cytokines in human RM

The predominant maternal immune response during pregnancy is humoral rather than cell-mediated (Wegmann *et al.*, 1993). Cell-mediated autoimmune diseases such as rheumatoid arthritis are ameliorated during human pregnancy, while antibody-mediated diseases such as systemic lupus erythematosus are aggravated, indicating a weakening of the cell-mediated and an enhancement of the antibody response, which also correlates with a down-regulation of Th1-type activity and an enhancement of Th2-type reactivity.

The evidence for an abnormal Th1 cellular immune response to reproductive antigens in women with RM is less convincing, although some studies do support this hypothesis. Peripheral blood mononuclear cells (PBMCs) taken before pregnancy from women with RM produce TNF $\alpha$  and IFN $\gamma$  in response to stimulation from trophoblast cell extracts, while cells from non-pregnant women with normal reproductive histories and men produced IL-10 in response to incubation with trophoblast cell extracts (Hill *et al.*, 1995). Further studies have shown that this abnormal Th1-type response is not seen in women with RM with chromosomally abnormal fetuses, or in women with a uterine

structural abnormality (Hill *et al.*, 1995). Supernatants from PBMCs taken from women with RM were also toxic to mouse embryos, and the toxicity was related to the presence of IFN $\gamma$  in the supernatants. Recently, the toxicity to human embryos was shown to originate from the CD3+ and CD56+ populations of peripheral blood leukocytes (Polgar and Hill, 2002).

Other studies have shown that PBMCs obtained from women with unexplained RM at the time of miscarriage and stimulated with trophoblast cell extracts produce decreased amounts of IL-6 and IL-10 and increased amounts of IFN $\gamma$  compared with stimulated PMBCs obtained at the time of delivery in women with a normal reproductive history (Raghupathy *et al.*, 1999). Similar studies have also shown decreased production of IL-4, IL-5, IL-6 and IL-10 and increased production of IFN $\gamma$ , IL-2, TNF $\alpha$  and TNF $\beta$  in supernatants of phorbol-12-myristate-13-acetate (PMA) -stimulated PBMCs obtained from women with RM at the time of miscarriage compared with stimulated PBMCs obtained during the first trimester of ongoing pregnancies in women with a normal reproductive history (Raghupathy *et al.*, 2000). Unstimulated PBMCs isolated from non-pregnant women with unexplained RM have also been shown to produce more IL-2 than either unstimulated PBMCs from women with no history of reproductive failure, or normal males (Hamai *et al.*, 1998). However, a recent study of cytokine production by peripheral blood cells of women with RM taken during early pregnancy before miscarriage, has shown opposite effects with increased IL-4 and IL-10 and decreased IFN $\gamma$  in women with RM (Bates *et al.*, 2002). In contrast to other studies, the results of this study are not complicated by comparing results from blood samples taken with and without miscarriage and during the first trimester of pregnancy and at birth, both of which are likely to affect cytokine production.

Differences in Th1 and Th2 cytokine production by T-cell clones derived from the decidua of RM women and decidua from women undergoing termination are also reported to show differences in the production of Th1 cytokines (Piccinni *et al.*, 1998). Significantly lower levels of IL-4 and IL-10 were produced by the isolated decidual CD4+ clones obtained during miscarriage from RM women after stimulation with PMA and CD3 antibody compared with stimulated CD4+ clones isolated from decidua from women undergoing termination. IL-4 production by stimulated CD8+ T-cell clones was also significantly lower in cells from RM women compared with cells from women undergoing termination. However, although these cells originated from the decidua, they underwent considerable in-vitro manipulations (cloning and artificial stimulation) before cytokine measurement. In addition, the T-cell population from which these clones were derived comprises only a small percentage of cytokine-producing cells in the decidua.

Th1 and Th2 cytokine mRNA expression in the endometrium of normal fertile women and RM women during the peri-implantation phase of the menstrual cycle has also been investigated (Lim *et al.*, 2000). This study showed that fewer women with RM had detectable levels of IL-6 in their endometrium, but more had detectable levels of TNF $\alpha$ , IFN $\gamma$ , IL-2 and IL-12, compared with control fertile women.

