Co-Evolution of NK Receptors and HLA ligands in humans is driven by Reproduction

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Abstract
Allogeneic individuals coexist during pregnancy in eutherian mammals. Maternal and fetal cells intermingle at the site of placental attachment in the uterus where the arteries are remodelled to supply the fetus with oxygen and nutrients. This access by placental cells to the maternal supply line determines the growth and birth weight of the baby and is subject to stabilizing selection. Invading placental trophoblast cells express HLA class I ligands (HLA-E, HLA-G and HLA-C) for receptors on maternal uterine NK and myelomonocytic cells, CD94/NKG2, LILR and KIR. Of these, only the KIR/HLA-C system is highly polymorphic. Different combinations of maternal KIR and fetal HLA-C variants are correlated with low birth weight and pre-eclampsia or high birth weight and obstructed labour, the two extremes of the obstetric dilemma. This situation might have has arisen because of the evolution of bipedalism and subsequently, in the last million years, larger brains. At this point the human system began to reach a balance between KIR A and KIR B haplotypes and C1 and C2 epitopes of HLA-C alleles that reflects a functional compromise between the competing demands of immunity and reproduction.

Introduction

In one episode of Downton Abbey, the period TV drama depicting the British upper class at play, Lady Sybil Crawley, married to the Irish ex-chauffeur, died of eclampsia after delivering her baby. This event was sufficiently important for august publications such as the Washington Post to comment, drawing considerable and welcome attention to this enigmatic disorder [1]. Eclampsia, ("a flash of lightening"), is manifest by convulsions occurring any time after 20 weeks of gestation and is the dramatic climax of pre-eclampsia, a collection of diverse signs and symptoms including hypertension [2]. Although full-blown eclampsia is now an uncommon event in high-income countries, the symptoms of pre-eclampsia occur frequently resulting in preterm delivery with its concomitant problems. In sub-Saharan Africa (SSA), eclampsia is ever present, depicted vividly in a poster from a hospital in Uganda (Figure 1). Although women in all populations are affected by this disorder, eclampsia/pre-eclampsia is an especially important disease throughout SSA and in emigrants from Africa to Caribbean, America and elsewhere [3]. Surprisingly, the selective pressures contributing to the co-evolution of KIR and HLA genes have emerged from studying pre-eclampsia.

Pre-eclampsia

It is hard to comprehend why there has been so little progress in understanding this tragic disease, particularly when it commonly affects young women in their first pregnancy. The reasons for lack of progress are numerous but an important reason is the confusion about the multiple stages of pathogenesis (Figure 2). The disease declares itself with the emergence of the dramatic systemic disorder whose features include edema, hypertension, edema, clotting problems, visual disturbances and epigastric pain. This is the tertiary stage and occurs sometime in the latter half of pregnancy after 20 weeks gestation. The symptoms of pre-eclampsia are all diverse manifestations of inflammatory dysfunction of systemic endothelial cells – hence all homeostatic functions are disturbed: movement of fluid between intra- and extravascular space, blood pressure control, and regulation of haemostasis and thrombosis. The triggers for this endothelial decompensation are essentially unknown although there are many candidates, eg VEGF and other cytokines, cell free DNA, microvesicles. Importantly they seem to emerge from a stressed placenta, the second stage of pathogenesis [4]. Indeed, the placenta is essential for the development of pre-eclampsia as it can occur in cases of hyatidiform mole, a developmental abnormality where the zygote contains two sets of paternal chromosomes with no maternal contribution, giving rise to an enlarged placenta but no fetal development. The placental stress generally develops when the fetal demand for nutrients and oxygen

exceeds the maternal blood flow through the uterine arteries that supply the placenta, although other causes of damage such as placental malaria may also trigger the syndrome. Pregnancy is a compromise between the mother and fetus and, for the continuation of parental genes, both mother and her baby must survive the pregnancy and the fetus develop and grow enough to survive the vulnerable first month after birth.

Pathogenesis of pre-eclampsia

To really understand why pre-eclampsia develops in pregnancy, it is therefore necessary to focus on the primary defect, the reduction in blood flow that gives rise to the stressed placenta. This is due to defective invasion of interstitial trophoblast cells into the uterine wall where they home to the spiral arteries to destroy and replace the smooth muscle media with acellular 'fibrinoid' material. Subsequently endovascular trophoblast cells move down the inside of the artery to replace the endothelium [5]. The invasion into arteries is essential to form high conductance vessels carrying sufficient oxygen and nutrients to the enlarging placenta and its fetus right until the end of gestation. These extravillous trophoblast cells (EVT) that invade maternal tissues are innately highly invasive. Trophoblast over-invasion (placenta percreta) in parts of the uterus or Fallopian tube where there is no formation of decidua (the progesterone-transformed endometrium), points to a crucial role for the uterine mucosal lining in controlling excessive invasion [6]. It seems that in pre-eclampsia, this control is 'too good' and EVT derived from the feto-placental unit are unable to adequately transform the uterine arteries. Thus, the crucial question is: how is trophoblast invasion regulated and a demarcation line made in the correct place in the uterus between the cells of two different individuals?

