

Killer immunoglobulin-like receptors (KIRs) and HLA-C allorecognition patterns implicative of dominant activation of natural killer cells contribute to recurrent miscarriages

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BACKGROUND: Decidual natural killer (NK) cells play key developmental roles at the feto–maternal interface. Individual differences in NK-cell interactions are dependent on the combinations of variable killer immunoglobulin-like receptor (KIR) and HLA class-I gene products. As different receptor–ligand interactions may result in altered NK-cell-mediated immunity against pathogens, it is proposed that the relationship between these genes may be important in a state such as recurrent miscarriage (RM). We had earlier reported that the predisposition to RM is influenced by the maternal KIR gene content. In the present study, we have attempted to extend our findings in the light of contribution from the paternal antigens on the outcome of pregnancy, since maternal NK cells may potentially encounter non-self-paternal HLA-C alleles on trophoblasts. All HLA-C allotypes fall into two major KIR epitopes—C1 (HLA-C*01/*03/*07/*08/*12/*14/*16) and C2 (HLA-C*02/*04/*05/*06/*15/*17/*18)—on the basis of a dimorphism at position 80 of the α I domain.

METHODS: PCR-sequence specific primer-based genotyping was used to determine the maternal KIR gene content and HLA-C genotypes down to allele level in couples experiencing RM and controls.

RESULTS: KIR2DL1 with both partners homozygous for HLA C2 was significantly higher in control couples when compared with the patients [$P = 0.0004$, odds ratio (OR) = 0.28, 95% confidence interval (CI) = 0.13–0.58]. The activating KIR2DS2 with both partners homozygous for HLA C1 was significantly higher in patients when compared with the controls ($P = 0.002$, OR = 2.83, 95% CI = 1.47–5.40).

CONCLUSIONS: Our results represented the ‘top-end’ of the activation spectrum of KIR-HLA-C compound genotype for NK cells and this may contribute to the immunological etiology of RM.

Key words: recurrent miscarriages / natural killer cells / KIR-HLA-C allorecognition / pregnancy

Introduction

Recurrent miscarriage (or RM) is the loss of three or more consecutive pregnancies, affecting ~1% of couples trying to conceive. RM remains unexplained in ~80% of cases, and despite the paucity of supporting evidences (Rai and Regan, 2006), the idea of RM having an immunological etiology is widespread. Natural-killer (NK)-like cells (large granulated lymphocytes with the CD3[−]CD16[−]CD56^{bright}) phenotype are the dominant decidual cell population from the first stages of pregnancy (King et al., 1996). Enhanced responsiveness of CD56^{bright} NK cells to stimulation of CXCR4 and CCR5 receptors by means of ligands secreted locally by cells in the decidua underlies

the recruitment of this subset to the decidua (Hanna et al., 2003). The possibility that decidual NK (dNK) cells interact with the fetal trophoblast is suggested by the observations that dNK cells have a series of receptors including the killer immunoglobulin (IgG)-like receptor (KIR) family and CD94/NKG2 family (Hiby et al., 1997). The ligands for these receptors include HLA-C, HLA-E and HLA-G, which are expressed on the trophoblast cells (Moffet and Loke, 2006). Of these, only HLA-C is highly polymorphic and interacts with the KIR expressed on the NK cell surface. The precise role of dNK cells is not clear; there are evidences that suggest their involvement in maternal blood vessel remodeling (Croy et al., 2003). Hanna et al. (2006) have demonstrated that the dNK cells regulate key

developmental processes at the human fetal–maternal interface, including trophoblast invasion by production of interleukin (IL)-8, interferon-inducible protein-10 and several chemokines. dNK cells are potent secretors of an array of angiogenic factors and induce vascular growth in the decidua. Such functions are regulated by specific interactions between dNK activating and inhibitory receptors and their ligands, uniquely expressed at the fetal–maternal interface. Moreover, Sharkey *et al.* (2008) have demonstrated the selective regulation of KIR expression over time *in vivo* in a normal physiological situation and suggested that KIR expression by dNK cells is regulated by the tissue microenvironment in the decidua.