### Pro-inflammatory cytokines

Several studies in mice have suggested the importance of pro-inflammatory cytokines, particularly LIF and IL-11, in successful

pregnancy outcome (Stewart *et al.*, 1992; Bilinski *et al.*, 1998; Robb *et al.*, 1998). Implantation does not occur in LIF knock-out mice, although transfer of homozygous LIF-negative blastocysts to pseudopregnant, wild-type mice results in normal implantation and pregnancy outcome (Stewart *et al.*, 1992). Endometrial LIF production is also decreased in women with unexplained infertility (Delage *et al.*, 1995; Laird *et al.*, 1997). Female mice with either an inactive or null mutation for the IL-11 receptor  $\alpha$  chain (IL-11R $\alpha$ ) are fertile, and their blastocysts implant and elicit an initial decidual response. However, only small decidua form and then subsequently degrade to result in pregnancy loss (Bilinski *et al.*, 1998; Robb *et al.*, 1998). The IL-1 family of proteins (IL-1 $\alpha$ , IL-1 $\beta$  and IL-1ra) are also thought to be important in successful pregnancy outcome. All three components are present in the endometrium and feto-placental unit and have been shown to be involved in the initial stages of implantation (Simon *et al.*, 1998).

### Endometrial expression of pro-inflammatory cytokines

Recently, immunocytochemical analysis has been used to compare expression of IL-1 $\alpha$ , IL-1 $\beta$ , LIF and IL-6 protein in the endometrium of women with RM and normal fertile women (Cork *et al.*, 1999). Immunostaining was compared in sections of endometrium obtained during the peri-implantation stage of the cycle from both groups of women. This study showed that, although some IL-1 $\alpha$  and IL-1 $\beta$  was expressed by stromal cells during the non-pregnant cycle, the epithelial cells are the major source of endometrial cytokines. The immunostaining for LIF and IL-6 was decreased compared with staining in sections from normal fertile women in 11/24 (46%) and 9/15 (60%) of RM women. Expression of IL-1 $\alpha$  and IL-1 $\beta$  was decreased in 5/12 (42%) and 2/10 (20%) of RM women respectively. The pattern of staining for IL-1 $\beta$  was extremely variable in the majority of biopsies from RM women, with some glands staining positive and others staining negative, and the luminal epithelium staining strongly. This pattern of expression was not seen in biopsies from normal women. These results agreed with those published elsewhere (von Wolff *et al.*, 2000), which showed decreased IL-1 $\beta$  and IL-6 mRNA expression in the endometrium of women with RM.

### Decidual cytokine production

One group (Piccinni *et al.*, 2001) has shown a decreased production of LIF by isolated decidual CD4+ clones obtained during miscarriage from women with RM after stimulation with PMA and CD3 antibody compared with stimulated CD4+ clones isolated from decidua from women undergoing terminations. These results suggest that decidual LIF might also be important in preventing miscarriage.

### Cytokine gene polymorphisms

Various studies have shown that polymorphism of cytokine genes influences cytokine production and may be associated with susceptibility to certain inflammatory and infectious diseases (McGuire *et al.*, 1994; Daser *et al.*, 1996; Wilkinson *et al.*, 1999; Mrak and Griffin, 2001). This has initiated the study of the presence of various cytokine gene polymorphisms in populations of women with RM. In particular, several reports have been made of various polymorphisms in the genes coding for members of the

IL-1 family of proteins (IL-1 $\alpha$ , IL-1 $\beta$  and IL-1ra). Three separate studies have shown no differences in distribution of a polymorphism created by a G→A base substitution at position +3594 in exon 5 of the IL-1B gene (which encodes IL-1 $\beta$ ) in women with RM compared with normal fertile controls (Helfer *et al.*, 2001; Reid *et al.*, 2001; Wang *et al.*, 2002). Although the polymorphism is not in the promoter region of the gene it has been shown to be in linkage disequilibrium with a polymorphism in the promoter region and is associated with elevated levels of IL-1 $\beta$  production *in vitro* (Pociot *et al.*, 1992). However, the results of another study (Helfer *et al.*, 2001) showed that there was no correlation between serum IL-1 $\beta$  levels and this IL-1B polymorphism in either normal fertile or RM women, suggesting that the polymorphism had little effect on IL-1 $\beta$  production *in vivo*. C→T base substitutions at positions -511 and -31 of the promoter region of the IL-1B gene have also been studied in women with RM. One study has shown an increased frequency of the IL-1B-511C and the IL-1B-31T alleles in women with unexplained RM compared with controls. There was also an increased frequency of the IL-1B-511C and IL-1B-31T alleles in women with RM whose PBMCs produced IFN $\gamma$  in response to trophoblast antigen stimulation compared with women with RM whose PMBCs did not produce IFN $\gamma$ ; this finding suggested an association with immune causes of RM (Wang *et al.*, 2002). However, another study on a larger number of women (130 compared with 59 in the study by Wang *et al.*) has shown no significant association between the IL-1B-511 polymorphisms and RM (Helfer *et al.*, 2002).

