

**THE SEARCH FOR LINKS
BETWEEN IMMUNOGENETIC
FACTORS AND RECURRENT
MISCARRIAGE**

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Abstract

Successful pregnancy is characterized by a shift toward Th2 type immune response and suppression of adaptive immune responses to ensure acceptance of the semi-allogenic fetal graft. Also the innate immune system plays a major role during pregnancy. Recurrent miscarriage is defined as three or more consecutive pregnancy losses. About 1% of all women will suffer recurrent miscarriage. The causes of recurrent miscarriage remain unexplained in half (50%) of the cases. Susceptibility to recurrent miscarriage is probably mediated by Th1 type immune response with pronounced expression and secretion of pro-inflammatory cytokines (e.g. TNF α and IFN γ) paralleled with decreased production of anti-inflammatory cytokines (e.g. IL-10). Factors that regulate immune response during pregnancy include hormonal factors (e.g. hCG and progesterone). Immunogenetic factors also contribute to this regulation. Several functionally important polymorphisms in various immunomodulatory genes have been identified during recent years. Some of these polymorphisms may be important in regulating the Th1/Th2 balance during pregnancy. Putative immune dysregulation caused by these polymorphisms has been researched intensively. Conflicting results have been published about associations between several of these polymorphisms and recurrent miscarriage.

In this study, HLA-G (exon 2 and 3), IL-10 (-1082A/G), IL-1RA (intron 2 VNTR) and CD14 (-159C/T) polymorphisms were studied in 38 Finnish women with RM. All of these polymorphisms have been associated with altered gene expression. Distribution of HLA-G**I*, *II*, *III* and *IV* were 0.577, 0.375, 0 and 0.048 respectively in the studied Finnish population. According to the present classification the G**I* allele group mostly consists of the allele 010101, while G**II* covers the combination of 010102, 010401 and 0105N, as well as some other rare alleles. There were no associations between recurrent miscarriage and the HLA-G, IL-10 and CD14 polymorphisms. However, in IL-1RA polymorphism, the rare IL1RN*3 allele was increased in women with recurrent miscarriage. It is not known, if this particular allele is associated with differences in IL-1RA or IL-1 production.

Although the study population was small, it may be supposed that quantitative differences in the production of single immunomodulatory molecules due to normal genetic variation may not be grossly harmful to the fetal allograft. This indicates the robustness and flexibility of the reproduction system. For survival, it is essential that minor variations are tolerated. Thus, large-scale studies focusing on the effect of a pro-inflammatory genetic profile based on the presence of several pro/anti-inflammatory genetic markers are needed to discover if immunogenetic factors predispose women to recurrent miscarriage.

Keywords: CD14 antigens, cytokines, genetic polymorphism, habitual abortion, HLA antigens

To my family

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Abbreviations

ANGPT-2	angiopoietin-2
ARMS	amplification refractory mutation system
Bp	base pair
C	complement
CD	cluster of differentiation
ConA	concanavalin A
CRP	C-reactive protein
Crry	complement receptor-related gene/protein y
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (nucleotide)
GM-CSF	granulocyte-macrophage colony stimulating factor
hCG	human chorionic gonadotropin
HLA	human leukocyte antigen
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
ILT	immunoglobulin like transcript
IvIg	intravenous immunoglobulin
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
mCD14	membrane-bound CD14
MHC	major histocompatibility complex
mHLA	membrane-bound human leukocyte antigen
MIC	macrophage inhibitory factor
mRNA	messenger ribonucleic acid
NK	natural killer cell
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory analgetics
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction

PG	prostaglandin
PIBF	progesterone-induced blocking factor
RCOG	Royal College of Obstetrics and Gynaecologists
RFLP	restriction fragment length polymorphism
RM	recurrent miscarriage
sCD14	soluble CD14
sHLA	soluble human leukocyte antigen
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
Th	helper T
TLR	toll like receptor
TNF	tumor necrosis factor
TORCH	toxoplasma, rubella virus, cytomegalovirus and herpesviruses
uNK	uterine natural killer cell
VEGF	vascular endothelial growth factor
VNTR	variable number of tandem repeats

List of original papers

This thesis is based on the following articles, which are referred to in the text by their corresponding Roman numerals.

- I Karhukorpi J, Ikäheimo I, Silvennoinen-Kassinen S & Tiilikainen A (1996) HLA-G polymorphism and allelic association with HLA-A in a Finnish population. *Eur J Immunogenet* 23:153–155.
- II Karhukorpi J, Laitinen T & Tiilikainen A (1997) HLA-G polymorphism in Finnish couples with recurrent spontaneous miscarriage. *Br J Obstet Gynaecol* 104:1212–1214.
- III Karhukorpi J, Laitinen T, Karttunen R & Tiilikainen A (2001) The functionally important IL-10 promoter polymorphism (-1082G→A) is not a major genetic regulator in recurrent spontaneous abortions. *Mol Hum Reprod* 7:201–203.
- IV Karhukorpi J & Karttunen R (2001) Genotyping interleukin-10 high and low producers with single-tube bidirectional allele-specific amplification. *Exp Clin Immunogenet* 18:67–70.
- V Karhukorpi J, Laitinen T, Kivelä H, Tiilikainen A & Hurme M (2003) IL-1 receptor antagonist gene polymorphism in recurrent spontaneous abortion. *J Reprod Immunol* 58:61–67.
- VI Karhukorpi J, Laitinen T & Karttunen R (2003) Searching for links between endotoxin exposure and pregnancy loss: CD14 polymorphism in idiopathic recurrent miscarriage. *Am J Reprod Immunol* 50:346–350.

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1 Introduction

Exact pathophysiological mechanisms leading to recurrent miscarriage (RM) are largely unknown. In some of these cases, the causes are believed to be immunological (Choudhury & Knapp 2001a). Many therapeutical trials have explored the benefits of immunomodulation on pregnancy failures (Omwandho *et al.* 2000). However, contradictory results have been obtained in these trials. Thus it is important to study basic immunological mechanisms of infertility in order to avoid unnecessary and costly therapies in cases where no effect can be obtained. Likewise, with the help of results from basic research, there are better chances to identify those couples that might benefit from different forms of immunotherapy, as reproduction failure may cause distress and lead to psychological problems. Currently, it seems that therapeutic immunomodulation during pregnancy has only limited or no value (Scott 2000).

Effective reproductive performance is vitally important for all species. Reproductive failure in animals may also cause economic problems. This is especially true in the livestock industry. Economic losses due to inefficient breeding have facilitated reproduction immunology research in the livestock industry (Lofthouse & Kemp 2003). Several animal models, mainly in mice and rats, have been used in reproduction studies. However, as the immunological systems between animals and humans are not identical, there is a need to specifically address reproductive immunological parameters in humans.

This study concentrates on immunogenetic factors, especially HLA and cytokines, which are possibly involved in altered immune response in women with recurrent miscarriage. As major histocompatibility complex antigens (MHC) may also operate in mate choice, obviously through olfaction, the literature highlighting these unconventional functions of MHC antigens is briefly reviewed.

2 Review of the literature

2.1 Key immunological changes during pregnancy

From an immunological point of view, the fetus represents a foreign graft (Medawar 1953). Immunoregulation is therefore essential in guaranteeing the acceptance of the semi-allogenic fetus by maternal immune cells. Generally the immune system may be divided into two compartments. The adaptive system relies on specific interactions between Major Histocompatibility Complex (MHC) antigen expressing target cells and T-cell receptor expressing lymphocytes. The innate immune system is more primitive lacking memory cells, but able to respond to various notorious stimuli. Both of these systems are probably important during pregnancy and both of them are controlled efficiently. Failure to control maternal immune response against fetal tissues is believed to lead to adverse pregnancy outcome such as miscarriage or preeclampsia.

During pregnancy it is noteworthy that local immune microenvironment at the implantation site may be totally different from that observed systematically. Moreover, regulation may be different at different stages of pregnancy (Choudhury & Knapp 2001a).

Locally at the placenta there are normally only a few maternal T and B cells that represent typical cell populations of adaptive immune response. They are considered important for normal reproduction as the elimination of T cells results in abortions and reduced placental growth in murine models (Athanasakis *et al.* 1987, Chaouat *et al.* 1988). The predominant immune cells belong to the innate immune system such as decidual macrophages. Natural killer (NK) cells are especially abundant in the decidua. They may recognize both self and non-self antigens especially when target cells are not expressing MHC molecules. The functions of uterine NK (uNK) cells are still somewhat unknown (Dociou & Giudice 2005). Experiments with NK-cell depleted knock-out mice have shown their importance in remodeling major decidual arteries. In knock-out mice the placental size is smaller. Additionally, midgestational fetal death occurs in > 50 % of pregnancies (reviewed in Erlebacher 2001).

As the uNK cells may be harmful when activated by fetally derived cells not expressing classical HLA antigens, it is important to regulate their action locally. It is believed that various inhibitory and activatory receptors of NK cells may interact with HLA-G, -E and

-C molecules on fetal cytotrophoblasts and by this action these HLA molecules might prevent lysis of fetal cells by maternal cells. HLA-G, -E and -C are expressed especially on extravillous cytotrophoblasts that invade maternal uterine tissues when arterial supply for growing placenta is modelled. Alternatively, the interaction between NK receptors and HLA molecules might influence the cytokine production of NK cells (Moffett-King 2002).

Besides the NK cells macrophages are also abundant in decidual tissues. These cells may have important immunoinhibitory effects at the materno-fetal interface. Decidual macrophages also express inhibitory HLA receptors (ILT2 and ILT4) suggesting that HLA-G from trophoblast may also suppress maternal immunity through interaction with these receptors (Petroff *et al.* 2002). Decidual macrophages produce IL-10 and tryptophan catabolizing enzyme indoleamine 2, 3-dioxygenase (IDO) (Heikkinen *et al.* 2003). IDO is one of those molecules that may suppress maternal T-cell activation. Inhibition of IDO in mice, especially in allogeneic mating combinations, results in fetal rejection with lethal inflammatory response, with T-cell infiltration and activation of complement. The role of IDO as a protective molecule at the materno-fetal interface seems to be especially important when there are sufficiently mismatched MHC antigens between mother and fetus (Mellor *et al.* 2001).

There are several constituents of the innate immune system which must be regulated at the feto-maternal interface. Inappropriate complement activation during inflammatory reaction may result in lysis of fetal cells as is the case in IDO deficiency (discussed above). Also, deficiencies in complement regulating proteins may cause adverse pregnancy outcomes. The murine Crry protein negatively regulates C3 and C4 complement proteins. In mice with non-functioning Crry, the complement regulation is severely affected and intrauterine embryo deaths are common due to C3 fixation on trophoblasts (Xu *et al.* 2000). Human trophoblasts also express several complement regulatory proteins: CD46 (membrane co-factor protein), CD55 (decay accelerator factor) and CD59 (reviewed in Weetman 1999).

The balance between several pro- and anti-inflammatory cytokines both locally and systemically may be critical during pregnancy. Pregnancy has been characterized as an immunosuppressive state dominated by Th2 cytokines (Wegmann *et al.* 1993, Shurin *et al.* 1999). The alleviation of rheumatoid arthritis symptoms, characterized by Th1 bias, could be viewed as clinically relevant evidence regarding the effect of overdominance of Th2 type cytokines. An opposite effect of pregnancy is evident in systemic lupus erythematosus (a Th2 type disease), which is often activated during pregnancy (Huizinga *et al.* 1999).

Alterations in systemic soluble immune mediators have been observed in normal pregnancies and miscarriages, though conflicting findings exist (Choudhury & Knapp 2001a). Decreased macrophage inhibitory cytokine 1 (MIC 1) concentration in serum precedes miscarriage. MIC 1 has Th2 type cytokine actions. Measuring MIC 1 concentrations during early pregnancy could possibly be used in evaluating a risk for future miscarriage (Tong *et al.* 2004).

Typical Th1/Th2 and anti-/pro-inflammatory cytokines are shown in Table 1.

Table 1. Classification of cytokines (Julkunen *et al.* 2003).

Th1 cytokines	Th2 cytokines	Proinflammatory cytokines	Anti-inflammatory cytokines
IL-2	IL-4	IL-1beta	IL-4
IL-12	IL-5	IL-1alpha	IL-10
IL-15	IL-10	IL-6	TGF-beta
IL-18	IL-13	TNF-alpha	IL-1RA
IL-21		IL-12	IL-18BP
IFN-alpha		IL-18	
IFN-gamma		IFN-gamma	

Recently the Th1/Th2 paradigm was challenged by findings showing that IL-10 and IL-4 deficient mice succeeded in completion of allogeneic pregnancy (Svensson *et al.* 2001). Moreover IFN-gamma, a typical Th1 cytokine, was found to contribute favourably to implantation and pregnancy outcome in mice (Ashkar & Croy 1999, Ashkar *et al.* 2000). The role of the innate immune system during pregnancy has been highlighted recently. It seems that intracellular cytokine production of PBMC does not fit into the Th2 paradigm either. PBMC are obviously primed to produce Th1 cytokine IL-12 after LPS stimulation (Sacks *et al.* 2003). Probably IL-12 has a beneficial role during pregnancy, as peripheral levels of IL-12 are down-regulated in women with miscarriage (Zenclussen *et al.* 2003). Despite these new interesting findings that question the Th1/Th2 paradigm in pregnancy, there is plenty of evidence suggesting that a shift to Th2 type immune response is important for successful pregnancy (reviewed in Shurin *et al.* 1999).

Several factors contribute to the carefully orchestrated balance between Th1 and Th2 type immune responses. Genetic factors are among the most important. In twin studies, it has been shown that production capacity of especially IL-1beta, IL-1ra, IL-10, IL-6 and TNF-alpha after ex vivo LPS stimulation was mainly (>50 %) genetically determined (de Craen *et al.* 2005). Some mouse strains also respond typically with either a Th1 (C57BL/6) or Th2 (DBA/2) bias (Butler *et al.* 2002).