There are no animal models for pre-eclampsia or for most other major obstetric disorders: fetal growth restriction, stillbirth, obstructed labour and post partum haemorrhage. This means that human pathologists rather than experimental scientists have been informative in pointing to what can go wrong and why. Apart from pre-eclampsia, the other disorders that arise from defective trophoblast invasion are fetal growth restriction (FGR), preterm labour and death in utero from hypoxia (stillbirth); these are known collectively as the 'Great Obstetrical Syndromes' [5]. However, obstructed labour - due to disproportion between the fetal head size and the pelvic bony canal it needs to pass through - is also a major cause of maternal and neonatal death. In these pregnancies, women undergo prolonged labour with the risk of a ruptured uterus (particularly if there has been a previous Caesarian section with a uterine scar), and atony of the myometrium after delivery leading to

post partum haemorrhage. Atony is failure of myometrial contraction necessary to compress and stem bleeding from transformed spiral arteries lacking smooth muscle media. Obstructed labour and its disastrous sequelae including obstetric fistulas are also more common in women from SSA than elsewhere in the world [3,7].

By studying natural childbirth in humans the concept of the 'obstetric dilemma' emerges; a balance between too much fetal placental cell invasion and too much maternal constraint [7-10] (Figure 3). A striking manifestation of this is the association of high neonatal and maternal morbidity and mortality with the extremes of birth weight; still births, small babies with FGR and maternal pre-eclampsia or large babies with obstructed labour, fetal asphyxia and maternal trauma and haemorrhage [11-13]. Furthermore, that loss of the uterine mucosa results in excessive invasion points to the decidua as playing a key role in this balancing act. Our focus has been on the decidual immune cells because amongst all the cellular elements present (epithelial glands, stromal cells, arteries), these will be the cells capable of mediating allo-recognition of fetal cells.

The involvement of the immune system in pre-eclampsia has been invoked for decades but in vague terms such as 'immunogestosis' [14] and 'immune maladaptation' [15]. This idea arose because of well-described epidemiological features such as the highest risk in first pregnancies (5% compared to 2% in subsequent pregnancies in large Norwegian cohort) [16]. This first pregnancy effect is not well understood. Young adolescents and elderly primipara are also at particular risk; in these women the cycling endometrium is often awry due to the immaturity or involution of hypothalamic-pituitary-ovarian axis [17]. This again points to the uterine lining and suggests that a normally cycling endometrium is essential for optimal control of placentation. Recurrence of pre-eclampsia after an affected first pregnancy carries a RR of 15 rising to ~40 after two affected pregnancies, pointing to involvement of maternal genetic factors [16]. A normal first pregnancy is a good prognostic sign unless there is a change of partner, although this may be confounded by the longer inter birth interval when there is a reassortment of couples [18]. These characteristics of the disorder resonated with immunologists and bring concepts like 'memory' of the first pregnancy and 'specificity' for particular male partners. Genetic studies also point to a clear role mainly for maternal genetic factors but also with a contribution from the father although the nature of these genetic variants is still unknown [19].

Dissecting exactly what immune maladaptation in reproductive success or failure means has not been easy because the field has been dominated by a persistent view of maternal-fetal immunological interactions that arose from considering the fetus as a classical allograft expressing non-self HLA antigens [20]. Because of this, the obvious cellular candidates invoked to explain the immunological aspects of pre-eclampsia were lymphocytes of adaptive immunity, T and B cells, which can respond to paternally mismatched HLA antigens. Indeed, the *systemic* maternal immune system does recognize a range of paternal alloantigens including HLA, Rhesus and platelet antigens. These maternal anti-fetal immune responses can have deleterious effects on the fetus such as haemolytic disease of the newborn, but there is no evidence that they are ever directed to extra-embryonic trophoblast. In the uterus, where *tissue* immune cells are in direct contact with EVT, a search for the elusive mechanisms that cause reproductive failure from maternal T cells attacking trophoblast has largely drawn a blank in humans. Presumably so many redundant mechanisms have evolved that protect trophoblast from maternal T cell activation, these rarely, if ever, all fail [21,22]. Instead, a closer inspection reveals that it is decidual Natural Killer (NK) cells and macrophages binding to HLA ligands expressed by the invading fetal trophoblast that may be responsible for the most relevant immunological events occurring at the maternal-fetal interface in utero.