The variable number tandem repeat (VNTR) polymorphism (two to five repeats of a 86 bp sequence) in intron 2 of the IL-1RN gene (which encodes the IL-1ra protein) has also been associated with altered production of IL-1 $\beta$  in mononuclear cells (Santtila *et al.*, 1998). There are contradicting reports on the associations of this polymorphism with RM. One study has shown an increased frequency of allele 2 (which has two copies of the 86 bp sequence) in women with unexplained RM (Unfried *et al.*, 2001), while others have shown no significant difference in the distribution of the IL-1RN alleles in women with RM and control fertile women (Wang *et al.*, 2002).

Other studies have shown no significant associations between RM and alleles at the -308 position of the TNFA gene (which encodes TNF $\alpha$ ), +874 position of the IFNG (which encodes IFN $\gamma$ ) or the -1082 position of the IL-10 gene promoter in women with RM and normal fertile women (Babbage *et al.*, 2001; Reid *et al.*, 2001).

These polymorphism studies have proved disappointing. Many have shown no significant correlation between alleles that might be expected to affect cytokine levels and RM, and for those that do show significant correlation there are other studies which show opposite results. This is a common phenomenon in allele association studies that are sensitive to selection criteria for patients groups and controls. Taken together, the studies reported do not support the hypothesis that genetic factors are a major determinant of cytokine production at the fetomaternal interface during pregnancy.

#### Other cytokines

Studies in mice have also suggested that GM-CSF and CSF-1 (also known as macrophage colony-stimulating factor, M-CSF)

are important in successful pregnancy outcome (Pollard *et al.*, 1991; Robertson *et al.*, 1999). Implantation is compromised in CSF-1 mutant mice (op/op mice) which have both a lower rate of implantation and fetal viability, both of which can be restored to normal by administration of exogenous CSF-1 (Pollard *et al.*, 1991). Decreased expression of utero-placental CSF-1 mRNA has also been shown in mice with spontaneous and induced pregnancy loss compared with control mice (Gorivodsky *et al.*, 1999). Decreased CSF-1 production by isolated decidual CD4+ clones obtained during miscarriage from women with RM after stimulation with PMA and CD3 antibody compared with stimulated CD4+ clones isolated from decidua from women undergoing termination has also been shown (Piccinni *et al.*, 2001). Abnormal placental function and development has been reported in GM-CSF-deficient mice (Robertson *et al.*, 1999); however, to date there are no reported studies on this cytokine in women with RM.

Levels of TGF $\beta$ 1 in the blood of women with RM at the time of miscarriage are reported as being significantly higher than levels in the blood of gestational week-matched normal women. In addition, levels of TGF $\beta$ 1 in women with a history of several consecutive first-trimester miscarriages were approximately 3-fold higher than those in non-pregnant controls (Ogasawara *et al.*, 2000). In contrast to increased peripheral TGF $\beta$ 1 blood levels, a deficiency in decidual lymphoid cells which produce TGF $\beta$ 2-suppressing activity has been reported in a subset of women with RM (Lea *et al.*, 1995).