KIR haplotypes on chromosome 19q13.4 are highly polymorphic, but can broadly be classified as group A or B. The classification is based on the number and type of genes encoding inhibitory and activating KIRs. The simpler group-A KIR haplotype comprises a fixed gene content of KIR3DL3-2DL3-2DL1-2DPI-3DPI-2DL4-3DL1-2DS4-3DL2. Haplotypes carrying any other combination of KIR loci are classified as group-B haplotypes (Rajalingam *et al.*, 2008). When considering HLA-C molecules as ligands for KIR receptors on NK cells, all HLA-C allotypes can be grouped into two major KIR epitopes: C1 (HLA-C*01/*03/*07/*08/*12/*14/*16) and C2 (HLA-C*02/*04/*05/*06/*15/*17/*18), on the basis of a dimorphism at position 80 of the α 1 domain. Group C1 (HLA-C^{asn80}) are ligands for inhibitory KIR2DL2/3 and activating KIR2DS2, and group C2 (HLA-C^{lys80}) are ligands for inhibitory KIR2DL1 and activating KIR2DS1 (Mandelboim *et al.*, 1996; Boyington and Sun, 2002; Igarashi *et al.*, 2004). The ligand-binding specificities of these inhibitory receptors are well characterized, but similar studies of the activating receptors have met with limited success (Vales-Gomez *et al.*, 1998; Winter *et al.*, 1998; Saulquin *et al.*, 2003), although clinical correlations point to their interaction with HLA class I (reviewed in Rajagopalan and Long, 2005). Certain combinations of HLA-C with KIR2DS1 and KIR2DS2 have been shown to correlate with autoimmune conditions (Yen *et al.*, 2001; Martin *et al.*, 2002; van der Slik *et al.*, 2003; Nelson *et al.*, 2004; Shastry *et al.*, 2008), leukemia (Middleton *et al.*, 2009) and inflammatory diseases (Boyton *et al.*, 2006).

Through the interaction with inhibitory KIRs, HLA-C molecules are able to modulate NK-cell function. Recent studies conducted in mice (Kim *et al.*, 2005) and humans (Anfossi *et al.*, 2006; Yu *et al.*, 2007; Fauriat *et al.*, 2010) have established that in order to become functional, the NK cells undergo a process of education and licensing during the course of their development through engagement of their inhibitory and/or activating KIRs with their cognate HLA class I ligands. NK cells with, for example, KIR2DL1 are only functional in individuals with group C2 alleles, but preferentially react against C2 negative targets. This ligand–receptor pairing is thus unusual in the immune system in-so-far as inheritance of different combinations of these polymorphic germ line sequences in populations imparts differential connectivity to NK-cell activation, with different effector cell outcomes capable of exerting a strong impact on disease susceptibility. The hypothetical framework for our study is that idiopathic RM, generally considered a disease of unknown etiology, may involve a genetic susceptibility to inappropriate or dysregulated NK cell activity in the decidua. Individual differences in NK-cell interactions are dependent on the combinations of variable KIR and HLA class-I gene products. As different receptor–ligand interactions may result in altered NK-cell-mediated immunity against pathogens, it is proposed that the relationship between these

genes may be important in a state such as RM. Moreover, it could be hypothesized that as there is a considerable genetic variation in the repertoire of KIR as well as polymorphic HLA-C antigens between different individuals, a particular KIR-HLA-C allorecognition pattern might influence the outcome of pregnancy.

In an attempt to characterize maternal KIR gene content with respect to the risk of RM, we have previously reported that the increased activating and decreased inhibiting KIR gene repertoire may have a predisposing effect on RM (Faridi *et al.*, 2009). In the present study, we have attempted to extend our findings in the light of contribution from the paternal antigens on the outcome of pregnancy, since maternal NK cells may potentially encounter non-self-paternal HLA-C alleles on trophoblasts. We have analyzed KIR-HLA-C allorecognition patterns in the light of recently described NK cell education theory, in a pool of well-characterized RM couples ($n = 177$ pairs) who have undergone at least three spontaneous early pregnancy losses of unknown etiology, with no successful live birth and compared the results with a group of 200 normal couples having at least two live births and with no history of previous miscarriages, pre-eclampsia, ectopic pregnancy or any other pregnancy-associated problems.