During pregnancy, several hormonal factors contribute to the Th2 bias. Especially progesterone is an important immunomodulatory molecule. Its effects are mediated by a lymphocyte-derived protein, progesterone-induced blocking factor (PIBF). In mice, treatment with anti-progesterone induces abortion. Also, neutralization of PIBF by PIBF-specific antibodies causes abortions in mice. The anti-abortive effect of PIBF is mediated by inhibiting NK cell activity (Szekeres-Bartho *et al.* 2001). Additionally, progesterone directly suppresses T cell differentiation into Th1 and enhances IL-10 producing Th2 cells (Miyaura & Iwata 2002, Piccinni *et al.* 1995). Relaxin, glycodelin and hCG are other factors that may modulate cytokine production during pregnancy (Carp *et al.* 2001, Seppälä *et al.* 2002, Mishan-Eisenberg *et al.* 2004).

To summarize the findings from recent research reports, it seems that the Th1/Th2 paradigm in pregnancy is probably an oversimplification that has served as a valuable tool in various research settings until today. From this point forward there will be a growing need to also focus on the role of the innate immune system. In addition to the suggested over-dominance of Th2 type lymphocytes, the systemic activation of the innate immune system may have important biological implications during pregnancy (Chaouat 2003). It

seems that the favorable Th1/Th2 balance is especially dependent on the stage of pregnancy. During implantation and early stages of pregnancy Th1 cytokines are needed, but when pregnancy proceeds, Th1 cytokines become more harmful until they are again needed during labour (Athanasakis *et al.* 2002).

2.2 Definition and causes of recurrent miscarriage

Miscarriage is a common event during early gestation. It is estimated to occur in ca. 15 % of all clinically recognized pregnancies (reviewed in Beydoun & Saftlas 2005). Recurrent miscarriage is defined as three or more consecutive pregnancy losses before the 24th week of gestation (Choudhury & Knapp 2001a). RM may affect up to 2-4 % of reproductive-age couples (Kutteh 1998). In another study it has been estimated that about 1.0 % of all women will suffer RM (Stirrat 1990). In the case of primary recurrent miscarriage there are no live born children, but in secondary recurrent miscarriage there may be usually first a live born child followed by a series of miscarriages.

The origin of RM is multifactorial. In about half of the cases the cause is unknown (Willcox *et al.* 1988). However, cytogenetic analysis of miscarriages has not been done routinely. Recent cytogenetic analyses of curettage specimens have shown cytogenetically abnormal embryos or fetuses in about 50 % of RM cases (Stephenson *et al.* 2002). In primary RM, 55 % of abortuses are abnormal and in secondary RM, 35 % are chromosomally abnormal (Clark *et al.* 2001).

Anatomical causes of RM include uterine anomalies, fibroids, intrauterine synechiae, and cervical incompetence. Also endocrine factors have been associated with RM (including luteal insufficiency, polycystic ovary syndrome, thyroid dysfunction, insulin resistance and diabetes).

As an adequate placental vascular network is vitally important for the growing fetus, all abnormalities that diminish blood flow to the placenta may result in gestational pathologies. Some of these changes may be anatomical abnormalities in blood vessel formation and some may be related to an increased tendency of thrombosis. Interestingly, local expression of vascular endothelial growth factor (VEGF) and its receptors the VEGFR-1 and -2 and the Tie-receptors, is diminished in recurrent miscarriage as compared to normal pregnancies (Vuorela *et al.* 2000). The angiopoietin gene family has been studied as a putative candidate gene for RM, as angiopoietin-2 is especially expressed in the placenta where vascular remodeling actively occurs (Holash *et al.* 1999). However, no association between ANGPT-2 polymorphism and RM was evident in an Austrian population (Pietrowski *et al.* 2003).

The antiphospholipid syndrome is the most common of immunologic/thrombotic causes of RM (Stirrat 1990, Vinatier *et al.* 2001). Genetic factors leading to thromboembolic complications may also be associated with RM. Increased frequencies of Factor V Leiden and prothrombin G20210A mutations have been found in women with RM (Grandone *et al.* 1997, Pihusch *et al.* 2001, Wramsby *et al.* 2000). In some studies, however, no associations have been found (Yamada *et al.* 2001, Hohlagschwandtner *et al.* 2003).

Recently an association between coeliac disease and miscarriages was suggested in an Italian population (Martinelli *et al.* 2000). However, in Finnish women with RM coeliac disease was not more prevalent than in women with normal fertility (Kolho *et al.* 1999).

Table 2. Table 2. Distribution of etiological factors in 545 with RM according to Kutteh & Carney 1999.

Factor	Frequency (%)
Genetic	3.1
Endocrinological	20.2
Anatomic	21.6
Immunologic	25
Infectious	5.8

As shown in Table 2, infections may cause 5.8 % of all RM cases. Conflicting results have been obtained concerning the role of genital tract infections in RM. Increased frequencies of antibodies to *Chlamydia trachomatis* were found in women with RM in some earlier studies (Quinn *et al.* 1987, Witkin & Ledger 1992). However, no association between *C.trachomatis* infection and RM was found recently in a Finnish population (Paukku *et al.* 1999). The role of TORCH infections (toxoplasma, rubella, other (congenital syphilis and viruses), cytomegalovirus and herpes simplex virus) is most probably unimportant in RM and routine screening is not recommended (RCOG 2003).

Bacterial vaginosis seems to be associated with miscarriage and especially mid-trimester pregnancy loss (Donders *et al.* 2000, Kurki *et al.* 1992). Also, association between bacterial vaginosis and first trimester RM has been reported (Ralph *et al.* 1999). Especially subclinical ureaplasma and mycoplasma infections in the genital tract may be the causative factors for recurrent miscarriage (Viniker 1999). Oral, but not vaginal clindamycin treatment of pregnant women with bacterial vaginosis has been shown to reduce the risk of late miscarriage and preterm delivery (Ugwumadu *et al.* 2003, Kekki *et al.* 2001, Rosenstein *et al.* 2000).

Some other environmental factors may also predispose women to spontaneous abortions though the association between these factors and RM is unclear. These include smoking, caffeine and alcohol consumption (Harlap & Shiono 1980, Kesmodel *et al.* 2002). Smoking causes hypoxia that is one of the regulators of ANGPT2 expression. Thus, smoking may also have an influence on placental vasculature (Haustein 1999, Larsen *et al.* 2002). In a recent Danish study, consumption of alcohol and caffeine was associated with an increased risk of miscarriage (Rasch 2003). Dietary factors other than smoking and alcohol consumption may also confer susceptibility to miscarriage. Especially a diet poor in green vegetables, fruits, milk and dairy products may confer susceptibility to miscarriage (Di Cintio *et al.* 2001).

Psychological factors, e.g. stress, may also predispose women to miscarriage and RM (Clark *et al.* 1999). Enhanced production of TNF-alpha was observed in those patients with first trimester miscarriages who had higher stress scores (Arck *et al.* 2001). Thus, psychoneuroimmunologic factors may play a role in the etiology of RM, as stress scores may be associated with Th1/Th2 balance.

Stress experienced by a pregnant woman may trigger alarm systems and lead to rejection of embryo. Evolutionally this makes sense as the mother is more prone to survive in extreme conditions without the growing conceptus. This is true also when a mother must deal with infection with vigorous Th1 type immune response (Clark *et al.* 1999). Immune activation may also inhibit sexual behavior. This was shown in female rats which were injected with endotoxin and IL-1. However, in male rats such an effect was not evident (reviewed in Avitsur & Yirmiya 1999).

Under conditions with a heavy pathogen load, the capability to respond with rigorous Th1 type cytokine profile may be consistent with survival. When infections were the major cause of early death in Britain there was an inverse association between family size and longevity of aristocratic women (Westendorp & Kirkwood 1998). This may indicate that factors favouring survival from fatal infections, such as cholera and tuberculosis during childhood (Th1 response), might have had a negative effect on reproduction later in life (Westendorp *et al.* 2001).

2.3 Treatment of recurrent miscarriage

Recurrent miscarriage is rare and only seldom presents a serious threat to a woman's physical health. Still it causes major psychological distress to those suffering from it. It was found that 32 % of the RM women were depressed and had higher than average levels of acute and chronic anxiety (Klock *et al.* 1997). Adverse psychological consequences of involuntary childlessness, including RM, affect both men and women (Whiteford *et al.* 1995). Thus, accurate diagnosis and treatment of RM are quite important.

In those cases where the underlying cause is found, the treatment regimen is directed to diagnosed disturbances. The situation is more complex when no reason for RM can be identified, though subsequent pregnancy is more likely to be successful in unexplained RM (Bricker & Farquharson 2002). Tender loving care can be used as the sole treatment for unexplained RM. It may be effective by lowering anxiety and stress scores and in this way modulating immune responses against the conceptus (Li 1998).

As immunological disturbances seem to be risk factors in many cases of RM, various immunotherapeutical interventions have been tested in RM. These include immunization with paternal or allogeneic leukocytes and intravenous immunoglobulin (IvIg). There is only limited or no benefit of lymphocyte immunization (Ober *et al.* 1999). Paternal or third party leukocyte immunization, trophoblast membrane infusion and intravenous immunoglobulin were found to be ineffective in a meta-analysis of 19 high quality trials (Scott 2003). However, in a recent randomized, placebo-controlled trial of IvIg in the prevention of RM, a therapeutic effect was obtained in women with secondary RM (Christiansen *et al.* 2002). New trials are still needed to confirm these results.

2.4 The role of MHC in reproduction

An important immunological function of the HLA molecules is to bind and present peptides to T lymphocytes. In HLA genes, polymorphic sites are found especially in those regions that encode amino acids around the peptide binding site, enabling HLA molecules to bind a wide array of different peptides.

HLA molecules establish the major obstacle for allogeneic transplantation as foreign HLA molecules recognized by T cell receptors of the host lead to rejection of the graft. As the HLA molecules are important in recognizing foreign cells and tissues, the role of HLA in reproduction has been elucidated intensively. The developing embryo inside the uterus is a semiallograft that would normally be rejected by the maternal immune system. During pregnancy, however, several different mechanisms act to suppress maternal immune attack against the embryo. One of these mechanisms is the lack of classical HLA molecules at the fetomaternal interface. Instead, a non-classical and rather non-polymorphic HLA-G is expressed and secreted by the trophoblasts (Choudhury & Knapp 2001a). The importance of MHC in reproduction may not be limited to the fetomaternal interface as several observations indicate that MHC molecules may also have a role in mate selection.

2.5 The HLA gene complex

The HLA (human leukocyte antigen) molecules are encoded by genes located within the major histocompatibility complex (MHC), referred to in man as the HLA gene complex. The HLA complex contains approx. 4 Mb of DNA on chromosome 6p21. The HLA region genes can be divided into three major classes, I, II and III. Class I genes encode cell-surface glycoproteins (alpha chains) expressed on nearly all nucleated cells. All of these class I products pair with beta-2 microglobulin, which is the product of a non-polymorphic gene on chromosome 15. HLA class I genes can be subdivided to classical genes HLA-A, -B and -C and non-classical HLA-G, -E, -F genes. Additionally, the class I region includes some pseudogenes, HLA-H, -J, -K and -L.

HLA class II loci (HLA-DR, -DQ, -DP, -DM, -DO) include genes for one alpha and at least one beta polypeptide. The HLA class II molecules are formed by one alpha and beta chain non-covalently linked together. HLA class II molecules are especially expressed on immunologically active cells, such as B lymphocytes, T cells, monocytes, macrophages and dendritic cells.

The HLA class III locus contains genes for complement components (C2, C4), TNF and heat shock proteins.

The HLA region is the most polymorphic region in the human genome. Especially class I and II loci show extensive allelic diversity. In some loci over 600 different alleles have been found (Schreuder *et al.* 2005).

2.5.1 Associations between HLA and recurrent miscarriage

Contradictory results have been obtained in studies focusing on associations between HLA locus and RM (Choudhury & Knapp 2001b). It has been argued that the observed HLA associations (especially those with class II) may be due to linkage disequilibrium with hypersecretory TNF-alpha alleles in the HLA class III region (Christiansen 1999). In a meta-analysis on Caucasian women with RM an association between HLA-DR1 and -DR3 antigens and RM was observed (Christiansen *et al.* 1999). Recently, a large case-control study showed that especially HLA-DRB1*03 is associated with RM in a Caucasian population (Kruse *et al.* 2004).

In the HLA class III region there are also many other immunologically important genes. Especially those coding for complement proteins are of interest with respect to recurrent miscarriage (Lokki & Laitinen 2001). The activation of the complement is tightly regulated at the materno-fetal interface. Finnish couples with recurrent miscarriage have an increased frequency of C4A and C4B null alleles compared to controls (Laitinen *et al.* 1991).

In a recent meta-analysis in which 40 observational studies were included, no consistent findings were observed in HLA allele sharing in couples with RM (Beydoun & Saftlas 2005). Increased parental HLA sharing may confer susceptibility to RM especially in isolated populations. In Finnish population, RM has been correlated with HLA-A and HLA-B sharing (Laitinen 1993). However, it has been speculated that even in a case of high degree of parental tissue incompatibility early miscarriage may actually be due to IDO insufficiency (Mellor *et al.* 2001). Furthermore, RM may be associated with certain HLA haplotypes (Laitinen 1993). Interestingly, 4 of these 12 risk haplotypes occurring in Finnish couples were found to segregate with RM in an extremely inbred Hutterite population, which is one of the most fertile human populations studied (Ober *et al.* 1998). The large family-sizes of Hutterites has offered the possibility to study the effects of HLA sharing on reproduction capacity, not only on pregnancy losses but also on birth intervals, as ten children in a family is not rare among Hutterites. In Hutterite couples with no shared HLA antigens, the time interval to needed produce 10 children was 13.7 years compared to 19 years for those sharing two or more antigens (Ober *et al.* 1983).

2.5.2 MHC in mate choice

Mate choice is an essential factor for success in reproduction. In some animal species, selection is based on rituals during which the fittest males are selected for mating. This guarantees that the most valuable genes are inherited by the next generation. For evolutionary purposes it is also essential to favour genetic diversity.