#### MHC molecules

##### HLA expression on trophoblast cells

Recognition of foreign cells occurs due to the expression of MHC molecules on the cell surface, and the maternal immune system should recognize fetal trophoblast cells as foreign if they express paternal MHC molecules. Fetal extra-villous cytotrophoblast cells do not express the classical MHC I molecules, HLA-A and HLA-B, and MHC class II molecules are also absent. Instead, they express the non-classical HLA-G and E molecules, together with low expression of HLA-C (King *et al.*, 1996; Le Bouteiller, 2000; van der Ven, 2000). Expression of HLA-G is particularly interesting because its expression is almost entirely limited to trophoblast cells, and is specifically expressed in invasive extra-villous cytotrophoblast cells, fetal endovascular endothelial cells and amnion cells (Le Bouteiller *et al.*, 1999), which are exactly the cells that will come into contact with the maternal immune system. Unlike other HLA genes, HLA-G shows an almost complete lack of polymorphism in its nucleotide sequence, which means that the HLA-G protein is essentially invariant in the human population (Bainbridge *et al.*, 2000), and because of this the maternal immune system is unlikely to recognize the trophoblast cells as foreign. In contrast, complete lack of expression of MHC class I molecules by a trophoblast cell might expose it to attack from NK cells.

Although HLA-G shows little polymorphism, its mRNA undergoes alternative splicing to produce five main forms of the molecule. HLA-G1 is the full-length form, which consists of  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3 domains with a cytoplasmic transmembrane tail. HLA-G2 consists of the  $\alpha$ 1 and  $\alpha$ 3 domains, while HLA-G3 contains

only the  $\alpha 1$  domain and HLA-G4 contains the  $\alpha 1$  and  $\alpha 2$  domains together with the cytoplasmic tail. A soluble form of the HLA-G1 molecule also exists which has a modified cytoplasmic tail sequence that allows secretion from the cell. A recent study has suggested that only HLA-G1 is expressed on the surface of the cell and is therefore of physiological significance (Bainbridge *et al.*, 2000).

HLA-G is also expressed by human embryos (Jurisicova *et al.*, 1996), and recent measurements of sHLA-G in culture supernatants of early embryos obtained by IVF before transfer have shown that its presence appears to be essential for successful pregnancy outcome (Fuzzi *et al.*, 2002).

The exact role of HLA-G in embryo implantation and fetoplacental development is not clear. HLA-G has been shown to be involved in cellular adhesion (Odum *et al.*, 1991), suggesting that it may play a role in attachment of the blastocyst to the endometrial epithelial cells. Its selective expression by extra-villous trophoblast cells, which are highly invasive and eventually invade the uterine arterial system and replace the endothelial cells lining the blood vessel walls, suggests that it plays an important role in the control of trophoblast invasion (Le Bouteiller, 2002). HLA-G-expressing cells are known to come into close contact with decidual immune cells, particularly uNK cells (Emmer *et al.*, 2002). Expression of HLA-G antigens has also been shown to protect cells from NK-cell-mediated lysis (Hunt *et al.*, 2000), suggesting that its expression may prevent NK cells attack on trophoblasts (Le Bouteiller, 2000). (See also section on possible mechanisms of NK-cell-mediated miscarriage.)

**HLA-G expression in women with RM**

A recent study has shown decreased expression of HLA-G immunostaining in extra-villous and endovascular cytotrophoblast tissue obtained after miscarriage compared with cytotrophoblast staining in tissue obtained from healthy pregnancies (Emmer *et al.*, 2002). This finding is in agreement with the results of a previous study which showed decreased HLA-G mRNA expression in women with RM who received immunotherapy compared with tissue from elective terminations (Fan *et al.*, 1999). However, the decreased expression seen in this study may have been due to the immunotherapy treatment.

Although polymorphisms of the HLA-G are limited, a number have been described and their presence in populations of women with RM have been studied. Three polymorphisms have been