Materials and Methods

Genomic DNA samples

All the samples were collected from patients attending the out-patients' department of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, and Queen Mary Hospital of CSM Medical University, Lucknow (Uttar Pradesh) India, for the evaluation of RM. All of them had a history of at least three spontaneous miscarriages (mean 4, range 3–7) and no previous successful pregnancy. All the patients were screened for various known causes of miscarriages, including parental chromosomes; Day 2 hormone levels of FSH (3–11 U/l), LH (3–12 U/l) and testosterone (0.5–3 nmol/l); antiphospholipid antibodies including lupus anticoagulant (positive likelihood ratio, 0.8–1.05) and anticardiolipin antibodies (IgG 0–12 IgG anticardiolipin units, immunoglobulin M 0–5 IgM anticardiolipin units) and prothrombotic risk factors including activated protein-C resistance (2.6–4.36 ratio), factor V Leiden and prothrombin mutations; investigation of luteal phase insufficiency; prolactin dosage; glycaemic curve, thyroid hormone levels; investigation of toxoplasmosis, cytomegalovirus, rubella, HIV, group B *Streptococci*, *Chlamydia trachomatis*, hepatitis B and C and bacterial vaginosis. The uterine cavity was investigated for cervical incompetence by hysteroscopy, hysterosalpingography and serial ultrasound. Of all the initially screened individuals, 39% ($n = 205$) had no known cause of RMs. Among these, there were a limited number of cases where the male partner consented to participate in this study. This limited our sample size and therefore the study has been conducted on 177 RM couples. All RM patients selected were primary aborters, not having any live child and belonged to four different caste groups, namely upper-caste Hindus (Brahmin, Vaishya, Kayastha and Kshatriya), backward class (OBC and SC/ST), Muslims (Shiya and Sunni) and others (Sindhi, Sikh, Jain and Christians). Number of patients in each of the caste populations was determined and limited by the stringent inclusion criterion of the RM patients. The control group consisted of 200 healthy parous couples having at least two live births and with no history of miscarriage, pre-eclampsia, ectopic pregnancy or preterm delivery. The inclusion of control couples' group was statistically matched for the ethnic distribution with the patients' group (Faridi *et al.*, 2009; Parveen *et al.*, 2010) and the differences in frequencies between the cases and controls for each caste group was analyzed for statistical significance at the

95% confidence interval (CI), using Fisher's exact test with Bonferroni correction. Blood samples (5 ml) from controls, RM women and their partners were collected in EDTA-coated collection vials, and DNA was extracted using Qiagen DNA extraction kits. This study was approved by the ethics committees of SGPGIMS and Queen Mary Hospital, CSM Medical University, Lucknow. Written informed consent to participate in this study was obtained from all the individuals.

KIR and HLA-C genotyping

Genotyping of maternal KIR genes was carried out by the PCR-sequence specific primer (PCR-SSP) technique as described previously (Faridi et al., 2009). In this study, we have additionally typed those women for their KIR gene content whose partners had also consented to participate in this study and excluded those who had no husband sample to analyze. High-resolution PCR-SSP genotyping of HLA-C was carried out as described by Bunce et al. (1996) for all participating women and their partners, wherein each sample was subjected to a set of 47 SSP reactions. The reliability and specificity of this system was established by confirming the genotype of random and dubious samples by commercially available PCR-SSP kits (AllSet™ Invitrogen, Madison, WI, USA).