Breeding with close relatives does not favour genetic diversity. Several cultural and social mechanisms exist to hinder pestilence in human populations. In addition, biological/physical factors may have an influence. One such factors is the smell of the partner (Potts & Wakeland 1993). In animal studies, it has been shown that female mice prefer to mate with males derived from a different strain that excrete non-familiar olfactory signals. In recognition of different smells, MHC gene locus or MHC antigens may play a role. Cur-

rently, it is believed that MHC may produce an odor of individuality, though this is a subject of great controversy among immunologists (Potts 2002).

Soluble MHC molecules are themselves too heavy to be candidates for odoriferous components (reviewed in Singh 2001). MHC molecules may serve as carrier molecules for small volatile compounds such as the pheromones. MHC polymorphism provides the functional basis for their ability to bind to a large array of different odoriferous molecules (reviewed in Wedekind & Penn 2000).

Other olfactory recognition mechanisms also exist. Individuality signals are mediated by major urinary proteins in wild house mice (Hurst *et al.* 2001). Perception of odors is mediated through olfactory receptors expressed in the main olfactory epithelium and vomeronasal organ. Interestingly, the largest of the olfactory receptor gene clusters is physically linked to MHC (Younger *et al.* 2001).

Humans may also produce MHC-specific odors. Women may detect differences on HLA alleles among male odor donors. Especially HLA alleles inherited from the father may influence this selection. The odors that share paternally derived alleles are more preferred (as more familiar) than those that don't share alleles (Jacob *et al.* 2002). Whether this has any effect on behavior such as mating preference is controversial. Some studies have shown that odor preferences are associated with HLA dissimilarities between the odor donor and the smeller (Wedekind *et al.* 1995, Wedekind & Furi 1997).

Currently there is no doubt about the significance of MHC in immunological self and non-self discrimination, but it remains a challenge to prove that MHC also play the role of an odor cue in mate selection, though this might be cost-effective both evolutionally and biologically.

2.5.3 HLA-G

Of the HLA molecules, HLA-G is of specific interest in respect to fetomaternal defense. The HLA-G gene is located telomerically to HLA-A on chromosome 6. Sequence data show that it is relatively non-polymorphic, though extensive polymorphism at this locus might exist in an African-American population (van der Ven & Ober 1994). According to the current nomenclature for factors of the HLA system, there are 15 different HLA-G alleles (Marsh *et al.* 2002). Only three of them code for new amino acid substitutions compared to the original G*0101 allele: G*0103 encodes a Thr to Ser substitution (ACG -> TCG) at codon 31 within exon 2 and G*0104 alleles encode a Leu to Ile substitution (CTC -> ATC) at codon 110 within exon 3. At codon 258 within exon 4, a Met to Thr substitution (ATG -> ACG) has recently been characterized in a Danish population (Hviid *et al.* 2001). Additionally, a cytosine deletion at the third base of codon 129 within exon 3 changes the reading frame from this position, yielding a stop codon at the beginning of exon 4 (Suarez *et al.* 1997). This "null" allele (0105N) thus gives rise to a truncated protein with an absence of the full-length, functional mHLA-G1.

The HLA-G molecule shares many structural similarities with other class I genes. HLA-G associates with beta-2 microglobulin (Kovats *et al.* 1990) and it is able to bind to CD8 on T lymphocytes (Sanders *et al.* 1991). HLA-G is also able to bind intracellular non-amer peptides (Diehl *et al.* 1996, Lee *et al.* 1995). These characteristics indicate that anti-

gen presentation is one of the functions of HLA-G. Another important function of HLA-G may be the regulation of HLA-E expression and thus the interaction and inhibition of NK cells (Le Bouteiller & Solier 2001).

Besides limited polymorphism in the antigen-binding region of HLA-G, it has several other features that may also have functional significance. HLA-G mRNA can be alternatively spliced to generate multiple isoforms of HLA-G molecules both membrane-bound (mHLA-G) and soluble ones (sHLA-G). Altogether, four different mHLA-G and three different sHLA-G isoforms can be generated (Paul *et al.* 2000). A unique feature in sHLA-G molecules is that part of intron 4 is also translated (Fujii *et al.* 1994).

2.5.3.1 Functional implications of HLA-G expression

Among the HLA molecules HLA-G is unique because it is expressed almost exclusively on trophoblasts (Kovats *et al.* 1990). It is widely believed that HLA-G has an important role to play in reproduction as a tissue-protective molecule, inhibiting inflammatory responses as it is expressed at the materno-fetal interface where expression of other HLA molecules is suppressed (Carosella *et al.* 2001). However, none of the supposed functions of HLA-G has been convincingly documented yet (Bainbridge *et al.* 2001).

HLA-G probably protects the fetal semi-allogenic graft by inhibiting maternal uNK cells through interaction with inhibitory receptors. In this process the HLA-E molecule is also important as it initially binds to the leader sequence peptide of HLA-G that is recognized by inhibitory receptors on NK cells (Rouas-Freiss *et al.* 1997a, Rouas-Freiss *et al.* 1997b, Carosella *et al.* 1999, Hofmeister & Weiss 2003). HLA-G also modulates T lymphocyte responses as it inhibits T cell allo-proliferation *in vitro* (Riteau *et al.* 1999). Soluble HLA-G is also secreted during pregnancy and sHLA-G is present in amniotic fluid (Hiby *et al.* 1999, Rebmann *et al.* 1999, Hamai *et al.* 1999). In addition to cytotrophoblasts, activated placental macrophages also secrete HLA-G (Chu *et al.* 1998).

In vitro, sHLA-G1 triggers apoptosis of activated CD8⁺ cells (Fournel *et al.* 2000). sHLA-G may stimulate the release of IL-10 and thus polarize immune response in the Th2 direction (Kanai *et al.* 2001). *In vitro* experiments show that in mixed lymphocyte culture, sHLA-G is secreted by allo-specific CD4⁺ cells from the responder population, thereby suppressing allogeneic proliferative T cell response (Lila *et al.* 2001).

Regulation of HLA-G expression differs markedly from that of other HLA class I molecules. This is probably due to unique structural characteristics of the HLA-G promoter region (reviewed in Le Bouteiller *et al.* 2003). Usually, HLA class I expression is down-regulated by IL-10. However, HLA-G in trophoblasts and monocytes is induced by IL-10, a potent anti-inflammatory molecule (Moreau *et al.* 1999). HLA-G up-regulation in some lymphomas and lung cancer are also associated with IL-10 production (Urosevic *et al.* 2001, Urosevic *et al.* 2002). Also, glucocorticoid hormones enhance levels of HLA-G transcripts in trophoblasts, though generally they down-regulate HLA class I molecules (Moreau *et al.* 2001).

HLA-G expression may have a protective role in organ transplantation. Allogenic heart transplant patients with HLA-G expression in endomyocardial biopsies and sHLA-G in serum showed reduced numbers of acute and chronic rejection episodes compared to those

patients with no HLA-G induction (Lila *et al.* 2000, Lila *et al.* 2002). Inhibition of human NK cell-mediated lysis of porcine endothelial cells transfected with HLA-G suggests that in the future HLA-G may have a role in preventing rejection even during xenotransplantation (Forte *et al.* 2001).

Because the classical HLA-A, -B and -C molecules are frequently down-regulated in human tumours similarly to the materno-fetal interface, HLA-G transcripts and proteins have been searched intensively in this setting as well. HLA-G up-regulation at the protein level has at least been detected at least in various lymphomas (Urosevic *et al.* 2002), melanoma (Paul *et al.* 1998), renal carcinoma (Ibrahim *et al.* 2001) and lung cancer (Urosevic *et al.* 2001). In human gliomas, aberrantly expressed HLA-G also shows functional activity by inhibiting alloreactive and antigen-specific immune responses (Wiendl *et al.* 2002). Thus, enhanced expression of HLA-G may be associated with down-regulation of host immune response against cancer. HLA-G up-regulation in cancer is currently controversial as no HLA-G expression in cancer has been found in some studies (Pangault *et al.* 1999, Real *et al.* 1999, Davies *et al.* 2001).

It seems that HLA-G expression in tumors is not a universal phenomenon, though inconsistent findings may in part result from differences in HLA-G specific antibodies used in some of the studies. Some antibodies may not detect all isoforms of HLA-G. Especially, commonly used 87G and 01G antibodies only detect mHLA-G1 isoforms and probably sHLA-G5. However, by using 4H84 other isoforms of HLA-G may also be detected (Paul *et al.* 2000).

HLA-G may also be expressed during various inflammatory and infectious conditions. In skin, HLA-G has been found in macrophages in psoriatic lesions (Aractingi *et al.* 2001). The function of HLA-G in connection with psoriasis may be to down-regulate T cells (reviewed in Carosella *et al.* 2001). Also, lung macrophages and dendritic cells expressed HLA-G in 25 % (2/8) of non-tumoral pulmonary diseases, including one case of pulmonary tuberculosis and bronchiectasis (Pangault *et al.* 2002).

Altered HLA-G expression has been found in some pregnancy pathologies. Attenuated HLA-G expression is especially evident in invasive trophoblasts of pre-eclamptic patients (Hara *et al.* 1996, Goldman-Wohl *et al.* 2000). Decreased HLA-G expression in trophoblast tissue from first trimester RM is also evident with immunohistochemical methods. Interestingly, this was paralleled by an increased expression of CD16+ uNK cells (Emmer *et al.* 2002). Previously it was reported that CD16+ uNK cell counts are elevated in recurrent miscarriage (Lachapelle *et al.* 1996). Thus, HLA-G/NK cell interaction may have an effect on the phenotype of NK cells.

The Leukemia Inhibitory Factor (LIF) is produced in high amounts by the human endometrium and it stimulates HLA-G production in JEG3 choriocarcinoma cells (Bamberger *et al.* 2000). LIF has an important role in implantation in mice. It has been shown that there is a lack of LIF and Th2 type cytokines in decidual T-cells of women with a history of recurrent miscarriage (reviewed in Piccinni *et al.* 2001). Possibly decreased expression of HLA-G in trophoblasts from first trimester miscarriages is partly due to defective production of LIF and IL-10.

The importance of HLA-G expression by embryonal tissues was recently shown in a study describing that in vitro fertilization procedures were successful only when embryonal cells produced sHLA-G (Fuzzi *et al.* 2002).

2.5.3.2 HLA-G polymorphism in various populations and disease associations

Initially, HLA-G was considered to be quite monomorphic. Then, a study describing a population of African origin found HLA-G to be extremely polymorphic (van der Ven & Ober 1994). In that study, 32 nucleotide sequence variations within exon 3 of the HLA-G gene were observed. However, in a recent analysis of 45 Zimbabwean subjects of African origin, only 5 nucleotide substitutions within exon 3 were evident (Matte *et al.* 2002). The original finding of van der Ven and Ober has since been suggested to be an artifact due to unspecific primers (Bainbridge *et al.* 1999, Ishitani *et al.* 1999). Controversy concerning HLA-G as being highly polymorphic aroused some confusion about the putative central role of HLA-G as a tolerance molecule. As it currently seems, this problem has been solved, but the changing nomenclature of HLA-G alleles and newly discovered alleles still causes some uncertainty when comparing allele frequencies of older and more recent studies in different populations (Matte *et al.* 2000). Data about the frequencies of some of the rarest and the newest alleles are not available from some populations due to the fact that methodologies have not allowed typing of all alleles.

It may be concluded that in Caucasian populations, HLA-G*010101 is the most prevalent allele (32-62 %), the second most prevalent allele being *010102 (27-36 %). Frequencies of *010105, *010106, *010107 and *0102 are not shown as these alleles were either not typed or not found in the study populations (Matte *et al.* 2000).

Table 3. Table 3. HLA-G allele frequencies in different populations (According to Matte *et al.* 2000).

Allele	Danish (n=144)	German/ Croatian (n=344)	Portuguese (n= 117)	Spanish (n=228)	Japanese (n=82)	Hutterite (n= 160)	African Shona (n=216)
010101	0.62	0.32	0.37	0.38	0.43	0.46	0.39
010102	0.27	0.36	0.31	0.22	0.14	0.21	0.14
010103	0.05	0.07	0.17	0.07	0.05	0.02	0
010104	nd	nd	nd	nd	nd	0.04	0
010108	nd	0.09	nd	nd	nd	nd	0.14
0103	nd	0.02	0.02	0	nd	0.04	0
010401	0.05	0.06	0.13	0.11	0.38	0.20	0.20
010403	nd	nd	nd	nd	nd	nd	0.004
0105N	0.007	0.023	0	0.03	0	nd	0.11

Abbreviation: nd = not determined

A linkage disequilibrium between HLA-G and HLA-A has been demonstrated in several previous studies (Alvarez *et al.* 1999, Ober *et al.* 1996, Morales *et al.* 1993).

HLA-G exhibits low polymorphism with only few alleles with differences in protein structure, thus interest has been especially focused on those alleles that are associated with differences in expression and activity of membrane-bound and secreted HLA-G isoforms. Especially the allele 0105N with a single base-pair deletion (1597ΔC) in exon 3 of HLA-G is interesting in reproduction studies as this mutation abolishes the expression of the

full-length protein HLA-G1. However, expression of the HLA-E molecule is not decreased. Thus, NK lysis of 0105N expressing cells is inhibited as in cells expressing full-length HLA-G molecules (Sala F *et al.* 2004). Individuals homozygous for 0105N allele exist and they seem to be healthy (Castro *et al.* 2000). This also indicates that mHLA-G1 is not mandatory for survival. Association between 0105N and pre-eclampsia or intrauterine growth retardation has neither been observed (Aldrich *et al.* 2000).

However, a recent finding suggests that the presence of an HLA-G*0104 or HLA-G*0105N allele in either partner is associated with unexplained recurrent miscarriage (Aldrich *et al.* 2001). Also, a significant increase in *010103 and *0105N alleles in patients with RM was observed in a German population, the frequencies being especially increased in those RM women with 5 or more miscarriages compared to controls (29 % vs. 4 %, $p=0.001$) (Pfeiffer *et al.* 2001). HLA-G*0105N and *010103 alleles were associated with lower sHLA-G plasma levels (Rebmann *et al.* 2001). Significantly lower sHLA-G levels in plasma and amniotic fluid have also been observed in subjects carrying HLA-A11 (Rebmann *et al.* 1999). This probably is due to linkage disequilibrium between HLA-A11 and HLA-G*010103.