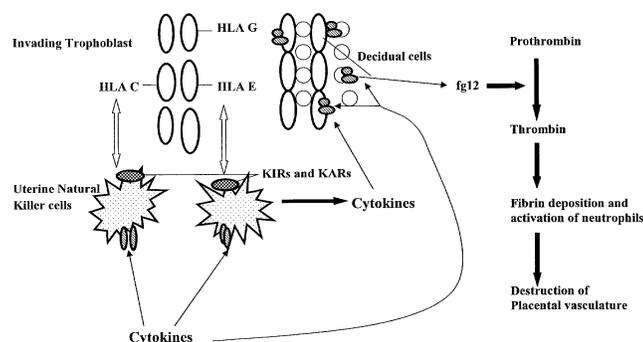
described which result in changes in amino acid sequences. The \*0103 polymorphism results in a Thr→Ser substitution at codon 31, while the \*0104 polymorphisms results in a Leu→Ileu replacement at codon 110. These are relatively conservative amino acid substitutions with respect to amino acid polarity and would not be expected to interfere with HLA-G structure and function. The \*0105 polymorphism is a frameshift mutation due to a single base deletion at codon 130, and leads to instability of HLA-G1 due to the loss of a disulphide bridge between residues 101 and 164 (van der Ven *et al.*, 2000). The presence of these various isoforms is associated with differences in levels of soluble HLA-G which is considered a useful indicator of HLA-G expression. \*0103 and \*0105 are associated with decreased HLA-G levels, while the presence of \*0104 is associated with increased expression (Rebmann *et al.*, 2001). Although earlier studies on small populations suggested that there are no differences in HLA-G polymorphisms in women with RM (Karhukorpi *et al.*, 1997; Penzes *et al.*, 1999), more recent studies have shown an association of the HLA-G\*0105N (van der Ven *et al.*, 2000; Aldrich *et al.*, 2001), the HLA-G\*0104 (Aldrich *et al.*, 2001) and the HLA-G\*0103 (Pfeiffer *et al.*, 2001) with repeated pregnancy loss in populations of women with RM.

**Possible mechanisms of pregnancy failure**

Although differences in expression of the various immune cells and cytokines described above have been shown in women with RM, little is known about the way in which their abnormal expression brings about pregnancy loss. Three different mechanisms have been postulated for their role in recurrent pregnancy loss: (i) increased activity of uNK cells and/or macrophages, resulting in attack on the invading trophoblast cells; (ii) direct effects of cytokines on trophoblast cells; and (iii) effects of cytokines on thrombotic events in the vasculature, resulting in decreased blood flow. These mechanisms are illustrated in Figure 1.

**Natural killer cell activation**

The trophoblast is resistant to killing by cytotoxic T lymphocytes, conventional NK cells and macrophages, and freshly prepared uNK cells are unable to lyse various cell types including trophoblast cells (Loke and King, 1991). The cytolytic activity of these NK cells is controlled by the interaction of cell-membrane receptors with their ligands. Three families of NK cell receptors have been described. The first family, killer inhibitory receptors (KIRs) and killer activatory receptors (KARs), belong to the immunoglobulin superfamily. Binding of ligands to KIRs inhibits cytolysis, while binding of ligand to KARs triggers cytolysis. The second receptor family consists of CD94/NKG2 heterodimers, while the third is the immunoglobulin-like transcript (ILT) family. While KIRs and KARs and CD94/NKG2 heterodimers are found exclusively on NK cells, ILT receptors are also found on macrophages and B cells (Loke and King, 2000). The ligands for these various molecules are the HLA class I molecules on target cells; for trophoblast cells this will be HLA-G, HLA-C or HLA-E. The lack of killer activity by uNK cells and decidual macrophages is postulated to be due to the interaction of these ligands with inhibitory receptors (Loke and King, 1997). One mechanism of pregnancy loss may therefore be



**Figure 1.** Possible roles of cytokines and immune cells in causing miscarriage. KIR = killer inhibitory receptor; KAR = killer activatory receptor.

due to a combination of paternal HLA-G, HLA-E or HLA-C molecules and maternal activation receptors that results in cell activation rather than inactivation.

Cytokines have also been postulated to play a role in uNK cell activation, and the receptors for various cytokines including IL-1, IL-2 and TNF $\alpha$  are found on uNK cells (Saito *et al.*, 1996; Jokhi *et al.*, 1997). It was originally proposed that the way in which Th1 cytokines brought about miscarriage was via activation of uNK cells (Wegmann *et al.*, 1993). In humans, IL-2 has been shown *in vitro* to induce cytotoxicity against trophoblast in uNK cells (Ferry *et al.*, 1991), and IL-4 inhibits the expression of IL-2R $\alpha$ , IL-2R $\beta$  and IL-2R $\gamma$  by decidual NK cells, thus preventing them reacting with IL-2 (Saito *et al.*, 1996).