Decidua and uterine NK cells

The great majority of immune cells in the decidua are lymphocytes with characteristics of NK cells [23-25]. Decidua is the transformed endometrium, a highly unusual mucosal surface because, under the control of the HPO axis, it undergoes cyclical breakdown and regeneration in women after puberty. During the estrogen-dominated proliferative phase of the menstrual cycle, glands, stromal cells and vessels regenerate from the basal layer. After ovulation, in the secretory phase, under the influence of progesterone derived from the corpus luteum, differentiation of these elements occurs. Uterine NK cells (uNK) are small and sparse before ovulation, but rapidly proliferate and differentiate into large cells with prominent cytoplasmic granules soon after ovulation. Early changes of decidualisation are seen in the late secretory phase, but if a blastocyst implants then the secretory endometrium continues to differentiate and forms decidua. uNK cells are an integral component of decidua accounting for ~30% of the cells in the stroma; they accumulate particularly around the infiltrating trophoblast cells in the decidua basalis [26]. Placental anatomists have described lymphoid cells with prominent cytoplasmic granules in many other species with invasive placentas in early pregnancy; these are always in close physical association with trophoblast

cells. Indeed, all species where decidualisation and uNK are found share the invasive haemochorial form of placentation. Here the trophectoderm breaches the uterine surface epithelium and embeds into the uterine wall. The close correlation between invasive haemochorial placentation with formation of decidua and uNK points to this maternal/fetal interface as key to controlled placentation and fetal growth. Neither decidua nor uNK are present in other forms of placentation such as epitheliochorial, which is characteristic of ruminants and pro-simian primates [27]. Here, the trophoblast cells attach to the surface epithelium of the uterus but do not invade; this is akin to a parasite hanging in the lumen of the gut without penetrating the mucosa. All this preamble points to a role for decidua and uNK in providing the balance between the nutritional needs of the fetus and the maternal requirements to provide a nurturing environment without risking her life. How might uNK cells contribute to this?

Uterine NK Receptors and Trophoblast ligands

NK cell function is regulated by NK receptors (NKR) that bind to MHC class I molecules, and other non-MHC ligands [28]. In contrast to the villous trophoblast that is bathed in maternal blood and is entirely HLA null, EVT have a unique HLA profile and do not express the polymorphic T cell ligands, class I HLA-A and HLA-B nor any HLA class II molecules [29]. Their absence precludes antigen presentation by EVT and provides one crucial mechanism for avoiding T cell recognition of invading fetal cells. EVT express two oligomorphic, non-classical HLA class I molecules (HLA-G and HLA-E) and one polymorphic classical class I molecule, HLA-C. HLA-E binds NKG2A and its activating counterpart NKG2C, and HLA-G binds to members of the LILR family. The only polymorphic molecules expressed by EVT are HLA-C allotypes and this means that the HLA-C alleles donated by the father will be different from the mother and specific for each pregnancy; this has obvious relevance to pre-eclampsia. Both maternal and paternal HLA-C allotypes are expressed at high levels in a fully conformed b2-m associated form at the EVT cell surface [30].

Some of the NKR that have potential ligands expressed by the invading EVT have now been defined (Figure 4). NKG2A and LILRB1 are inhibitory, whilst NKG2C and DNAM-1 are activating. Whereas there is no evidence for expression of ligands on EVT for NCR (NKp30, NKp44 or NKp46), the DNAM-1 ligand PVR is present [29]. A major activating NKR also expressed by uNK is NKG2D, but again NKG2D ligands, which belong to stress-induced MHC-like glycoproteins MIC-A and MIC-B and ULBPs, have not been found on EVT in normal pregnancies. Whether they are upregulated in failing pregnancies caused by infection or chromosomal abnormalities is not known [31]. NKG2D

ligands on stromal cells however may regulate uNK cells [32] and pbNK cells are reported to be affected by placental-derived soluble forms of these ligands [33].