A 14 bp deletion polymorphism in the 3' untranslated region of the HLA-G gene is associated with HLA-G mRNA expression and low sHLA-G levels (Hviid *et al.* 2003, Rebmann *et al.* 2001). This polymorphism is also associated with fetoplacental growth (Hviid 2004), but not with RM (Hviid *et al.* 2002). Additionally, several polymorphic sites have been observed in the 5' promoter region in Hutterites. Carriage of the G-allele in the -725 site was found to be associated with increased risk of miscarriage. This allele may also down-regulate HLA-G transcription (Ober *et al.* 2003). In some studies no association between the subset of HLA-G alleles and RM have been observed (Penzes *et al.* 1999, Yamashita *et al.* 1999).

2.6 IL-10

2.6.1 The IL-10 gene, protein and function

IL-10 is a pleiotropic cytokine secreted by monocytes and T lymphocytes. IL-10 potently inhibits the production of e.g. IL-1 α and IL-1 β , IL-6, IL-12, IL-18, GM-CSF and TNF. Anti-inflammatory effects of IL-10 are especially due to its inhibitory activity on IL-1 and TNF production (reviewed in Moore *et al.* 2001). IL-10 enhances the production of anti-inflammatory IL-1 receptor antagonist after stimulation of monocytes with LPS (Jenkins *et al.* 1994). IL-10 also inhibits HLA class II expression of human monocytes (de Waal Malefyt *et al.* 1991), promotes B cell survival, proliferation and differentiation; inhibits cytokine production by Th1 cells and macrophages and is the effector molecule of some regulatory T cells (Fiorentino *et al.* 1991a, Fiorentino *et al.* 1991b, Akbari *et al.* 2003). Thus, IL-10 is an important regulatory cytokine involved in diverse areas of the human immune system.

The human IL10 gene is localised on chromosome 1 and contains five exons. After the discovery that several polymorphisms in the 5' promoter region of the human IL-10 gene may be associated with IL-10 production capacity, a considerable number of studies describing IL-10 genotype and allele frequencies have been published on a variety of diseases. Polymorphism at -1082G to A is associated with differences in *in vitro* IL-10 production. Here, lymphocytes with the GG genotype responded with significantly enhanced IL-10 secretion after ConA stimulation compared to those with the AA genotype (Turner *et al.* 1997). Furthermore, decreased IL-10 plasma levels in a Caucasoid population are associated with the -1082A allele (Koss *et al.* 2001). Other polymorphic markers in the 5' proximal promoter region have been found, of which -819C/T and -592C/A are in linkage disequilibrium with the -1082 site. In general, three different haplotypes have been observed in different populations (ATA, ACC and GCC) (Temple *et al.* 2001).

In the vicinity of the IL-10 gene there are two microsatellites with different numbers of CA tandem repeats 1.2 kb and 4.0 kb upstream from the transcription start site, which are associated with differences in IL-10 secretion (Eskdale *et al.* 1998).

Unaffected family members of SLE patients produce high levels of IL-10 (Llorente *et al.* 1997). Genetic influence on IL-10 production may thus be crucial in autoimmune diseases, but also in infectious diseases, as the family members of patients who had died from meningococcal infection showed significantly increased *in vitro* IL-10 production after LPS stimulation compared to the relatives of patients that had survived. Studies on monozygotic twins show that 75 % of the variation in IL-10 production is genetically determined (Westendorp *et al.* 1997).

IL-10 attenuates clearance of various pathogens. The polymorphisms that might be associated with genetic regulation of IL-10 are thus especially interesting in regard to infectious diseases.

Detected associations between IL-10 promoter polymorphisms and diseases range from classical autoimmune and infectious diseases to schizophrenia and longevity (Chiavetto *et al.* 2002, Lio *et al.* 2002).

In the Finnish population, plasma IL-10 concentrations were higher in patients with Sjögren syndrome carrying the GCC haplotype than in non-carriers (Hulkkonen *et al.* 2001). On the other hand, the ATA haplotype was associated with increased plasma IL-10 levels in healthy Finnish subjects (Helminen *et al.* 1999, Kilpinen *et al.* 2002).

Initially it was suggested that especially the G allele at -1082 and GCC haplotype would be associated with enhanced IL-10 secretion, but subsequently higher IL-10 plasma levels were found to be associated with the -1082A allele in the Dutch population (Huizinga *et al.* 2000). The -1082A allele confers a two fold increase in transcriptional activity of the IL-10 promoter (Rees *et al.* 2002). However, in another study a slight increase in transcriptional activity of the GCC haplotype was observed (Crawley *et al.* 1999).

New distal SNP markers in the promoter region of IL-10 gene may be more informative than the markers in the proximal promoter region used earlier. Using these markers (-3575T/A, -2849G/A and -2763C/A) together with -1330, -1082, -819 and -592, new extended haplotypes were found in association with high (T-G-C) or low (A-G/A-A) IL-10 production after LPS stimulation (Gibson *et al.* 2001). In healthy Dutch population -2849A/G genotypes were associated with differences in IL-10 production (GG high, GA medium, AA low) (Westendorp *et al.* 2001). The -2849G allele co-segregates with the -1082A allele in extended haplotypes described in this Dutch population (haplotype fre-

quency 0.45) and the -2849A allele segregates with the -1082G allele (frequency 0.33). In only 23 % of haplotypic combinations -2849G is found together with -1082G. Thus, the relative frequencies of these haplotypic combinations may explain the finding of the association of -1082A allele with enhanced production of IL-10 after LPS stimulation (Huizinga *et al.* 2000).

Indeed, contradictory findings have been published about the association of various alleles and haplotypes regarding IL-10 production. The differences may be due to ethnic variations or different stimuli (LPS, ConA etc.) used in *in vitro* experiments.

2.6.2 IL-10 in pregnancy

Enhanced secretion of anti-inflammatory Th2 cytokines is a characteristic feature in normal physiologic pregnancy. In RM, however, defective production of IL-10 and other Th2 cytokines has been shown in humans.

In normal pregnancy, abundant IL-10 is considered important. In RM, on the contrary, maternal decidual T-cells and mitogen-activated peripheral blood mononuclear cells show decreased expression of IL-10 (Piccinni *et al.* 1998, Marzi *et al.* 1996). There is also evidence of a diminished Th2 type immune response to placental and trophoblastic antigens in RM (Hill *et al.* 1995, Raghupathy *et al.* 1999). Serum IL-10 levels are also low in pre-eclampsia, another common disorder of pregnancy (Hennessy *et al.* 1999). Thus, IL-10 could be an important anti-inflammatory cytokine contributing to the maintenance of pregnancy.

During pregnancy IL-10 is also produced locally in the fetoplacental unit by cytotrophoblasts (Hanna *et al.* 2000) and it upregulates the HLA-G expression of cytotrophoblasts at the feto-maternal barrier. This has been suggested to protect the fetus from rejection (Moreau *et al.* 1999). IL-10 has important modulatory effects against pro-inflammatory cytokines, especially IFN- γ and TNF- α , which have been shown to be detrimental to the fetoplacental unit in a murine model (Chaouat *et al.* 1995). High serum TNF- α values have been reported in RM women (Mallmann *et al.* 1991, Szekeres-Bartho *et al.* 1996, Deneys & De Bruyere 1997). However, contradictory results have also been published (Schust & Hill 1996).

Genetic polymorphisms associated with high and low production of IL-10 may also play a role in RM. This question has been addressed in only a few studies thus far. However, no significant associations between IL-10 promoter haplotypes and RM were found in individual studies (Babbage *et al.* 2001, Senghore *et al.* 2002, Daher *et al.* 2003).

2.7 IL-1 complex

2.7.1 *IL-1 genes, proteins and functions*

The human IL-1 genes are located on chromosome 2, in position 2q13. There are three members of the IL-1 gene family, two agonists, IL-1 α and IL-1 β and one antagonist, IL-1 α . All three molecules bind to IL-1 receptors. IL-1 α and β are potent pro-inflammatory cytokines while IL-1 α is an anti-inflammatory cytokine and competes with IL-1 α and β for binding to IL-1 receptors. Secretion of IL-1 leads to a pro-inflammatory cascade, including Th-1 proliferation, production of TNF- α , IFN- γ , IL-2 and IL-12. IL-1 was the first endogenous pyrogen to be identified and it is involved in the induction of fever (reviewed in Arend *et al.* 1998, Mantovani *et al.* 1998). Together with IL-6, it stimulates the liver to produce acute-phase proteins such as CRP and fibrinogen (Dinarello 2000).

2.7.2 *IL-1 in reproduction*

Cytokines in the IL-1 system are produced at the fetomaternal interface during early pregnancy (Robertson *et al.* 1994). IL-1 has been implicated in implantation, trophoblastic growth and invasion (Simon *et al.* 1998, Librach *et al.* 1994).

RM patients may have elevated serum IL-1 β levels during the first trimester compared to women with a normal pregnancy outcome (Shaarawy & Nagui 1997). During the non-pregnancy state, no differences were evident in the IL-1 β serum levels between RM women and non-pregnant controls. Unfortunately, no information about the timing of the serum sampling with respect to the menstrual cycle was given in the study (Hefler *et al.* 2001). IL-1 β serum levels vary during the menstrual cycle, being highest during the secretory phase (Cannon & Dinarello 1985). In the mid-secretory phase, IL-1 β mRNA levels are lower in RM women compared to normal subjects (von Wolff *et al.* 2000).

Mice lacking a functional type 1 IL-1 receptor exhibit no profound reproduction deficiencies. Only litter sizes are slightly reduced (Abbondanzo *et al.* 1996). IL-1 agonist and antagonist levels vary among individuals and have been associated with common variations in IL-1 genes (Shirodaria *et al.* 2000, Pociot *et al.* 1992, Hurme & Santtila 1998).

An 86-bp tandem repeat has been described in the intron 2 of the IL1RN gene. At least 5 alleles (with 2 to 6 repeat units) have been detected (Tarlow *et al.* 1993). The most frequent allele is the four-repeat allele (IL1RN*1). The second commonest allele in different populations is the two-repeat allele (IL1RN*2), which has been associated with the severity of several inflammatory and autoimmune diseases, such as ulcerative colitis, multiple sclerosis, rheumatoid disease, psoriasis, alopecia areata and coronary heart disease (reviewed in Tazi-Ahnini *et al.* 2002). It is also associated with increased activity of IL-1 β , but the underlying mechanism is not known (Santtila *et al.* 1998).

An association between IL1RN*2 and RM has been recently reported among Austrian women (Unfried *et al.* 2001). The IL1RN*2 association in the Austrian population suggests

that IL-1 β has a functional role in RM, as subjects with IL1RN*2 tend to show enhanced IL-1 β activity (Santtila *et al.* 1998). It is also possible that increased IL-1ra production associated with the IL1RN*2 allele is responsible for abortions, as IL-1ra prevents embryonic implantation in a murine model (Simon *et al.* 1998).

Other polymorphisms in the IL-1 signalling pathway have also been shown to correlate with IL-1beta production. One of these, the G \rightarrow A nucleotide substitution at +3954 of IL1B, has been recently examined in 17 British and 131 Austrian RM patients. However, no differences in allele frequencies were found in either of the studies (Reid *et al.* 2001, Hefler *et al.* 2001). A recent study showed no evidence of an association between a polymorphism in the promoter region of the IL1B gene (position -511) and RM in a Caucasian population (Hefler *et al.* 2002). However, in another population that consisted of women of US Caucasian origin, -511C and -31T in the IL1B promoter region were shown to be associated with RM. Women with RM carrying the -511C allele also had increased IFN-gamma production compared to non-carriers when their PBMC were stimulated with a protein extract from trophoblast cell line Jeg-3 (Wang *et al.* 2002).

In this study however, no association between IL1RN*2 and RM was observed. Thus, partly conflicting results have been reported on associations between IL-1 complex polymorphisms and RM.

2.8 CD14

2.8.1 The CD14 gene, protein and function

The CD14 gene is localized on chromosome 5q31.1. CD14 is a pattern recognition receptor for several microbial products and apoptotic cells (Gregory 2000, Kaisho & Akira 2001). It is mainly expressed in monocytes, neutrophils and hepatocytes. Additionally, a soluble form of the CD14 molecule (sCD14) may be found in serum due to shedding from cell membranes. Especially hepatocytes may be a source for sCD14 production (Liu *et al.* 1998, Pan *et al.* 2000).

The activation of monocytes/macrophages by LPS mainly takes place through a CD14-dependent pathway (Wright *et al.* 1990). sCD14 mediates the effects of lipopolysaccharide (LPS) and also of other microbial products to the cells lacking membrane-bound CD14 (Haziot *et al.* 1993, Loppnow *et al.* 1995, Pugin *et al.* 1993). sCD14 is elevated in many chronic infectious and inflammatory conditions including borreliosis, tuberculosis, periodontal disease and Kawasaki disease (Hayashi *et al.* 1999, Juffermans *et al.* 1998, Lin *et al.* 2000, Takeshita *et al.* 2000). CD14 levels in plasma rise rapidly by 45 to 75 % during endotoxemia, which is compatible with the criteria for an acute-phase protein (Landmann *et al.* 1995, Landmann *et al.* 1996).

sCD14 is a multifunctional molecule. In addition to being a receptor to LPS and other bacterial structures, it may modulate LPS-triggered apoptosis (Frey & Finlay 1998, He *et al.* 1998). sCD14 may also regulate T and B lymphocyte activation and function (Arias *et al.* 2000, Rey Nores *et al.* 1999). sCD14 may regulate human B cell function by enhancing

IgG1 and suppressing IgE production in activated tonsillar B cells and Ag-stimulated PBMC through CD40-dependent interaction (Arias *et al* 2000).