Statistical analysis

Gene and genotype frequency of KIR was determined by direct counting. Frequency differences between the RM and control groups for KIR2DL1, 2DL2, 2DL3, 2DS1, 2DS2, 2DS4 and 2DS5 were tested for significance at 95% confidence limits using Fisher's exact test with Bonferroni correction. Allele and genotype frequencies of HLA-C were determined similarly, and were grouped into C1 and C2 groups prior to analysis. Statistical analysis of KIR–ligand interactions was based on the presence of a putative HLA-C genotype of both the partners in the presence of its cognate maternal KIR. The magnitude of the effect was estimated by odds ratios (ORs) and their 95% CIs (Windows 11.0.0.2001; SPSS Inc.). The significance levels of average inhibitory or activating KIR–ligand interactions between RM patients and control couples were tested, and a linear model with a logistic link was used to test the association between increasing/decreasing extent of activating/inhibiting interactions and the prevalence of RM. $P \leq 0.05$ were considered significant.

Results

Using sequence-specific PCR amplification, we have determined the maternal KIR and parental HLA-C genotypes of 177 couples experiencing RM and 200 healthy parous couples. Carrier frequencies of

individual KIR genes were determined previously (Faridi et al., 2009). In the present study, we have additionally typed those women whose partners consented to participate in the study and excluded those who had no husband sample available for analyses. We observed little or no deviation in the maternal KIR genotype patterns from that reported previously. Here, we present only those variable KIRs that are specific for HLA-C alleles. The frequencies of KIR2DL1, 2DL2, 2DL3, 2DS1, 2DS2, 2DS4 and 2DS5 in RM women and controls, and their statistical associations are presented in Table I. Furthermore, we have analyzed the results of HLA-C genotyping in combination with the maternal KIRs that recognize HLA-C alleles.

Frequency distribution of HLA-C among RM and control couples

Upon analyzing the frequency of HLA-C groups C1 and C2 in RM and control couples, we observed that the HLA-C1 and C2 frequencies in RM and control women and their partners were not significantly different (Table II). We have evaluated whether individual HLA-C alleles were associated with RM. All women and their partners were typed by high-resolution PCR-SSP. There were no significant differences in the allele frequencies in RM couples ($n = 177$ pairs) when compared with the control couples ($n = 200$ pairs).

Combinatorial effects of KIR and their putative HLA-C ligands

Frequencies of parental HLA-C genotypes in the presence of their cognate maternal KIR in cases and control groups were analyzed for statistical association with RM (Table III). Upon analyzing various KIR–HLA combinations, we found that there was a significantly higher proportion of control group pairs that was characterized by the presence of maternal inhibitory KIR2DL1 with both the partners being homozygous for HLA-C2 when compared with the patient group pairs ($P = 0.0004$, $OR = 0.28$, $95\% \text{ CI} = 0.13–0.58$), whereas maternal 2DL1 without HLA-C2 (both partners homozygous for HLA-C1) was represented significantly more often by the patient couples when compared with the control couples ($P = 0.018$, $OR = 2.06$, $95\% \text{ CI} = 1.13–3.74$). On the other hand, the activating KIR2DS2 with homozygous HLA-C1 in both partners was significantly higher in patients when compared with the controls ($P = 0.002$,

Table I KIR carrier frequencies among patients and controls (KIRs specific for HLA-C are shown).

S. no	Gene	Control (n = 200)	Patients (n = 177)	P value*	OR	95% CI
1	KIR2DL1	0.95	0.69	<0.0001	0.11	0.04–0.31
2	KIR2DL2	0.49	0.54	0.571	0.81	0.46–1.42
3	KIR2DL3	0.84	0.82	0.851	1.15	0.55–2.41
4	KIR2DS1	0.39	0.45	0.474	0.78	0.44–1.37
5	KIR2DS2	0.35	0.50	0.045	1.85	1.05–3.28
6	KIR2DS4	0.73	0.53	0.005	0.41	0.23–0.75
7	KIR2DS5	0.54	0.60	0.048	1.27	0.72–2.23

*The difference in frequencies between the case and control groups was analyzed for statistical significance at the 95% CI using Fisher's exact test with Bonferroni correction. ORs were calculated and reported within 95% confidence limits. Logistic regression analysis was performed with different gene carrier frequencies as the independent factors, with the risk of RM being the dependent variable. P values < 0.05 were considered significant.