Informed by the epidemiological studies of pre-eclampsia, our focus has centred on the NKR that can bind to paternal HLA-C allotypes, killer-like immunoglobulin receptors (KIR). KIR are the most variable receptors on uNK with diversity in gene number between individuals and additional allelic diversity at individual *KIR* loci [34]. This means that in a population different women will inherit a particular set of *KIR* genes. KIR with long (L) cytoplasmic tails bearing ITIM motifs are inhibitory, whereas KIR with short (S) intracellular tails are activating and transduce signals through the ITAM-containing adaptor, DAP12. HLA-C has emerged as the most important known ligand for members of the KIR family. HLA-C-binding KIR are expressed at high frequencies by uNK and will be confronted by variable paternal HLA-C allotypes. Therefore, in a reproducing population a wide range of maternal *KIR* and paternal *HLA-C* combinations will occur. All *HLA-C* alleles can be assigned to C1 or C2 groups, based on a dimorphism at position 80 of the a1 domain located at the KIR-binding epitope of HLA-C. All individuals will therefore have C1 and/or C2 epitopes that will bind KIR; C1 are bound by KIR2DL2/3 and C2 by KIR2DL1 and activating KIR2DS1 (Figure 5). This is in contrast to the other two KIR epitopes that are only present in a minority of individuals in a population: A3/A11 present in some HLA-A allotypes and Bw4 present in some HLA-B and HLA-C allotypes.

Combinations of paternally-derived C2 epitopes and maternal KIR genotypes at the extremes of the obstetric dilemma

This focus on interactions between KIR and HLA-C arose because both the genetic and epidemiological features of pre-eclampsia point to variable interactions between maternal and paternal genes in conferring susceptibility to poor placentation. It is only during pregnancy and especially the intermingling of maternal and fetal cells in the decidua that cells from two genetically different individuals naturally come into direct contact in mammals. Because one of the two maternal HLA-C allotypes is absent on EVT, this situation has some resonance with NK functions in tumours and viral infections where NK cells discern 'missing self'. A paternally-inherited allogeneic HLA-C molecule is present though, so the fetus can be considered by an NK cell as both 'missing self' and 'non-self' [35]. The closest clinical situation that compares with placentation is haploidentical haematopoietic cell transplantation (HCT), in which the donor is half-HLA matched with the

patient [36]. Subsets of donor NK cells expressing inhibitory KIR not engaged by the mismatched host HLA-C allotypes could be sensing the missing self HLA-C and, free from inhibitory constraints, would attack and eliminate residual leukaemia cells and help prevent relapse [37].

Because *KIR* and *HLA* are the two most variable gene families in humans, they define individuals as 'self'. Fifteen human *KIR* genes and two pseudogenes have been identified [38], all located on Chromosome 19 within the *KIR* locus in the Leukocyte Receptor Complex (LRC) on 19q13.4. A broad distinction of *KIR* haplotypes into A or B has proved useful in genetic epidemiological studies. Indeed, in Europeans, only 11 haplotypes are found in 94% of individuals [39]. *KIR A* haplotypes have a fixed gene content of 7 *KIR* that are all inhibitory, apart from *KIR2DS4* that is frequently disabled. *KIR B* haplotypes have variable number of additional genes, many of these are activating, although the product of only one, *KIR2DS1*, has any known ligand – HLA-C allotypes bearing C2 epitopes. A recombination hot spot divides the centromeric (cen) from the telomeric (tel) segment of the *KIR* locus and the two segments can re-assort in different haplotypes containing permutations of these regions, designated cen-A, cen-B, tel-A, tel-B [40]. Moreover, each *KIR* gene has multiple alleles, varying from 15-18 for the least polymorphic *KIR2DS1, KIR2DS3* and *KIR2DS5* to 110-112 for the most polymorphic *KIR3DL1, KIR3DL2* and *KIR3DL3*. In addition to haplotypic and allelic variations, copy number variation also contributes to *KIR* gene diversity [41].

We have compared maternal *KIR* genotypes in combination with fetal *HLA-C* genotypes in cohorts of women with pregnancy disorders (pre-eclampsia, fetal growth restriction or recurrent miscarriage) compared with women who had a normal first pregnancy [35,42-45]. A consistent finding in UK, Norwegian and African populations is that the frequency of maternal *KIR AA* genotypes in combination with a paternally-derived *HLA-C* allele bearing a C2 epitope is increased in women with disorders of pregnancy associated with poor placentation. This association of pre-eclampsia with *KIR* and *HLA-C* genes is still the only robust genetic association found for pre-eclampsia. In all our genetic studies it is also emerging that the maternal *C1* or *C2* status has an effect. The risk of pre-eclampsia is especially pronounced in these pregnancies with maternal *KIR AA*/fetal *C2* genotypes when the mothers lack any *C2* alleles themselves and are homozygous *C1C1* [35]. This points to a role for NK education and a beneficial effect for *C2* alleles in the mother compared with the detrimental effect seen in the fetus.