2.8.2 CD14 polymorphism and disease associations

A C-to-T transition polymorphism at position –159 in the promoter region of the CD14 gene has been shown to influence sCD14 levels (Baldini *et al* 1999, Karhukorpi *et al.* 2002) and also mCD14 expression on monocytes (Hubacek *et al.* 1999). CD14 –159 polymorphism is associated with Crohn's disease, which suggests that genetically determined differences in the innate immune response against bacteria or bacterial endotoxins may be involved in the pathogenesis of inflammatory bowel disease (Klein *et al.* 2002, Klein *et al.* 2003). An association between the TT genotype of the CD14 gene and myocardial infarction has been recently reported in different populations (Hubacek *et al.* 1999, Shimada *et al.* 2000, Unkelbach *et al.* 1999). Conflicting data have also been presented, as no association between CD14 –159 polymorphism and the risk of myocardial infarction was observed in rather large studies (Koch *et al.* 2002, Koenig *et al.* 2002, Nauck *et al.* 2002, Longobardo *et al.* 2003). No association between carotid atherosclerosis and CD14 polymorphism was observed in one study (Ito *et al.* 2000). However, another study suggests that carotid atherosclerosis is associated with the CC genotype in smoking subjects (Risley *et al.* 2003).

CD14 polymorphism, being putatively an important risk factor for atherosclerosis or myocardial infarction, might provide new evidence for a role of lipopolysaccharides and gram-negative bacteria in pathogenesis of atherosclerosis (Kane & Havel 1999). Similarly, as endotoxin may confer risk to RM, it is important to study CD14 polymorphism in the recurrent miscarriage setting as well.

2.8.3 The possible role of endotoxin in recurrent miscarriage

Although immunostimulation by endotoxin may have beneficial effects during pregnancy such as accelerated maturation of fetal lungs (Bry & Lappalainen 2001), it may also have adverse effects during pregnancy. A theory of the deleterious role of endotoxin also in human pregnancies has also been presented (Clark *et al.* 2002).

The activated state of the innate immune system during pregnancy may also modulate the immune response against endotoxin. Supposing that the decrease in lymphocyte responsiveness during pregnancy would not be compensated for by activation of the innate immune system, pregnant women would perhaps be even more susceptible to infections (Sacks *et al.* 1999). Thus, the monocyte/ macrophage and the granulocyte have a more important role to play in pregnancy related immunological adaptation than generally anticipated. Pregnant animals responding vigorously and often fatally to injection of endotoxin have been presented as a hallmark of the activated immune system during pregnancy (Vizi *et al.* 2001).

During pregnancy, there are several inflammatory changes in cell populations of the innate immune system. These include increased numbers and phagocytic capacity of monocytes and granulocytes (Koumandakis *et al.* 1986) and enhanced expression of CD11b, CD14 and CD64 (Sacks *et al.* 1998, Faas *et al.* 2000). Thus, activation of the innate immune system during pregnancy has been observed. This may be due to pregnancy related hormonal factors. *In vitro* experiments suggest that human placental lactogen, which is normally produced only during pregnancy, increases significantly CD14 expression on monocytes (Cranny *et al.* 2002).

It has been proposed that endotoxin derived from bacteria of the normal intraluminal flora might play a role in RM as endotoxin is a potent inducer of the pro-inflammatory immune response (Clark *et al.* 2001). Thus, no manifestations of clinical infection may be present and still there is increased endotoxin exposure due to endotoxin leakage from the alimentary tract.

Harmful effects of endotoxin on a growing conceptus have been shown in animal models. Endotoxin has been used to induce abortions especially in mice. In BALB/c mice, LPS-induced nitric oxide (NO) production leads to fetal rejection (Athanasakis *et al.* 2000). NO production after LPS stimulation is mediated by CD14 receptors (Moon *et al.* 2000). Generally, NO has a beneficial role in implantation and decidualization, but in high concentrations it has toxic effects and causes embryonic resorption (Ogando *et al.* 2003). Trophoblasts in the placenta also express CD14 molecules and are thus vulnerable to the LPS attack (Okada *et al.* 1997). LPS may also suppress the endocrine functions of trophoblasts (Okada *et al.* 1997).

In an animal model, endotoxin treatment of pregnant mice resulted in enhanced TNF-alpha production and decreased IL-10 production demonstrating that systemic stimulation with endotoxin causes Th1 type innate immune response that may be detrimental to the fetus (Vizi *et al.* 2001). However, in humans, decidual macrophages did not respond with pro-inflammatory IL-1beta and TNF-alpha production upon endotoxin stimulation (Heikkinen *et al.* 2003). This shows, that locally at the fetomaternal interface, the innate immune system also has immunoinhibitory functions.

Gut-derived endotoxin may have a role in some other pathologic conditions including heart failure and alcoholic cirrhosis (Niebauer *et al.* 1999, Thurman 1998). It seems that genetically determined immune response to endotoxin contributes to the development of tissue injury in alcohol liver disease. In the Finnish population, high-producing CD14 genotypes confer susceptibility to the effects of gut-derived endotoxin in alcoholic liver disease. Especially the TT-genotype associated with enhanced expression of CD14 confers a 3- to 4- fold risk of liver damage (Järveläinen *et al.* 2001). Earlier studies had already shown that, in alcoholic liver injury, gut-derived LPS may contribute to hepatic pathology by binding to the CD14 receptors on Kupffer cells and monocytes and hence initiate the cascade that leads to severe inflammation and release of cellular activation products such as proinflammatory cytokines IL-1 and TNF-alpha (reviewed in Thurman 1998). A correlation between endotoxin levels and liver pathology has also been observed in experimental alcoholic cirrhosis (Nanji *et al.* 1993). In animal models, both acute and chronic ethanol exposure increases CD14 expression in liver showing increased sensitisation to endotoxin (Wheeler & Thurman 2003).

In animal models, it has been shown that Kupffer cell CD14 expression and plasma endotoxin levels are significantly higher in female compared to male rats on an

ethanol-containing diet. This is due to the effect of estrogen (Kono *et al.* 2000). Thus, especially women may be at an enhanced risk of developing detrimental effects caused by gut endotoxin. Estriol treatment in animal models increases gut permeability to endotoxin (Enomoto *et al.* 1999). During pregnancy, sensitivity to endotoxin *in vivo* is probably even increased when estrogen levels are high (Aplitz 1935).

Women develop alcohol-induced liver injury more rapidly than men. Even moderate alcohol consumption during pregnancy is associated with an increased risk of first-trimester spontaneous abortions (Windham *et al.* 1997, Kesmodel *et al.* 2002). Besides teratogenic effects that may lead to fetal alcohol syndrome, chronic alcohol use during pregnancy stimulates fetal cytokine synthesis and secretion. Especially IL-1 beta, IL-6 and TNF-alpha production is induced (Ahluwalia *et al.* 2000). Thus, it was suggested that immunological mechanisms are related to early pregnancy loss, fetal death, fetal growth impairment, and preterm labor which all are common sequelae of alcohol use during pregnancy. It is not clear how alcohol use results in increased cytokine expression, but increased endotoxin permeability is one of those factors that might be responsible for this (Ahluwalia *et al.* 2000, Nair *et al.* 1994).

As pregnancy-specific hormonal factors (placental lactogen and increased estrogen) enhance CD14 expression and increase gut permeability to endotoxin, it is intriguing to speculate about the role of endotoxin also in RM.

Treatment with non-steroidal anti-inflammatory analgetics is related to reproduction failures in animals. This may be due to endotoxin effects. Mucosal lesions caused by indomethacin treatment may also lead to increased leakage of endotoxin from the gut into the peritoneal space and cause abortion in rodents (Clark *et al.* 1993). In a large Danish study, recurrent miscarriage in humans was associated with the use of non-steroidal anti-inflammatory analgetics (Nielsen *et al.* 2001). Whether adverse outcome of pregnancy during the use of NSAIDs is due to endotoxin leakage from the gut is currently unknown. Other explanations may also exist as prostaglandins have an effect on Th1/Th2 balance. PGE2 may suppress Th1 type immune response by stimulating IL-10 release. Indomethacin treatment inhibits PGE2 synthesis and IL-10 production at least in elderly people (Bour *et al.* 2000). Thus, indomethacin may lead to an imbalance between Th1/Th2 type immune response contributing to an increased risk of early pregnancy loss by this mechanism.

If the theory about the link between miscarriage and endotoxin holds true, it could be argued that adverse pregnancy outcomes should be more common in some common chronic infective conditions in which chronic endotoxin exposure might take place. Periodontal diseases and *H.pylori* infections are common infectious diseases in humans. Bacterial products, such as LPS and pro-inflammatory cytokines IL-1 beta, TNF-alpha and other inflammatory mediators (PGE2) originating from periodontal tissues, may affect tissues distant from oral cavity. Periodontal disease of the mother is associated with low birth weight in newborns (Li *et al.* 2000).

At first, chronic *H. pylori* infection was not considered to have any major negative impact on reproduction capacity because the infection typically manifests itself only after reproductive age (Blaser 1997). Recently however, *H.pylori* has been linked as a causative factor for idiopathic short stature possibly due to its role in causing iron deficiency anemia (Choe *et al.* 2000). Chronic *H. pylori* infection of the mother may be also associated with fetal growth restriction (Eslick *et al.* 2002). In a mouse model, *H.pylori* infection caused abortions and fetal growth restriction. *H.pylori* infection may locally activate endometrial

macrophages and alter Th1/Th2 balance which may lead to fetal resorption (Rossi *et al.* 2004). Whether *H.pylori* contributes to early pregnancy loss in humans is not clear. Two studies suggest that *H.pylori* is probably not able to colonize the vagina and thus cannot cause bacterial vaginosis (Minakami *et al.* 2000, Martin-De-Argila *et al.* 1998). Despite of these negative results, in a hypothetical model, *H.pylori* might exist in the vaginal niche and even be transmitted through oral-genital contact (Eslick 2000).

Mucosal damage due to *H.pylori* infection or due to NSAID use may be associated with systemically increased sCD14 levels (Karhukorpi *et al.* 2002). Whether this phenomenon is related with increased leakage of endotoxin through gut and systemic immunostimulation is not known.

3 Aims of the study

The aims of the study were as follows:

1. To examine associations between recurrent miscarriage and polymorphisms of the immunomodulatory genes that may play a role during pregnancy. The genes chosen for this study were those that are either expressed at the fetomaternal interface (HLA-G) or those whose production may be impaired in women with recurrent spontaneous miscarriage (IL-10). Additionally, a polymorphism of the IL1RN gene was analyzed as there is some evidence that it is linked to recurrent miscarriage in a Caucasian population other than Finns.
2. As there was only scarce information about HLA-G in Caucasian populations at the time the study was initiated, the allelic distribution of HLA-G and its linkage disequilibrium with the HLA-A locus was studied in two-generation families of Finnish origin.
3. As the methods initially used to genotype the IL-10 promoter region polymorphism were cumbersome, an attempt was made to develop new methods for studying IL-10 -1082A/G polymorphism.
4. Lipopolysaccharide (LPS) derived from gut microbial flora may be a pathogenic factor in recurrent miscarriage and increased sCD14 levels may be associated with Th1 bias. Therefore CD14 (LPS-receptor) genotypes of women with recurrent miscarriage were determined to find out whether CD14 polymorphism is associated with recurrent miscarriage.

4 Subjects and methods

4.1 Study populations

For study I, DNA samples from twenty-six, two-generation Finnish families were collected. The families consisted of a mother, father and 1-2 children. All the families, consisting of 97 subjects altogether, had been recruited earlier for immunogenetic studies performed at the Department of Medical Microbiology, University of Oulu, Finland.

Thirty-eight couples with a history of at least three unexplained consecutive recurrent miscarriages in early pregnancy were included in studies II, III, V and VI. These couples belong to the original study group of the 59 couples with recurrent miscarriage (Laitinen *et al.* 1993). All women (mean age 33 yrs, range 26-46) with miscarriages (mean number of miscarriages three, range 3-8) had regular menstrual cycles and were healthy. Parental karyotypes were analyzed but no fetal cytogenetic analyses were done. The women underwent careful clinical examinations including hysteroscopy and serial endometrial biopsies, as well as analyses of tissue antibodies and autoantibodies. Plasma glucose and thyroid stimulating hormone concentrations were also measured. In addition, a possible infectious aetiology was studied by assessing the immunoglobulin (Ig)G and IgM antibodies to cytomegalovirus and *Toxoplasma gondii*. Moreover, microbiological samples were obtained from the cervix and uterine cavity during hysteroscopy, and *Chlamydia trachomatis*, *Neisseria gonorrhoea*, *Listeria monocytogenes* and herpes simplex viruses were cultured according to Tulppala and her co-workers (Tulppala *et al.* 1993). No cytogenetic analysis was done for fetal tissues.

No obvious cause for RM was found in our study group and the miscarriages were thus classified as unexplained. A total of 27 women had no previous children and 11 had had one to two children before the consecutive miscarriages.

Couples of study I were chosen as a reference group representing a population with normal reproduction capacity for the HLA-G polymorphism study (II). In studies III, IV and VI, healthy adults of Finnish origin served as the reference group. These subjects belong to the original study group of 199 subjects of whom various immunogenetic and infectious parameters had been determined in earlier studies (Karhukorpi *et al.* 1999). In study V, the

reference group consisted of 800 healthy adult (18-60 years of age) blood donors of whom the IL1RN genotype had been determined earlier at University of Tampere (Hurme 1998).

The samples from couples with recurrent miscarriage were studied with the approval of the joint ethical committee of the Oulu University Hospital and the University of Oulu and with informed consent of the subjects belonging to the reference groups in studies II, III, IV, V and VI.

Table 4 summarises the subjects and immunogenetic methods used in this study.

Table 4. Study and reference populations.