Human uNK cells are also known to produce a variety of cytokines including CSF-1, TNF $\alpha$ , IFN $\gamma$ , IL-1 $\alpha$ , TGF- $\beta$ , LIF and GM-CSF (Saito *et al.*, 1993; Jokhi *et al.*, 1994; 1997), suggesting that the major function of activated uNK cells is the production of cytokines rather than cell lysis (Loke and King, 2000). These cytokines may affect trophoblast cell growth and function directly, or they may cause activation of macrophages which could attack the trophoblast (Hunt and Robertson, 1996). Incubation of uNK cells with K562 or B- lymphoblast cell lines transfected with HLA-G results in altered cytokine production compared with incubation with non-transfected cells, suggesting that interaction of trophoblast HLA-G with KIRs and KARs on uNK cells may alter cytokine production. These cytokine responses may be different in women with RM and normal fertile women, and this may result in pregnancy loss (Kanai *et al.*, 2001; Rieger *et al.*, 2002).

Although the cytolytic activity of decidual NK cells has been compared in tissue from spontaneous miscarriage and elective terminations (Vassiliadou and Bulmer, 1998b), few studies have compared the function and activity of decidual or endometrial uNK cells in women with RM and normal fertile women. This may be due to difficulties in obtaining adequate decidual or endometrial tissue samples for the preparation of the active forms of these cells from women with RM. However, such studies would aid our understanding of the role of these cells in the mechanism of pregnancy failure. These studies could include comparison of: (i) cytokine production by CD56+ cells; (ii) expression of the various KIRs, KARs and other activity receptors by CD56+ cells; and (iii) cytolytic activity of CD56+ cells from women with RM and control women.

#### **Direct effect of cytokines on trophoblast cell growth and function**

The receptors for the various cytokines postulated to show abnormal production in RM women, including those for IL-1, IFN $\gamma$ , TNF $\alpha$ , TGF- $\beta$ , CSF-1, GM-CSF, LIF, IL-4 and IL-6, are present on trophoblast cells (Mitchell *et al.*, 1991; Hampson *et al.*, 1993; Yelavarthi and Hunt, 1993; Simon *et al.*, 1994; de Moraes-Pinto *et al.*, 1997; Jokhi *et al.*, 1997; Sharkey *et al.*, 1999) showing that cytokines have the potential to directly affect trophoblast cell growth and function.

Various studies have shown that TNF $\alpha$  and IFN $\gamma$  can inhibit human placental trophoblast cell growth and metabolic activity and stimulate apoptosis *in vitro* (Yui *et al.*, 1994; Ho *et al.*, 1999; Knofler *et al.*, 2000). The pro-apoptotic effects of TNF $\alpha$  and IFN $\gamma$  are completely neutralized by epidermal growth factor (EGF) and partially neutralized by basic fibroblast growth factor (bFGF),

insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) (Smith *et al.*, 2002), showing that it is the balance of cytokines which is important. A decreased expression of TNF $\alpha$  and in the pro-apoptotic bcl-2/bax ratio in syncytiotrophoblasts from first-trimester placenta from failing pregnancies (both sporadic and recurrent) has been reported, suggesting that altered apoptosis of trophoblast cells may be important (Lea *et al.*, 1999).

IL-4, IL-6 and LIF have been shown to stimulate hCG secretion by trophoblast cells (Sawai *et al.*, 1995; Saito *et al.*, 1997). LIF is also proposed to drive differentiation of cytotrophoblast cells away from syncytiotrophoblast towards a non-invasive extravillous trophoblast type with decreased production of matrix metalloproteinases (MMPs) (Bischof *et al.*, 1995; Nachtigall *et al.*, 1996). IL-1, IL-6 and TNF $\alpha$  have also been shown to stimulate MMP-2 and MMP-9 activity in cytotrophoblast cells (Meisser *et al.*, 1999a,b). TGF $\beta$  is known to affect growth and differentiation of first-trimester trophoblast cells (Graham and Lala, 1991; Morrish *et al.*, 1991), and also inhibits integrin expression, MMP-9 production and hCG secretion by human trophoblast cells *in vitro* (Morrish *et al.*, 1991; Irving and Lala, 1995; Song *et al.*, 1996; Meisser *et al.*, 1999b).