Table II HLA-C allele and group frequencies among RM patients and controls.

S. no	Alleles	Group	Patients (n = 177 pairs)		Controls (n = 200 pairs)		P value*	OR (95% CI)
			Group frequency	Allele frequency	Group frequency	Allele frequency		
1	C*01:02	C1	0.45	0.04	0.48	0.04	0.918	1.013 (0.82–1.24)
2	C*03:02			0.07		0.06		
3	C*03:12			0.06		0.05		
4	C*07:01			0.09		0.11		
5	C*07:02			0.08		0.10		
6	C*07:03			0.03		0.02		
7	C*14:02			0.08		0.08		
8	C*02:02	C2	0.55	0.04	0.52	0.02		
9	C*04:01			0.01		0.04		
10	C*04:03			0.02		0.01		
11	C*04:07			0.07		0.05		
12	C*06:02			0.07		0.06		
13	C*12:02			0.12		0.14		
14	C*15:02			0.07		0.09		
15	C*15:05			0.12		0.11		
16	C*17:01			0.03		0.02		

*Statistics same as in Table I.

OR = 2.83, 95% CI = 1.47–5.40). KIR2DS1 positive women homozygous for HLA-C1 with their partners being homozygous for HLA-C2 were observed more often in patients group (10.2%) and were of rare occurrence in the control group (3.0%; $P = 0.005$, OR = 3.66, 95% CI = 1.41–9.44). Our results showed that the ‘top-end’ of the activation spectrum of NK cells was seen among RM patients with reduced inhibitory and increased activating KIR-HLA combinations, when compared with controls, where the reverse combinations were more prevalent.

Discussion

The present study is the first to attempt the characterization of the KIR-HLA-C allorecognition patterns and its effects on idiopathic RM among North Indians. In this study, we have attempted to extend our previous findings pertaining to predisposing effects of KIR repertoire on RM (Faridi *et al.*, 2009), taking into account the contribution of paternal antigens on determining the outcome of pregnancy. Using sequence-specific PCR amplification, we have determined HLA-C genotype and maternal KIR content among couples experiencing RM and healthy parous couples. Upon analyzing the individual allele frequencies as well as the frequency of HLA-C1 and C2 group, we did not find any significant difference in RM couples compared with the control couples. Although the HLA-C alleles were invariably distributed among the control and the patient groups, the differences were seen when analyzed in association with the presence of maternal KIR gene content. The RM group contained a significantly increased number of pairs of individuals, where both partners expressed HLA-C group I with 2DS2, whereas the pairs of individuals wherein both partners expressed HLA-C2 in the presence of 2DL1 were

higher in controls than in patients. This may be considered at the ‘top-end’ of the activation spectrum for NK cells for the sake of risk assessment of the disease. For example, individuals who are 2DL1⁺2DS1⁺ were found to be more prevalent in the patient’s group (14.7%) when compared with controls (2.4%). This reflects a particularly high-risk group if the fetus carries HLA-C2. On the other hand, individuals who are 2DL1⁺2DS1[−] were more prevalent among controls (58.5%) than in patients (38.4%).