Series	Number of study subjects	Number of reference subjects	Methods
I	97, belonging to 26 two-generation families	None	HLA-G genotyping with PCR-RFLP
II	38 couples suffering from RM	26 random healthy couples with at least one child from study I	HLA-G genotyping with PCR-RFLP
III	38 women with RM	131 healthy Finnish adults	IL-10 genotyping with ARMS-PCR method
IV	94 healthy Finnish adults	To validate the method 36 DNA samples belonging to control group in study III were sequenced	IL-10 genotyping with one-tube PCR method
V	38 women with RM	800 blood donors	IL1RN genotyping with PCR
VI	38 women with RM	127 healthy Finnish adults	CD14 genotyping with PCR

4.2 Methods

4.2.1 DNA extraction methods

Genomic DNA was extracted from peripheral blood lymphocytes by using the guanidium-isothiocyanate method used at the Department of Medical Microbiology, University of Oulu. DNA extraction from couples with recurrent miscarriage had been previously conducted at the Finnish Red Cross Blood Transfusion Service, Tissue Typing Laboratory using the salting-out method (Miller *et al.* 1988). Likewise, DNA from the 800 blood donors in study V had been previously extracted at the Department of Microbiology and Immunology, University of Tampere Medical School.

4.2.2 Genotyping methods

4.2.2.1 HLA-G genotyping (I, II)

HLA-G genotyping was done by a PCR-RFLP method (Morales *et al* 1993). With this method four different HLA-G alleles can be distinguished according to the presence of the restriction site for each enzyme in individual alleles. Conditions for PCR were as follows: The reaction volume was 50 µl, primer concentrations were 0.5 µM, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, pH 8.3, 0.2 mM of each dNTP and 2U Taq-polymerase (Boehringer Mannheim, Germany). The average amount of DNA used was 0.5µg per each PCR reaction. The cycling conditions were as follows: an initial 5-min denaturation at 94°C, followed by 32 cycles at 94°C for 1 min and 60°C for 1 min. Amplified products were digested with allele-specific restriction endonucleases: exon 2 products with HinfI and MspI and exon 3 products with AclI according to the protocol recommended by the manufacturer (Boehringer Mannheim, Germany).

When performing studies I and II for typing the allele frequencies in Finland and comparing the frequencies between RM and control subjects, the allele classification was based on GI-IV groups. Later on the grouping has been abandoned and the alleles are numbered separately using 4 to 6 numbers as the allele codes. In HLA-G**I* (representing 010101), the MspI site is present at codon 57 of exon 2, the HinfI site is not present at codon 31 of exon 2 and the AclI site is present at codon 107 of exon 3, yielding the restriction pattern (+,-,+). The pattern for HLA-G*0103 is +,+,+ and that for HLA-G*010103 is -,,-. Additionally, allele HLA-G**II*, which is not an officially recognized allele, yields a restriction pattern -,,-+. The restriction patterns were determined by analyzing the digestion results in UV-light in ethidium bromide stained 1.5 % agarose with 150 V. HLA-A typing was done using a standard lymphocytotoxicity assay.

According to the current nomenclature of HLA-G alleles the HLA-G**I* is equivalent for 010101, G**II* for 010102, G**III* for 0103 and G**IV* for 010103 (Marsh *et al*. 2002). However, HLA-G**I*, *II* and *IV* may include several other alleles that are quite rare in Caucasian populations (Matte *et al*. 2000). Two officially recognized alleles (01042 and 01015) remain totally untypable with the method used here (Table 5).

Table 5. Officially recognized HLA-G alleles corresponding to allele groups detected in this study. Sequence alignments according to Marsh SG (2005). http://www.anthonynolan.com/HIG/seq/nuc/text/g_nt.txt

G* <i>I</i>	G* <i>II</i>	G* <i>III</i>	G* <i>IV</i>	Untypable
010101	010102	0103	010103	010402
010104	010108		010107	(codon 57: CCC)
010106	010401			010105
0102	0105N			(codon 57 as in 010101, but
010403	0106			codon 107 as in 010103)

4.2.2.2 *IL-10* genotyping (III, IV)

IL-10 -1082G→A genotyping was done using the ARMS-PCR method in which both alleles were amplified in separate PCR-tubes. Also, a method in which both alleles could be amplified simultaneously in one-tube was developed.

The two-tube method:

The target sequences for the primers were obtained from GenBank (accession number X78437). An additional mismatch (underlined) was inserted into the 3' penultimate nucleotide of allele-specific forward primers to prevent unspecific amplification of the opposite allele.

The A allele was amplified with the forward primer *fpena* (5'-AAC ACT ACT AAG GCT TCT TTG GGT A-3') and the G allele was amplified with the primer *fpeng* (5'-AAC ACT ACT AAG GCT TCT TTG GGT G-3'). The primer *grev2* (5'-GTA AGC TTC TGT GGC TGG AGT C-3') was used as a reverse primer in both reactions. These reactions amplify allele-specific fragments of 161 bases of the promoter of the *IL-10* gene. An additional upstream sense primer, *afor3* (5'-TTT CCA GAT ATC TGA AGA AGT CCT G-3'), was included in both PCR reactions to amplify an internal control band (313 bp) with the reverse primer *grev2*. PCR was performed in a total volume of 10 µl (100 ng of genomic DNA, 1 x PCR buffer with 1.5 mM MgCl₂, 200 µM of each nucleotide, 0.8 U Taq polymerase and 0.5 µM of each primer). The cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec and 63°C for 30 sec. The final extension step was at 72°C for 5 minutes. The PCR products were visualized under UV light with ethidium bromide stain after agarose gel electrophoresis.

One-tube method:

Blood samples were obtained from 94 healthy persons, and genomic DNA was extracted using the standard protocol. The -1082 A and G alleles of the *IL-10* promoter region were amplified in a single PCR reaction tube with the following primers (position shown in brackets):

<i>afor3</i>	(-1258/-1234):	5'-TTTCCAGATATCTGAAGAAGTCCTG-3'
<i>fpena</i>	(-1106/-1082):	5'-AACACTACTAAGGCTTCTTTGGG <u>T</u> A-3'
<i>revp1</i>	(-990/-966):	5'-TCTAAAGTTAAAAGATGGGGTGGGA-3'
<i>rpeng</i>	(-1082/-1059):	5'-CTCTTACCTATCCCTACTTCCCG <u>C</u> -3'

The target sequences for the primers *revp1* and *rpeng* were similarly obtained from GenBank sequence data (accession number X78437) as in the two-tube method. One additional mismatch (underlined) was inserted into the 3' penultimate nucleotide of the allele A-specific forward primer and the allele G-specific reverse primer to prevent non-specific amplification of an opposite allele.

The PCR conditions were as follows: 1 x PCR buffer with 1.5 mM MgCl₂, 200 µl of each dNTPs, 0.8 U Taq polymerase (all reagents supplied by Perkin Elmer), 0.5 µM each primer and approximately 100 ng of genomic DNA. Reaction volume was 20 µl. The following cycle conditions were used: 95°C, 5 min; 30 cycles of 95°C, 30 s, 60°C, 30 s; 72°C, 5 min with a Perkin Elmer 9600 thermal cycler. The PCR products were separated on a 1.5 % agarose gel and visualised in UV light with ethidium bromide staining. The primers *fpena* and *revp1* amplify a 141 bp product for the A allele, while the primers *afor3* and

rpeng respectively amplify a 200 bp product for the G allele, and the outermost primers afor3 and revp1 amplify a 293 bp product, serving as an internal control.

4.2.2.3 Validation of IL-10 genotyping methods.

The two-tube and one-tube mismatch PCR methods were validated by comparing the PCR results of 36 samples with the results obtained by sequencing. The IL-10 promoter region was first amplified (between -1258 and -936) with the primers grev2 (position -957/-936): 5'-GTA AGC TTC TGT GGC TGG AGT C-3' and afor3 (sequence and position shown above). The PCR conditions were as above, except that only the primers grev2 and afor3 were used. The PCR products were purified with the Quickstep PCR purification kit (Edge Biosystems), and cycle sequencing was done with the reverse primer grev2, a cycle sequencing ready reaction kit and a genetic analyzer (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and ABI 373 DNA Sequencer, respectively; Perkin-Elmer, Foster City, CA).

4.2.2.4 IL-1RA genotyping (V)

The following primer pair was used for PCR amplification: 5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3' (Tarlow *et al.* 1993). The samples were denatured for 5 min at 94°C and subjected to 35 cycles, each of 1min at 94°C, 1min at 60°C, and 1 min at 70° C, and finally elongated for 5 min at 72°C. The products were resolved on 2 % agarose gel electrophoresis, stained with ethidium bromide and photographed under UV illumination. The four-repeat allele (IL1RN*1) corresponds to the 410 bp product, the two-repeat allele (IL1RN*2) to the 240 bp product and the five-repeat allele (IL1RN*3) to the 500 bp product. No other alleles were found in the Finnish sample sets. The genotyping of 800 control samples was done at the Department of Microbiology and Immunology, University of Tampere Medical School.

4.2.2.5 CD14 genotyping (VI)

A bidirectional allele-specific single-tube PCR method was set up to determine -159C/T alleles in the promoter region of the CD14 gene of the study subjects using same principle as in the IL-10 genotyping. Target sequences for partly overlapping allele-specific primers (cfors for the C allele and trevs for the T allele) were obtained from GenBank (Accession number X74984). An additional mismatch was inserted at the penultimate 3' nucleotide (underlined) of the allele-specific primers to increase the specificity of the PCR reaction. Additionally, two previously described outer primers were used (Hubacek

et al. 1999). The method has been described in our earlier study (Karhukorpi *et al.* 2002). The primer sequences were as follows:

cfors: 5'-CTC CAG AAT CCT TCC TGT TAC GAC-3'

trevs: 5'-TGT AGG ATG TTT CAG GGA GGG GTA-3'

cdp1: 5'-TTG GTG CCA ACA GAT GAG GTT CAC-3' (Hubacek *et al.* 1999)

cdp2: 5'-TTC TTT CCT ACA CAG CGG CAC CC-3' (Hubacek *et al.* 1999)

PCR was performed in a total volume of 10 μ l (50-100ng of genomic DNA, 1 x PCR buffer with 1.5 mM MgCl, 0.2 mM of nucleotides, 0.5 U Taq polymerase and 0.5 μ M of CD14 primers). The following reaction conditions were used: an initial denaturation at 95° C for 5 min followed by 30 cycles at 95° C for 30 s, at 60 C for 30 s, and at 72° C for 1 min. The final extension step was at 72° C for 5 min. The PCR products were visualized by electrophoresis in 2 % (w/v) agarose gel stained with ethidium bromide. The PCR method was validated by determining the CD14 alleles of 50 samples with the PCR-RFLP method described earlier (Baldini *et al.* 1999).

4.2.3 Statistical methods

Fisher's exact test and Chi-square test were used in allele and genotype frequency comparisons. Bonferroni correction was used in study II. Odds ratios with 95 % confidence intervals were calculated in studies V and VI.

5 Results

5.1 HLA-G polymorphism in a Finnish population and in couples with recurrent miscarriage (I, II)

All of the 97 DNA samples could be genotyped and the allele frequencies were calculated from parental DNA samples (n = 52). Linkage disequilibrium with HLA-A could be determined by comparing inherited combinations of HLA-A and HLA-G in the second generation samples.

Table 6. Distribution of HLA-G allele frequencies in a Finnish population and linkage disequilibrium between HLA-A and HLA-G loci in a Finnish population % (n). Current HLA-G allele equivalents are shown in parenthesis according to Marsh et al. 2002.

Alleles	GI (010101)	GII (010102)	GIII (0103)	GIV (010103)	Frequency
A1	0	0.134	0	0	0.134
A2	0.356	0	0	0	0.356
A3	0.211	0	0	0	0.211
A9=A23,24	0	0.095	0	0	0.095
A10	0	0.029	0	0	0.029
A11	0	0	0	0.048	0.048
A28	0	0.058	0	0	0.058
A30	0	0.048	0	0	0.048
A31	0	0.010	0	0	0.010
A32	0.010	0	0	0	0.010
Frequency	0.577 (60)	0.375 (39)	0	0.048 (5)	1.00 (104)

There were two common alleles, namely G*I and G*II, the distributions of which were 0.577 and 0.375. The frequencies of G*I and G*II are quite equivalent to the present alleles 010101 and the combination of 010102, 010401 and 0105N.

HLA-G*I (010101) was in linkage disequilibrium with HLA-A2 and -A3. All five HLA-G*IV (010103) positive subjects had HLA-A11 antigen showing association bet-

ween *010103 and HLA-A11. G*II (010102) was associated with several serological HLA-A specificities indicating that G*II contains several alleles. No HLA-G*III (0103) alleles was present here (Table 6).

No statistically significant differences in allele frequencies were observed between couples with recurrent miscarriage and the reference group (Table 7). No (G*IV) G*010103 positive women with recurrent miscarriage were detected. Thus carriage frequencies of *010103 positive women in recurrent miscarriage group and reference group were 0 % vs 15.4 % (uncorrected $p=0.024$, corrected $p=ns$).

Table 7. Allele frequencies of HLA-G in 38 couples with recurrent miscarriage and in 26 reference couples. Values are given as % (n).

Allele	Couples with recurrent miscarriage		Reference couples		
	Women	Men	Women	Men	All
GI	71 (54)	53 (40)	54 (28)	63 (32)	58 (60)
GII	29 (22)	39 (30)	38 (20)	35 (20)	37 (39)
GIII	0	0	0	0	0
GIV	0	8 (6)	8 (4)	2 (1)	5 (5)
All	100 (76)	100 (76)	100 (52)	100 (52)	100 (104)

HLA-G allele sharing between couples with recurrent miscarriage and reference couples:

No significant differences in rates of allele sharing were observed in the two groups. The mean number of shared HLA-G alleles was 1.2 in the RM group and 1.1 in reference group respectively.

5.2 IL-10 -1082 polymorphism in women with recurrent miscarriage (III,IV)

The IL-10 polymorphism was analyzed in 38 women with recurrent miscarriage and 131 ethnically compatible reference subjects. There were no significant differences in the IL-10 (-1082G→A) genotype or allele frequencies between the two groups (Table 8).

The allelic frequencies of IL-10 -1082 in our reference population closely resembled those published earlier in Finland (Helminen *et al.* 1999).

Table 8. Allele and genotype frequencies of the IL-10 –1082 polymorphism in women with recurrent miscarriage and in the reference group. There were no significant differences between the groups.