CSF-1 is known to stimulate trophoblast cell proliferation (Jokhi *et al.*, 1995; Hamilton *et al.*, 1998). Both CSF-1 and GM-CSF have also been shown to stimulate differentiation of cytotrophoblast cells into syncytium *in vitro* (Garcia-Lloret *et al.*, 1994). CSF-1 also stimulates hCG, human placental lactogen, MMP-2, tissue inhibitor of metallo proteinase (TIMP)-1, fibronectin and the  $\alpha_5 \beta_1$  integrin expression by various types of cytotrophoblast cells (Garcia Lloret *et al.*, 1994; Hamilton *et al.*, 1998; Omigbodun *et al.*, 1998). GM-CSF also stimulates MMP-9 and hCG production by trophoblast cells (Garcia Lloret *et al.*, 1994; Meisser *et al.*, 1999b).

Thus, the abnormal production of any of these cytokines in women with RM may lead to abnormal placental growth and function and subsequent miscarriage.

#### **Activation of thrombotic events**

Recent studies have suggested that the way in which Th1 cytokines bring about pregnancy loss is via up-regulation of a newly described pro-coagulant, fg12. In mice, anti-fg12 antibodies completely prevent spontaneous abortion and dramatically reduce the effects of TNF $\alpha$  and IFN $\gamma$  on abortion rates (Clark *et al.*, 1998), while TNF $\alpha$  and IFN $\gamma$  have been shown to up-regulate the production of fg12 by both fetal trophoblast and maternal decidua (Clark *et al.*, 2001a). In humans, increased expression of fg12 in trophoblast cells from failing pregnancies with chromosomally normal embryos, but not in trophoblast tissue from chromosomally abnormal embryos, have also been reported (Clark *et al.*, 1999; Knackstedt *et al.*, 2001). Fg12 converts prothrombin to thrombin, which in turn leads to deposition of fibrin and activation of polymorphonuclear leukocytes that can destroy the vascular supply to the placenta (Clark *et al.*, 2001b).

#### **Future studies**

Although the evidence presented in this review suggests that there may be differences in the some immune cells and molecules in women with RM, the findings are often contradictory. Whilst

much of this variation occurs due to difficulties in obtaining the optimum tissue, some will be due to study design. A population of women with unexplained RM is likely to comprise subsets with RM of different aetiologies. As most published studies only involve a small number of women it is possible that some differences are also due to the fact that different populations of women with RM have been selected. This is inevitable because the recruitment of patients is difficult. However, other factors which might account for differences are the compartment (peripheral blood, endometrium or decidua) in which the cells or molecules are measured. The measurement of factors such as cytokines (which are known to act locally) in peripheral blood may have little significance as this compartment is far removed from where the important interactions are taking place. In addition, the peripheral blood cell population is considerably different to that in the endometrium and decidua. The timing of sampling is also important, both with respect to the point in the menstrual cycle and pregnancy and whether it is at the time or just after miscarriage, as both of these factors will affect the expression of these cells and molecules. The population of RM women should also be clearly defined. Some studies have included women with only one or two miscarriages, which is not an accepted definition of RM, whereas in other cases it is unclear whether the studied population fits into the unexplained RM group. Karyotyping of the fetus is also important, and should be carried out in future studies so that miscarriages which result from chromosomally abnormal pregnancies can be considered separately from those resulting from chromosomally normal pregnancies. Care should also be taken in extrapolating information obtained from studies in mice to the human situation, as the implantation process is very different in these two species.

## Conclusions

Although much of the evidence obtained is contradictory, the results of these studies do suggest that differences exist in the expression of some immune cells and molecules in women with RM. There appears to be differences in the CD56+ population of cells, and there is also some evidence for an alteration in the ratio of Th1 and Th2 cytokines produced by PBMCs and clones of decidual CD4+ cells. There is also some evidence for differences in endometrial cytokine production, and in particular a decreased production of pro-inflammatory cytokines such as IL-6. The exact way in which the abnormal expression of these cells and molecules results in miscarriage in humans is not clear, but experiments in mice have suggested that there may be an initiation of coagulation or abnormal interactions between HLA molecules and uNK cells.

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