Analysis of the KIR and HLA-C allorecognition has been pursued as a new approach in studying the immunological etiology of pregnancy disorders including pre-eclampsia and RM, and studies reporting the correlation of appropriate NK-cell activation/inhibition and the outcome of pregnancy have recently been reported (Hiby *et al.*, 2004, 2008; Witt *et al.*, 2004; Varla-Leftherioti *et al.*, 2005, 2010; Flores *et al.*, 2007; Wang *et al.*, 2007; Vargas *et al.*, 2009). Moreover, HLA-B alleles are believed not to be expressed on the trophoblast, but a lack of HLA-B alleles with the Bw4 epitope has been observed in RM couples in one of the earliest attempts to study the association of HLA class-I alleles with RM. Consistent with our observations (Table II), Christiansen *et al.* (1997) reported an equal prevalence of the HLA-C alleles belonging to the HLA-C1 and C2 groups in RM couples and controls. Our findings are in concordance with the results of 15th International Histocompatibility Workshop Reproductive Immunology Component (Varla-Leftherioti *et al.*, 2010), in which the ‘strong inhibiting’ KIR2DL1-HLA-C2 combination (when compared with ‘less inhibiting’ KIR2DL2/3-HLA-C1 combination; Parham, 2005) was found to be lower in the RM group in comparison with that in the control group. Our understanding of the immunological etiology of RMs, however, does not support the NK-mediated ‘killing’ of the fetus. Our findings reflect a shift toward the

Table III KIR and their ligands among RM patients and controls.

KIR and their ligands			Patients, <i>n</i> = 177 pairs (%)	Controls, <i>n</i> = 200 pairs (%)	<i>P</i> value*	OR	95% CI
Maternal	Paternal						
KIR	HLA-C	HLA-C					
Inhibitory KIR-ligand associations							
2DL1	C2/C2	C2/C2	10 (5.6)	35 (17.5)	0.0004	0.28	0.13–0.58
2DL1	C2/C2	C1/C1	37 (20.9)	38 (19.0)	0.699	1.12	0.67–1.86
2DL1	C1/C2	C2/C2	0 (0.0)	5 (2.5)	–	–	–
2DL1	C1/C2	C1/C1	0 (0.0)	30 (15.0)	–	–	–
2DL1	C1/C1	C2/C2	30 (16.9)	38 (19.0)	0.687	0.87	0.51–1.47
2DL1	C1/C1	C1/C1	33 (18.6)	20 (10.0)	0.018	2.06	1.13–3.74
2DL2/3	C2/C2	C2/C2	40 (22.6)	38 (19.0)	0.445	1.24	0.75–2.05
2DL2/3	C2/C2	C1/C1	36 (20.3)	38 (19.0)	0.796	1.09	0.65–1.81
2DL2/3	C1/C2	C2/C2	0 (0.0)	5 (2.5)	–	–	–
2DL2/3	C1/C2	C1/C1	0 (0.0)	8 (4.0)	–	–	–
2DL2/3	C1/C1	C2/C2	30 (16.9)	33 (16.5)	1.000	1.03	0.60–1.77
2DL2/3	C1/C1	C1/C1	33 (18.6)	27 (13.5)	0.204	1.46	0.84–2.55
Activating KIR-ligand associations							
2DS1	C2/C2	C2/C2	19 (10.7)	15 (7.5)	0.286	1.48	0.72–3.01
2DS1	C2/C2	C1/C1	19 (10.7)	18 (9.0)	0.606	1.21	0.61–2.29
2DS1	C1/C2	C2/C2	0 (0.0)	0 (0.0)	–	–	–
2DS1	C1/C2	C1/C1	0 (0.0)	13 (6.5)	–	–	–
2DS1	C1/C1	C2/C2	18 (10.2)	6 (3.0)	0.005	3.66	1.41–9.44
2DS1	C1/C1	C1/C1	13 (7.3)	8 (4.0)	0.181	1.90	0.76–4.70
2DS2	C2/C2	C2/C2	16 (9.1)	23 (11.5)	0.499	0.76	0.39–1.49
2DS2	C2/C2	C1/C1	9 (5.1)	9 (4.5)	0.813	1.13	0.44–2.93
2DS2	C1/C2	C2/C2	0 (0.0)	0 (0.0)	–	–	–
2DS2	C1/C2	C1/C1	0 (0.0)	4 (2.0)	–	–	–
2DS2	C1/C1	C2/C2	6 (3.4)	11 (5.5)	0.456	0.60	0.21–1.66
2DS2	C1/C1	C1/C1	33 (18.6)	15 (7.5)	0.002	2.83	1.47–5.40

Inhibitory KIR and activating KIR genes are shown top to bottom. The HLA-C genotypes in the presence of their maternal KIRs in cases and control groups were analyzed for statistical association with RM.