Allele	Women with recurrent miscarriage (n=38)		Reference group (n=131)	
	n	%	n	%
A	42	55.3	152	58.0
G	34	44.7	110	42.0
Genotype				
AA	13	34.2	44	33.6
AG	16	42.1	64	48.9
GG	9	23.7	23	17.5

All the samples in the series could be genotyped with the PCR method described. The PCR method was validated by determining the IL-10 -1082 alleles of 36 samples by sequencing (ABI Prism, Perkin Elmer).

The genotyping results of the PCR methods and sequencing were identical in all 36 samples that were sequenced. In all, 11 of 23 A/A homozygous, 11 of 24 G/G homozygous and 14 of 47 A/G heterozygous samples were sequenced. All ninety-four samples were typable with the one-tube method in the first run, and no confirmatory PCR was hence needed in this material.

5.3 IL-1RA intron 2 polymorphism in women with recurrent miscarriage (V)

The IL1RN polymorphisms were successfully analyzed in 37 women with recurrent miscarriage and 800 ethnically compatible healthy control subjects (Table 9). Genotyping of women with recurrent miscarriage was done in two different occasions by different persons in a blind fashion. The results obtained in both experiments were identical. One DNA sample of the original sample set of 38 women with RM was not typable because of the low amount of DNA in the diluted sample/low quality of DNA. Genotype frequencies did not significantly deviate from the Hardy-Weinberg expectation. There were no significant differences in the frequencies of IL1RN*1 and IL1RN*2 between the RM women and the reference group. The least frequent IL1RN*3 allele, however, was significantly more frequent among women with recurrent miscarriage (4/37=11%) compared to the reference group (17/800=2%, $P=0.006$; OR 5.6; 95 % CI 1.5-19.0). The frequency of IL1RN*1 not homozygosity was not significantly lower among women with recurrent miscarriage compared to the reference group (32 vs. 47 %, $P=0.12$).

Table 9. Genotype and allele frequencies of the IL1RN polymorphism among Finnish women with recurrent miscarriage and adult blood donors. Values are given as % (n).

IL1RN geno- type	Women with recurrent miscarriage (n=37)	Controls (n=800)	Odds ratio (95 % CI)	P value
1/1	32.4 (12)	46.8 (374)	0.5 (0.2-1.2)	0.12
1/2	51.1 (19)	42.9 (343)	1.4 (0.7-2.9)	0.40
2/2	5.4 (2)	8.3 (66)	0.6 (0.04-2.8)	0.70
1/3 or 2/3	10.8 (4)	2.1 (17)	5.6 (1.5-19.0)	0.006
IL1RN allele	74 chromosomes	1600 chromosomes		
1	62.2 (46)	69.0 (1104)	0.7 (0.4-1.2)	0.3
2	32.4 (24)	29.9 (479)	1.1 (0.7-1.9)	0.7
3	5.4 (4)	1.1 (17)	5.3 (1.5-17.4)	0.006

5.4 CD14 –159 polymorphism in women with recurrent miscarriage (VI)

No association between recurrent miscarriage and CD14(-159C/T) polymorphism was evident in this study. CD14 allele and genotype frequencies in women with recurrent miscarriage and the healthy reference population are shown in Table 10.

Table 10. Allele and genotype frequencies of the –159C/T polymorphism in the CD14 gene promoter in women with RM and in the control group. There were no significant differences between the groups.

	Women with recurrent miscarriage (n=38)		Reference group (n=127)		Odds ratio (95 % CI)	P value
	n	%	n	%		
Allele						
C	45	59.2	164	64.6	0.797 (0.471-1.346)	0.417
T	31	40.8	90	35.4	1.255 (0.743-2.122)	0.395
Genotype						
C/C	16	42.1	55	43.3	0.952 (0.457-1.982)	1.000
C/T	13	34.2	54	42.5	0.452 (0.330-1.498)	0.452
T/T	9	23.7	18	14.2	1.879 (0.765-4.617)	0.210

6 Discussion

6.1 The rationale for measuring immunogenetic parameters in recurrent miscarriage

There still is controversy about the functional significance of different immunological factors in reproduction. Genetic studies may provide clues for the evaluation of the importance of different immunological molecules also in recurrent miscarriage. This is true especially when frameshift mutations that abrogate the functioning molecule are focused upon. An example of this is the occurrence of deletion polymorphism in HLA-G exon 3 (codon 130) that results in abrogation of full length HLA-G1 protein (Suarez *et al.* 1997). As subjects homozygous for this allele (0105N) are healthy and reproducing normally, it may be estimated that the full length HLA-G1 molecule is not essential for reproduction (Ober *et al.* 1998). Also, less dramatic situations occur when some alleles may be associated with quantitative differences in a gene product. This is the case in some other HLA-G alleles (010103), but also in IL10, IL1RN and CD14 polymorphisms. Recently, it was estimated that 53 % and 62 % of IL-1ra and IL-10 production capacity is genetically determined (de Craen *et al.* 2005).

Molecular and genetic studies are often more standardized and provide good reproducibility compared to many immunological assays. Results obtained in *in vitro* assays may not be equal to the *in vivo* situation. Therefore, it has been estimated that immunogenetic studies may provide useful information about involvement of immunoregulation in recurrent miscarriage. Especially studies in closely related human populations and studies in animal models have been encouraging (Choudhury & Knapp 2001a, 2001b).

The results obtained in the majority of studies describing genetic polymorphisms in recurrent miscarriage are conflicting. Many of the reported associations have not been reproduced in later studies. This is partly due to the fact that the associations may vary in different ethnic populations and they may be biased because of small sample sizes. This also may be true in the present study. One reason for a small sample size is that recurrent miscarriage is relatively uncommon, affecting only 1 % of women.

Collaboration between research groups may resolve the problem of small sample sizes, but comparison of results from different ethnic backgrounds has its weaknesses, such as

population stratification bias (Cardon & Palmer 2003). Under the circumstances the study and control populations should remain as homogenous as possible. The Finns are an ideal population in this respect. Plans to collect large cohort or case-control DNA samples that could be used in genetic association studies are ongoing in several European countries. These projects (Biobank UK and others) may circumvent statistical difficulties encountered in studies with small sample size.

6.2 Methodological aspects

DNA samples of couples with recurrent miscarriage used in this study originated from the Finnish Red Cross Blood Transfusion Service, Helsinki. The samples had been collected during 1990's. Thus, no cytogenetic analysis of fetal products was available at that time. This is an obvious shortcoming in this study. Recently, the Royal College of Obstetricians and Gynaecologists has published guidelines for management of recurrent miscarriage, including recommendation of karyotyping of all fetal products as women with recurrent miscarriage are not protected from a sporadic fetal loss due to chromosomal abnormalities. However, the guidelines point out, that chromosomal abnormalities would probably be less frequent in abortuses in recurrent miscarriage (RCOG 2003).

Genetic abnormalities in fetal tissues have been observed in ca. 50 % of all miscarriage cases. This may also be true in recurrent miscarriage (Stern *et al.* 1996). However, in some studies the frequency of embryonic karyotype aberrations has been as low as 29 % (Carp *et al.* 2001) and in some studies as high as 70 % (Rubio *et al.* 2003). The frequency of genetically abnormal embryos has been higher in studies in which also women with only two consecutive miscarriages have been included (Carp *et al.* 2001). The normal karyotype rate significantly increases with the increasing number of previous spontaneous abortions (Ogasawara *et al.* 2000). Recurrent miscarriage may result from different mechanisms. Even women with antiphospholipid syndrome may have miscarriages due to chromosomal anomalies in conceptuses (Takakuwa *et al.* 1997).

Thus in a series of miscarriages, both genetically normal and abnormal embryos may be included. Obviously there must have been genetically abnormal, rejected embryos in the study group presented here, but in the absence of fetal karyotyping their number is not known. In most countries fetal karyotype analysis is not routinely done for miscarriages unless for research purposes (Carp *et al.* 2001). This is also true in Finland.

Bacterial vaginosis may be associated at least with late miscarriage, whereas its role in the first trimester miscarriages is not generally accepted (RCOG 2003). However, some evidence supporting its association with an increased risk of first trimester miscarriage has been published (Ralph *et al.* 1999). Although infections as an etiological factor for miscarriages were excluded in the present material, genital ureaplasma and mycoplasma infections cannot be ruled out because specific diagnostic tests were not done to detect these pathogens.

Genotyping methods are becoming more standardized and accurate. The high cost of genotyping remains a problem especially when performing association studies with large sample sizes. Today, commercial kits for SNP genotyping are available. Introduction of automatic DNA amplification workstations in laboratories will reduce the significance of

manual primer design and manual genotyping. However, even modest developmental achievements obtained in manual methods may still benefit smaller laboratories working without modern facilities.

It is obvious that laboratory workload may considerably decrease if the amount of PCR tubes to be handled during the course of the experiment can be reduced. This is especially true in large-scale typing studies should automatic PCR workstations not be available. For instance, a one-tube PCR method was developed here for IL-10 and CD14 genotyping. In high quality DNA samples the one-tube method was found to be reliable and the typing results from sequencing and PCR-RFLP were identical to those obtained with the one-tube PCR method. However, for routine use the two-tube version of IL-10 method was found to be more practical. This method has been used later in one study in this laboratory (Kinnunen *et al* 2002).

Especially for samples with lower DNA quality the two-tube method is more reliable. Simultaneous detection of two alleles in the same PCR reaction with partly overlapping allele-specific primers running on opposite directions may not be achievable if the DNA strand in the vicinity of polymorphic site contains unfavourable sequences (palindromic sequences, too high GC content etc) either in 3' or in 5' directions. However, when only one allele is to be amplified at a time, one can decide the most practical direction of amplification.

Allele-specific PCR may offer some benefits compared to the PCR-RFLP method. This may be important for SNP genotyping in laboratories lacking facilities to analyze DNA samples using more advanced techniques (pyrosequencing, real-time PCR etc). As no restriction enzyme digestion is needed in AS-PCR, the costs of the DNA analysis may be cheaper and the results may be obtained within half a day. The lengthy incubations in restriction enzyme digestion may delay the completion of the genotyping and the results may only be seen in the next working day when using PCR-RFLP. However, this is usually of minor importance.

In the PCR-RFLP analysis, there occasionally is incomplete digestion of the PCR product. Thus, faint bands may be observed in agarose gel electrophoresis. Faint bands also occur in allele-specific PCR. They may be due to unspecific priming. In designing allele-specific primers for SNP genotyping, the principle to also modify the penultimate 3' nucleotide was found to be useful as this considerably inhibits non-specific amplification of the opposite allele. In our laboratory, unspecific PCR products were regularly seen when AS-PCR for TNF -308A/G polymorphism was determined following a previously published protocol in which primers were not modified (Brinkman *et al.* 1994). With the help of additional mismatch, the annealing temperature in PCR reaction can be lowered and the amount of amplification product increases (unpublished results).

The same principle was followed in genotyping the CD14 -159C/T alleles. Primarily a two-tube method with modified allele-specific primers was developed and later the one-tube method was developed as well.

6.3 HLA-G

Interpreting HLA-G typing results is somewhat cumbersome because the typing protocols used in recent years do not allow typing of all relevant HLA-G alleles. Papers I and II were published in 1996 and 1997. At that time only a limited number of HLA-G alleles were known and thus there are some limitations in the results of these papers that deserve closer consideration.

As HLA-A had been suggested to be in linkage disequilibrium with HLA-G in one Caucasian population (Morales *et al.* 1993) the HLA-A locus was also studied here. The HLA-A typing results are important when HLA-G typing results are explored. Some data may be deduced from the observed HLA-A antigen frequencies, as by now there are some well-known linkages between HLA-G alleles and HLA-A antigens.

HLA-A antigen frequencies of our study population closely reflected the previously published distribution of HLA-A antigens in another Finnish population of 188 random individuals showing that also the HLA-G allele frequencies studied here can be used to represent a normal distribution of HLA-G alleles in the Finnish population (Hietarinta *et al.* 1994). According to the current nomenclature of HLA-G alleles the HLA-G*I is equivalent for 010101, G*II for 010102, G*III for 0103 and G*IV for 010103 (Marsh *et al.* 2002). However, HLA-G*I, II and IV may include several other alleles that are quite rare in Caucasian populations (Matte *et al.* 2000). Two officially recognized alleles (01042 and 01015) remain totally untypable with the method used here.

*G*I*

Though G*I includes several different alleles it may be assumed that majority of G*I positive typing results indicate the presence of true 010101. This assumption is based on previous typing results from other Caucasian populations: 010104 is extremely rare in Caucasian populations studied so far (frequency <0.02). In Danish subjects, the frequency of 010403 that corresponds to G*I in this study was found to be <0.01 (Hviid *et al.* 2002). Furthermore, in some studies 010401, 010402, 010403 alleles may have been grouped as 0104 alleles together with a frequency of about 0.08 (Aldrich *et al.* 2001). These studies indicate that the 010403 allele is probably also quite rare in the Finnish population.

Typing results for frequency estimations for 0102 are not available. Moreover, in some studies, typing protocols have not allowed reliable discrimination of 010101 and 010106 alleles. Allelic association with the HLA-A locus and the frequency of the G*I allele closely resembled that observed in other studies on Caucasian populations (Morales *et al.* 1993).

*G*II*

G*II includes several different alleles of which 010108 is rare (<0.02). The frequency of 010401 in German/Croatian population is 0.06 (van der Ven *et al.* 1998). In 228 healthy Danish couples the 0106 allele frequency is 0.05 (Hviid *et al.* 2001). In another study, the frequency of 010401/2 was 0.011 (Pfeiffer *et al.* 2001). Thus, it may be estimated that majority of G*II typing results belong to 010102 in this study.

An important pitfall with the typing protocol used here is that the 0105N allele remains untypable. The frequency of 0105N allele in Caucasian populations has ranged from zero in the Portuguese population to 0.03 in the Spanish population (Aldrich *et al.* 2002). In the Danish population the frequency of 0105N is 0.007. Evolutionally an interesting finding is that in African populations the 0105N allele frequency is as high as 0.11. This may indicate that this allele may be beneficial especially in regions with high pathogen loads (Aldrich C *et al.* 2002).