The candidate risky *KIR* on the *A* haplotype is *KIR2DL1*, a KIR that has a strong and specific inhibitory interaction with C2 [46]. This contrasts with C1-binding KIR where the inhibitory interaction is weak and less specific so that C1-binding KIR can also bind some C2 allotypes. A maternal *KIR AA* genotype will impart strongly inhibitory function to uNK because there are two copies of *KIR2DL1*. In women with KIR B haplotypes this strong NK inhibition is lost. Furthermore, there are many alleles of *KIR2DL1* that vary in their inhibitory potential on binding to HLA-C2 allotypes and when present on the *KIR* cen-B region (eg *KIR2DL1*004*), these bind much less well than KIR2DL1 cen-A alleles (eg *KIR2DL1*003*) [46]. It is likely that when larger cohorts have been studied certain *KIR2DL1* alleles will be found that confer most risk than others. All the genetic evidence therefore suggests that excessive inhibition of uNK cells may be detrimental for healthy placentation [22].

In line with this model, we have also found that women with a *KIR B* haplotype have a lower risk of developing pre-eclampsia [35]. The activating *KIR* genes linked to this lower risk of pre-eclampsia vary in different populations. In Europeans, protection is associated with the tel-B region where the activating *KIR* for C2, *KIR2DS1* is found [35]. In contrast, in a Ugandan cohort, particular cen-B regions that are unique to SSA and characterized by the present of *KIR2DS5* are protective [44]. There are no known HLA ligands for KIR2DS5 but all experiments performed to date used the single European *KIR2DS5*002* allele, an activating *KIR* always found in European populations in tel-B. The *KIR2DS5*006* allele is the protective allele present in cen-B in SSA and might be activated after binding to common African HLA-C2 allotypes. In addition, because *KIR2DL1* alleles present on cen-B regions have much weaker inhibition than *KIR2DL1* alleles present on cen-A [46], there are at least two possible mechanisms to explain how this cen-B region could weaken the strong inhibition from cen-A (Figure 5). This study illustrates the informative power of studying genetic associations in populations with richer *KIR* diversity where the disease of interest is more prevalent and has a stronger impact on natural selection.

A striking finding is that maternal *KIR* and paternally-inherited *HLA-C2* genotypes are also associated with the extremes of the human birth weight distribution [12] (Figure 3). It is therefore not just in pre-eclampsia and FGR that paternally-deried C2 may result in problems in pregnancy. In a large cohort of Norwegian pregnancies, women with *KIR AA* tend to have very small babies (<5th centile), whereas women with *KIR B* haplotypes that include *KIR2DS1* are likely to have large babies (>10th centile). This *KIR* association at both these

extremes of birth weight is associated with a paternal C2 epitope present in the fetus [12].

It is interesting to compare these findings with HCT where, although treatment regimes between different centres vary considerably, especially in type of donor and T cell content, a consensus is emerging: in acute myelogenous leukaemia patients receiving unrelated HCT from donors with a *KIR B* haplotype have better clinical outcome especially when these recipients have a C1 epitope and are not *C2C2* homozygous [47]. Both cen-B and tel-B *KIR* contribute to this effect and, extrapolating from our data, this might be different in European compared to African Americans as the ethnicity of the cases is not clear in most of these studies. The parallel between pregnancy and HCT, which share NK cell-mediated allorecognition, is that *KIR AA* genotypes are a problem in both when combined with an allogeneic C2 epitope [48].

Expression and function of KIR on uNK cells

How do these genetic findings translate into biological events occurring at the maternal-fetal interface during placentation? Stratified onto the inherited KIR and HLA-C diversity is the repertoire diversity generated somatically, resulting in variegated expression of KIR in individual NK cells. Each individual cell can express none, one, two up to five KIR and the expression is monoallelic, with NK cell clones cultured in vitro remaining stably committed to one allele, although one of the two parental alleles is randomly chosen by different cells of the same individual. In other words, any given population of NK cell expressing KIR is a mosaic of maternally and paternally expressed alleles [49]. This variegated KIR expression depends on host HLA and KIR genotypes, epigenetic regulation at the promoter level, with additional influences from KIR gene copy number and on whether individuals are positive for cytomegalovirus. KIR expression is a late event in NK cell development and it occurs because NK cell-committed progenitors acquire responsiveness to IL-15 after expression of the b chain of the IL-15 receptor (CD122, IL2Rb). IL-15 is produced by stromal cells in the uterine microenvironment in response to progesterone and is required for uNK cell differentiation [50]. Because uNK develop from progenitors in utero [51], their repertoire forms in response to local cues that may include signals from fetal EVT, maternal leucocytes, epithelial or stromal cells.