*Statistics same as in Table I.

overactivation of NK cells mediated by the KIR-HLA compound genotype, the effect of which might not be directly lethal, but may contribute to the generation of a cytokine milieu that may be deleterious to the growth and differentiation of fetal trophoblast cells.

The NK cell surface density of CD56 (neural cell adhesion molecule, 140 kDa isoform) discriminates between two functionally distinct NK cell subsets: cytolytic CD56^{dim} NK cells and cytokine-producing CD56^{bright} NK cells (Cooper *et al.*, 2001). NK cell activity is governed by the combinatorial engagement of activating and inhibitory cell-surface receptors which determines whether NK cells will or will not kill target cells and/or produce cytokines during their effector phase of activation (Vivier *et al.*, 2004). Based on the recent studies conducted in mice (Kim *et al.*, 2005) and humans (Anfossi *et al.*, 2006; Yu *et al.*, 2007; Fauriat *et al.*, 2010), it has now been established that in order to become functional, the NK cells undergo a process of education and licensing, during the course of their development through engagement of their inhibitory and/or activating KIRs with

their cognate HLA class I ligands. According to the education theory, NK cells with, for example, KIR2DL1 are only functioning in women with group C2 alleles, but preferentially react against C2 negative targets (e.g. on the trophoblast). Our data and its association with the outcome of pregnancy fits into this model to a large extent (Table III). Couples where both partners were homozygous for HLA-C2 in the presence of maternal KIR2DL1 were significantly more prevalent in the control group (35%) when compared with the patients (10%). This genotype is in theory associated with maximum inhibition of the NK cell toward the trophoblast. Similarly the 2DS2 + C1/C1/C1/C1 genotype, which is associated with the NK-cell activation, was found to be significantly increased in patients (33%) when compared with the controls (15%). Comparisons of the frequencies of these compound genotypes between patients and controls have yielded the lowest *P* values as presented in Table III. However, it remains as yet undefined that how dNK cells are educated, licensed and regulated in the uterine microenvironment. In

the NK cell education studies, it has been observed that the NK cells that readily respond to IL-12 or IL-15 belong to the CD56^{bright} NK cell subset, and the licensing effect was less prominent in pre-activated NK cells, suggesting that licensing requirements could be bypassed under certain circumstances (Kim *et al.*, 2005, Anfossi *et al.*, 2006). dNK cells express highest amounts of CD56 (Koopman *et al.*, 2003) and express receptors and respond to IL-15 (Verma *et al.*, 2000). The invading trophoblast have been shown to promote the recruitment of the dNK cell subset to the fetal–maternal interface by secreting a specific set of cytokines (Drake *et al.*, 2001; Hanna *et al.*, 2003, 2006). Furthermore, in one study (Hiby *et al.*, 2004), the association with maternal KIR AA–fetal C2 combination in pre-eclampsia is seen whether or not the mother herself has a C2 gene, implying that the HLA-C molecules displayed by the trophoblast must be special and distinct from those found on somatic cells (King *et al.*, 2000).

As a result of the stringent inclusion criteria for the selection of study group couples, there was a limitation of sample size in the present study; also we have attempted to minimize the limitation of the resultant mixed ethnicity of patients' group by including ethnically matched control group couples. Our results support the hypothesis that an element of the pathogenesis in RM relates to excessive activation (or lack of appropriate inhibition) of NK cells. This implication needs to be further pursued through functional studies based on the new insights on the regulation of NK cell activity in the deciduas (Hanna *et al.*, 2006), the unique expression pattern of dNK cell regulatory receptors (Sharkey *et al.*, 2008), and in this case, the KIR-HLA-C allorecognition patterns.

Authors' roles

R.M.F. conducted all the experiments, analyzed data and wrote the paper. S.A. supervised the project contributed intellectual inputs and finalized the manuscript.

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