The frequency of 0105N in women with recurrent miscarriage is increased in two different Caucasian populations (Aldrich *et al.* 2001, Pfeiffer *et al.* 2001). However in a recent Danish study, no association between 0105N and recurrent miscarriage was observed (Hviid *et al.* Tissue Antigens 2002). In Caucasian and African populations the 0105N allele is in disequilibrium with the HLA-A30 antigen (Aldrich *et al.* 2002).

In the present study, the 0105N allele would have been typed as GII. There were five subjects in the control group who had the HLA-A30 antigen and all of them were also carriers of G*II. Interestingly no subjects in the recurrent miscarriage group had the HLA-A30 antigen. Especially HLA-A30 positive subjects of African descent carry the mutated HLA-G0105N allele (Aldrich *et al.* 2002). It remains uncertain whether this allele exists in the Finnish population as efforts for developing a new typing method for this allele were hampered by a lack of a 0105N- positive control sample.

*G*III*

The G*III or 0103 allele was not found in the Finnish population. The frequency of the 0103 allele has been generally low in populations studied so far. In Hutterites, the frequency of the 0103 allele is 0.04, but in all other populations the frequency varied between 0 and 0.02 (Matte *et al.* 2000). Thus, the results obtained herein are in accordance with the other reports on the 0103 allele.

*G*IV*

G*IV typing results most probably represent the 010103 allele. Another allele that would be typed as G*IV is 010107 which is rare with a frequency of <0.02 (Aldrich *et al.* 2001). Finnish women suffering from recurrent miscarriage do not have an increased frequency of the 010103 allele associated with sustained HLA-G expression. On the contrary, carriage rate of the 010103 allele seems to be decreased in women with recurrent miscarriage compared to the reference group (0 vs. 15.6 %, p=ns). In one study an association between 010103 and recurrent miscarriage was observed (Pfeiffer *et al.* 2001). However, other studies have failed to confirm this (Aldrich *et al.* 2001, Hviid *et al.* 2002). This indicates that women with low producing 010103 allele may be at an increased risk of recurrent miscarriage in some ethnic populations only. In the present study, all Finnish subjects with the 010103 allele had the HLA-A11 antigen. Thus, previously detected association between A11 and G010103 was confirmed in the present study (Morales *et al.* 1993).

Overall, an important finding in the present study is the observed similar linkage disequilibrium between HLA-A and HLA-G loci in Finnish and Spanish populations (Morales *et al.* 1993).

6.4 IL-10

It is relevant to ask whether association studies with a limited number of study and control subjects are meaningful because the power to detect associations is poor (Ioannidis *et al.* 2003). To study the power of this sample set, e.g. how small a genetic effect it would be able to exclude from the data set presented here, a permutation test was used for the IL-10 study (III). The power of study III to detect an allele association was good enough to exclude a risk rate (RR) of > 1.4 for the locus in recurrent miscarriage. If the locus had had an RR of 1.6, an association at P level < 0.00001 would have been found, and if the locus had had an RR of 1.4, an association at P level < 0.02 would still have been found. Very small effects, however, could have been missed.

Interestingly, in a meta-analysis published after completion of study III, all thus far published IL-10 -1082 and recurrent miscarriage studies ($n=3$) were combined. A statistically significant association between the IL-10 -1082G/G genotype and recurrent miscarriage was observed ($p=0.03$, OR 1.75 (1.06-2.91) (Daher *et al.* 2003). In this meta-analysis, the study and the reference groups from paper III were also included. In all three study populations (British, Brazilian and Finnish), there was a tendency of an association between the IL-10 -1082G/G genotype and recurrent miscarriage, while none of the single studies had sufficient power to detect the weak association.

In the same meta-analysis statistically a significant association was also found for the IFN-gamma +184T/T genotype and recurrent miscarriage ($p=0.04$, OR 1.92 (1.02-3.63) (Daher *et al.* 2003). Combining the study populations from different ethnical groups also has its weaknesses, but under the circumstances the IL-10 genotype frequencies were similar in all populations and there was no evidence of heterogeneity. Thus, the meta-analysis may be justified. Although statistically significant, the association between the IL-10 -1082G/G genotype and recurrent miscarriage remains to be confirmed in a larger study population.

The results of the meta-analysis are in accordance with the finding that, in a Dutch population reproductive success was associated with the IL-10 -2849 genotype. Westendorp and co-workers (2001) compared -2849 genotypes of 73 women with at least three consecutive miscarriages before 16 weeks of gestation and those of 323 women with normal reproductive capacity. They found that the -2849A/A genotype was associated with lower IL-10 responsiveness upon stimulation with endotoxin. The same low-producing IL-10 genotype was also twice as prevalent in women with recurrent miscarriages compared to controls. In a Dutch population, the IL-10 promoter region haplotypes show that -2849G co-segregates with -1082A with a frequency of 0.45 and -2849A with -1082G with a frequency of 0.33 (Gibson *et al.* 2001). The association of the -1082GG genotype with recurrent miscarriage in combined Brazilian, British and Finnish populations might be due to lower IL-10 production capacity, especially after endotoxin stimulation. This holds true assuming that the haplotypic combinations are similar in these populations and in the Dutch study.

Currently, there is no information about these distal-proximal haplotypes of the IL-10 promoter region from populations other than the Dutch population. The -1082A allele containing ATA haplotype in the proximal region of the IL-10 promoter was connected with higher IL-10 plasma levels in a Finnish population (Helminen *et al.* 1999), suggesting that the -1082GG genotype might be associated with decreased IL-10 production capacity.

There may be ethnic variations though, as in the Spanish population the -1082G allele is associated with the highest serum IL-10 concentrations (Suarez *et al.* 2003).

As IL-10 probably has an important role during pregnancy, it would be important to resolve whether the association between the IL-10 genotype and recurrent miscarriage is an association with a “low” or a “high” producer genotype. This also has significance when evaluating the validity of the Th1/Th2 paradigm of pregnancy. Currently, it may be too early to decide which of the genotypes or alleles are eventually related to the “high” IL-10 production capacity, although the majority of research reports published so far have classified the IL-10 -1082G allele as the IL-10 “high” producing allele.

6.5 IL-1RA

The putative association between the IL1RN*3 allele and recurrent miscarriage is difficult to interpret. As the frequency of this allele is low in the general Finnish population, it will be challenging to explore whether there are any differences in IL-1beta production in IL-1RN*3 carriers compared to those with the IL1RN*1 allele. The results obtained in Austrian women with recurrent miscarriage could not be confirmed here (Unfried *et al.* 2001).

One interesting issue regarding the distribution of IL1RN alleles and genotypes in Caucasian populations is the high variability in the observed frequencies of IL1RN*2 alleles (Table 11). The IL1RN*2 allele and genotype frequencies in the Finnish population are among the highest. On the contrary, both IL1RN*2 allele and genotype frequencies in the control group in the study of Unfried *et al.* (2001) are considerably lower than those observed in other Caucasian populations. However, the IL1RN*2 allele and carrier frequencies in Austrian women with RM are not different from those reported from several other Caucasian populations.

Table 11. Frequencies in the IL1RN*2 allele in various Caucasian populations.

	Allele 2 frequency (%)	Allele 2 carriage rate (%)	Reference
Controls in the study of Unfried <i>et al.</i>	6.6	11.0	Unfried <i>et al.</i> 2001
Women with RM in the study of Unfried <i>et al.</i>	21.4	34.0	Unfried <i>et al.</i> 2001
Scottish	7.4	7.8	McGarry <i>et al.</i> 2001
Czech	15.8	26.2	Vencovsky <i>et al.</i> 2001
English	17	32	Whyte <i>et al.</i> 2000
English	20	33	McKibbin <i>et al.</i> 2000
Italian	Not reported	52	Tountas <i>et al.</i> 1999
Italian	20	36	Whyte <i>et al.</i> 2000
French	22.8	38.4	Cantagrel <i>et al.</i> 1999
Dutch	25.5	41.8	Schrijver <i>et al.</i> 1999
Spanish	28.8	46.0	Garcia-Gonzalez <i>et al.</i> 2001
US Caucasian	27.4	46.4	Rider <i>et al.</i> 2000
Finnish	29.9	51.6	Present study, paper V

6.6 CD14

CD14 is upregulated on monocytes during pregnancy and it is a key molecule in LPS binding and signaling. The association between enhanced CD14 production and the -159T allele has been observed earlier in the Finnish population (Karhukorpi *et al.* 2002). However, no association between CD14 polymorphism and recurrent miscarriage was found in this study suggesting that genetic differences in CD14 production are probably not important in conferring susceptibility to recurrent miscarriage.

This does not rule out the possibility that endotoxin might also be an etiologic factor in recurrent miscarriage in humans. (Clark *et al.* 2002). It would be of interest to study polymorphisms of other genes coding for molecules involved in endotoxin signaling. Preeclampsia is another pregnancy disorder in which polymorphisms of genes involved in the endotoxin signaling pathway would be of interest to study as preeclampsia shares many characteristics akin to sepsis (Sacks *et al.* 1998). Interestingly, TLR4 polymorphism appears to be associated with an increased risk of gram-negative sepsis (Agnese *et al.* 2002). Thus, especially genetic association studies with TLR4 should also be warranted in pregnancy disorders.

6.7 Search for genetic traits predisposing women to reproductive failure

Taken together, the studies describing putative associations between immunomodulatory gene polymorphisms and recurrent miscarriage show that the associations are generally weak and many of them have not been confirmed (Choudhury & Knapp 2001b). This was also true in this study. Essentially all the results presented in this communication were eventually negative. An exception is the IL1RN*3 allele which was associated with recurrent miscarriage in the Finnish population studied. Such an association has not been reported before (Unfried *et al.* 2001) and, therefore, the present findings need to be confirmed.

Inconsistent replication is a common problem in genetic association studies (Lohmueller *et al.* 2003, Ioannidis *et al.* 2003). Many of the suggested associations between immunogenetic factors and recurrent miscarriage probably result from publication bias and chance. Realizing this fact allows for one to conclude that the reproduction process itself is quite tolerant to modest quantitative differences in various immunomodulatory molecules. At least genetic variations that were described herein were found to be quite insignificant risk factors for recurrent miscarriage.

It may be supposed that quantitative differences in the production of immunomodulatory molecules due to normal genetic variation are not grossly harmful to the fetal allograft. This indicates the robustness and flexibility of the reproduction system. For fetal survival, it is essential that minor variances are tolerated. Thus, a pro-inflammatory genetic profile based on carriage of several cytokine genetic markers might be needed to predispose to inefficient reproduction (Costeas *et al.* 2004). In the Dutch population high TNF-production capacity in combination with low IL-10 production capacity were associated with recurrent miscar-

riage (Westendorp *et al.* 2001). This underlines the impact of deleterious genetic combinations on reproduction. The role of pregnancy-specific factors and also environmental factors cannot be overlooked in maintaining the favorable balance between pro- and anti-inflammatory cytokines during pregnancy.

In this study, genetic variants leading to inefficient reproduction were sought. Studying variants or genetic combinations that would provide an advantage in reproduction would be an alternative approach to the subject. Unfortunately this strategy may not work any more because large family-size is rare in modern societies despite of woman's high natural capacity for procreation (Juntunen *et al.* 1994).

Communities in which no birth control methods are in use have offered a unique chance to study effects of genetic polymorphisms on reproduction capacity, not only on pregnancy losses but also on birth intervals. A well-known example of this kind of community is the Hutterite community as ten children in a family is not rare among them (Ober *et al.* 1983).

Whether genetic variants that provide superior reproductive capacity are advantageous for survival of the species is an interesting question. In some historical cohorts reproduction has been found to negatively impact the longevity of mothers (Westendorp & Kirkwood 1998). In Sami women, giving birth, especially to sons, reduced a mother's longevity. This may be due to immunosuppressive effects of elevated testosterone levels during pregnancies with male fetuses (Helle *et al.* 2002).

The inverse association between family size and longevity of mothers in historical cohorts is related to selection by early fatal infections. Only those who could respond favorably to infection with Th1 type immune response survived, but later this Th1 type skewed response might have negatively impacted reproduction capacity (Westendorp *et al.* 2001). Also, mortality in febrile infections is increased in subjects with a high IL-10/TNF alpha ratio (van Dissel *et al.* 1998).

Thus, low IL-10 production capacity (Th1 bias) may enhance survival of fatal infections, but the cost may be inefficient reproduction in some cases. However, in this study no evidence of genetically determined Th1 bias was observed in women with recurrent miscarriage.

7 Future remarks

There still are no definitive immunological therapies available to those suffering from recurrent miscarriage because key immunological factors associated with acceptance/rejection of fetal allograft are still widely unknown. Favourable modulation of the immune system is still quite useless in recurrent miscarriage despite considerable research efforts (Scott 2003).

New technological innovations have made it possible to gain ever-deeper insights into reproduction and infertility. Through genetic studies, many of the open questions related with infertility and recurrent miscarriage may eventually be answered. It is emphasized that there is always a risk for inappropriate use of the information obtained through genetic testing.

From an evolutionary perspective, reproduction is the most important task for an individual. Recurrent miscarriage affects only ca. 1 % of all women. Thus, it probably has no large-scale biological consequences to the human race. Still, it is important to try to find ways to help those suffering from it, as recurrent miscarriage may cause severe psychologic stress and despair (Klock *et al.* 1997). Looking at the future, rapidly growing knowledge of key biological factors in reproduction will probably lead to more effective cures for childlessness in those cases in which the genetic makeup of the fetus is not severely compromised. When efficient and high-throughput technologies, such as genomics and proteomics with microchip-arrays, allow large-scale studies, research will hopefully proceed very rapidly (Lander 1999, Pandey & Mann 2000). While waiting for these new scientific innovations, it is helpful to appreciate that unexplained recurrent miscarriage has an excellent prognosis, with a probability of over 75 % of successful future pregnancy with tender loving care alone (Stray-Pedersen & Stray-Pedersen 1984).

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