KIR determine NK cell function in the context of other receptor-ligand interactions and permutations of 28 NKR result in at least 10,000 different NK cell subsets in one given individual [52]. The inhibitory NKR that play a role in uNK-trophoblast interactions, however are only 5: KIR2DL1, KIR2DL2/3,

NKG2A and LILRB1, resulting in a repertoire of 32 different NK cell subsets. CD94/NKG2A is present on almost all uNK and LILRB1 is found on ~30-40%. The KIR repertoires of uNK and pbNK are quite different when taken from the same woman at the same time. In uNK taken at 8-10 week of gestation there are far more frequent KIR2DL1/S1^{+} and KIR2DL2/3/S2^{+} than in pbNK, so that the majority of uNK will potentially bind to C1 and C2 epitopes of HLA-C expressed by EVT [45]. In contrast, KIR3DL1/S1^{+} cells are low in uNK and as frequent as in pbNK. This dominance in the decidua of uNK expressing C1- or C2-binding KIR is not so obvious in endometrial uNK isolated from non-pregnant women, suggesting repertoire adaptation to pregnancy, rather than tissue-specific features. Whether there is any skewing after the mid-secretory phase when implantation would occur, however, is not yet clear [53]. KIR are able to bind to HLA-C molecules expressed by EVT and HLA-C tetramers bind specifically to KIR on uNK [54]. All these observations indicate that this is a mechanism for maternal immune recognition of fetal trophoblast. Of note is that such an interaction has not been shown for any receptor/ligands between maternal T cells and trophoblast in humans in normal or failing pregnancies.

During NK development when NK cells are educated, the *HLA-C* genotypes can affect the KIR repertoire of pbNK, most obviously seen in TAP-deficient patients [55]. NK cell subsets that express inhibitory NKR binding to self HLA molecules acquire functional competence and become more responsive to perturbation of HLA. The stronger inhibition mediated via KIR2DL1/C2 interactions means pbNK are more responsive in individuals with a C2 than those who are C1C1. Our genetic findings suggest that there is an effect of the maternal C1C1 or C2X genotype in women who have a *KIR AA* genotype and are then confronted with a paternal C2 ligand [35]. This raises the question whether these tissue uNK are similarly educated during their differentiation in utero. Unpublished findings indicate that maternal C1 or C2 status does affect KIR2DL1/S1 expression. The lower frequency of KIR2DL1^{+} uNK in women who possess an HLA-C allele bearing a C2 epitope supports the view that C2 is beneficial when present in the mother compared with the detrimental effect of a paternal C2 in the father.

There is certainly a close spatial and temporal interaction between KIR-expressing uNK and EVT-expressing HLA-C in the decidua basalis during placentation [35]. It has been difficult, however, to determine the functional responses of uNK following KIR ligation to EVT because of ethical and practical issues in isolating primary cells from first trimester human pregnancies. Our own findings have initially focused on activation of *KIR2DS1*, the *KIR* gene that provides protection from pre-eclampsia [45]. A range of chemokines and

cytokines (eg GM-CSF) are produced following ligation of KIR2DS1 to C2 and these may act to increase invasive capacity of EVT, more arterial transformation and thus more fetal growth.

LILR and HLA-G in utero

Only two of the polygenic family of *LILR* have been studied on decidua and endometrial leukocytes. The decidual myelomonocytic cells, which include mainly CD14+, HLA-DR+ macrophages and a small population of dendritic cells (DC), express both LILRB1 and B2 and, in comparison with blood monocytes, the expression levels are higher [56]. LILRB1 is also found on a subset of uNK cells (~40%). Although LILRB1 and LILRB2 bind to all HLA class I, there is stronger binding to HLA-G molecules, shown using HLA-G tetramers, recombinant HLA-G and surface plasmon resonance [57-59]. Uniquely amongst HLA class I molecules, HLA-G is found at the cell surface both as a monomer and, because there is an unpaired cysteine residue at position 42 in the a1 domain, as dimers [60,61]. These dimers bind with increased avidity to LILRB1 in their fully folded b2m-associated form and this translates into highly efficient signalling. A comparison of crystal structures show differences between LILRB1 and LILRB2 binding to HLA-G dimers; LILRB1 binds more to b2m whilst LILRB2 binds more to the a3 domain, explaining why the latter is able to bind to free heavy chains. Other unusual features of HLA-G are a prolonged half-life at the cell surface because of a truncated cytoplasmic tail promoting recycling through the endocytic pathway and retention in the ER. Furthermore, HLA-G transcripts for several isoforms exist including a soluble form; whether EVT produces protein products of these isoforms remains controversial [62].

Obviously these findings have particular relevance to the trophoblast/decidual interface where HLA-G dimers are present at the EVT cell surface and are bound by LILRB1-Fc fusion proteins [56]. Several lines of evidence point to the function of this interaction in regulation of uterine mucosal APC. In mouse models prolongation of allograft survival occurs [63]. Strong binding of HLA class I molecules to LILRB results in impaired antigen presentation and alteration in cytokine secretion. In humans, antibody blocking of LILRB on dermal CD14+ cells (that have some similarities to decidua CD14+ macrophages), leads to stimulation of CD8+ responses and deviation of cytokine production [64]. Other data show association of specific HLA-B alleles with response to HIV; those allotypes that have a higher affinity to LILRB inhibit DC function mediated by the increased binding to APC [65].

An intriguing idea arises that both trophoblast and HIV-infected cells have evolved to escape generation of effective T cell responses by binding strongly

to inhibitory LILRB on APC. Thus, a fetal molecule, HLA-G, specifically expressed by EVT, will only affect those HLA-DR$^+$ APC that fetal cells come into direct contact with in the uterus. This is a further effective mechanism for avoidance of damaging maternal T cell responses to fetal allogeneic molecules. Maternal APC elsewhere in the body do continue to respond efficiently to pathogens so there is no global maternal immuno-suppression. During primate evolution, the necessity to deviate uterine mucosal APC towards a tolerogenic phenotype became a particular issue when trophoblast adopted an interstitial form of invasion deep into the uterine stroma. This occurs in chimpanzees and gorillas and it is only in these species that HLA-G orthologues are non-polymorphic and able to form a homodimer due to the presence of the cysteine at position 42. This correlation of HLA-G dimerisation with extensive EVT interstitial invasion lends support to the role of HLA-G in diverting maternal uterine APC towards a tolerogenic function, preventing any maternal T cell activation when there is extensive contact in the tissues between fetal HLA-G+ trophoblast and maternal cells in the decidua.

HLA-G also provides a high affinity peptide derived from its leader sequence for presentation by HLA-E. CD94/NKG2A, expressed at high levels on the great majority of uNK cells, provides a strongly inhibitory signal to uNK when bound by HLA-E on trophoblast [66]. Uniquely amongst HLA class I leader sequences, when HLA-E is bound to the HLA-G signal peptide, it will bind to CD94/NKG2C and induce cytotoxic responses in pbNK cells [67,68]. CD94/NKG2C is the activating counterpart of NKG2A and CD94/NKG2C+ NK cells are expanded in HCMV seropositive individuals [69]. African children lacking the NKG2C gene may have poorer control of HCMV infections [70]. Although no isolates have been identified with the HLA-G leader sequence from the HCMV protein, UL40, which provide an HLA-E binding peptide, there are some identical to HLA-C leader sequences [71]. Interestingly, the HLA-G leader sequence also has a higher affinity than any UL40 isolates. As HLA-A and HLA-B genes are not transcribed in trophoblast this is another parallel between an infected cell and trophoblast.

KIR2DL4, one of framework genes, codes for an unusual KIR which has a single ITIM and a positively charged residue in the transmembrane region that associates with FceRg. KIR2DL4 has also been reported as being triggered by soluble HLA-G, although direct binding has been hard to detect in vitro [72]. KIR2DL4 has a D0 and D2 domain unlike the HLA-C1 and C2 binding KIR that have a D1 and D2 structure. In addition, whether KIR2DL4 is expressed in early endosomes or at the cell surface of uNK is still not clear. A recent crystal structure has been described showing that KIR2DL4 oligomerizes depending

on residues in the D0 domain; no binding to the monomeric form of HLA-G or other HLA class I molecules was found [73]. Heparan sulphate may be a non-HLA ligand for KIR2DL4 (as shown for other NKR) causing NK activation but this has not been studied in uNK [74]. The role of KIR2DL4 in reproduction therefore remains unresolved.

Murine models of NKR and MHC

Although there are many differences between mice and human, particularly in relation to the immune and reproductive systems, the mouse does have an invasive haemochorial form of placentation with a dense accumulation of uNK cells around the ectoplacental cone as it moves into the decidua (Figure 6). What is lacking is deep interstitial invasion with trophoblast transformation of spiral arteries; the rat and guinea pig resemble humans more closely in this respect [75]. Nonetheless, informed by the human genetic studies, murine models have been used to show that paternal MHC influence placentation [76] and that MHC-dependent inhibition of certain Ly49 (the mouse analogue of KIR) on uNK cells impedes both vascular remodelling in the decidua and fetal growth [77]. The impact of NK cell education can be studied in vivo in mice and these studies suggest that only maternal MHC class I molecules educates uNK cells [77], and yet paternal MHC class I molecules affects the outcome of uterine vascular adaptation to pregnancy and fetal growth [76,77]. 'Humanized' transgenic mice are now available that express KIR and HLA-C in a homogeneous genetic background. These mice will be instrumental to study the role of individual KIR and HLA-C in isolation.

Co-Evolution of KIR/MHC in primates in relation to placentation, birth weight and childbirth

The immunogenetic findings point to a pivotal role for interactions between paternal HLA-C allotypes carrying the C2 epitope with maternal KIR expressed by uNK in determining the extent of placental access to oxygen and nutrients necessary for optimal fetal growth. *KIR A* and *KIR B* haplotypes are present in all extant human populations although their frequency differs [34,38]. Strikingly, the frequency of *KIR A* haplotypes is inversely correlated with the frequency of *C2* alleles in these same populations, a situation that may have evolved to prevent too many pregnancies occurring with the risky *KIR AA*/paternal *C2* combination [78]. So far there has been no indication that paternal *HLA-C* alleles carrying the C1 epitope have any impact on pregnancy outcome (C1C1 fetus) whatever *KIR* genotypes the mother possesses. Thus, in reproduction the fine-tuning of the maternal supply line pivots only on C2 and not C1 epitopes interacting with different maternal *KIR* genotypes. How has this situation arisen during human evolution?

Time spent in clinical observation in a maternity hospital in SSA is both a salutary experience and a stimulus in thinking about evolution of immune system genes. Maternal mortality rates are very high throughout SSA at ~600/100,000 compared with <10 in high income countries, a clear historic selective pressure [79]. Indeed, it has been estimated that ~20% of pregnancies will result in maternal or fetal death in communities where there is no access to medical care, particularly intervention by caesarian section. The major causes of death are pre-eclampsia/eclampsia, sepsis and cephalo-pelvic disproportion. This means that more than half the deaths are occurring in women and their babies whose pregnancies are at the extremes of the obstetric dilemma – either from poor placentation (preterm labour, pre-eclampsia, FGR) or obstructed labour (post partum haemorrhage, birth trauma and asphyxia). It is at these two extremes that a paternal C2 epitope is playing a role, interacting with either very strong inhibition (*KIR AA* genotype) or activation (*KIR B* genotypes).

However, our glance at reproduction in the present is too narrow a focus to answer the key evolutionary questions, because the innovation in current human populations has relied upon and been constrained by what they have inherited from the past: the 20-38 million years of *KIR* and *MHC* class I interactions and the haemochorial placenta characteristic of all simian primates, which have both contributed to primate reproductive biology. There is a clear correlation between forms of placentation in different primates, (particularly the extent and pattern of trophoblast invasion), with the extent and onset of decidualisation and presence of uNK [80]. Pro-simian primates have an epithelial non-invasive type of placenta, no decidual formation, no KIR nor uNK. In Old World Monkeys there is limited decidual formation with uNK, EVT do infiltrate the uterine lining but only down the arterial wall and not through the stroma. It is only in great apes, chimpanzees and gorillas that trophoblast invasion also infiltrates extensively through the stroma and in humans moves threateningly deep into the myometrium. The increased invasion increases the blood supply to the feto-placental unit by increasing the extent of arterial transformation by trophoblast [34]. Other changes that occurred during hominin evolution result in two conflicting demands on the pelvis, bipedal locomotion and childbirth, that have both resulted in considerable changes to female pelvic anatomy. It is only in modern humans that the fetal head needs to rotate to squeeze through the cramped space of the birth canal [9,10]. Amelioration of this is seen from the correlation of head size, stature and pelvic dimensions [8]. The result is that there have been considerable fitness costs associated with child birth in humans.

Key events that contributed to the evolution of primate placentation were the evolution of the C1 epitope from a *MHC-B* ancestor in orangutans and its expression by EVT (although the latter remains an assumption in all species apart from humans) [81] (Figure 7). The increased interstitial EVT invasion observed in hominids that is absent in Old World monkeys correlates with the emergence in orangutans of C1 and C1-recognizing KIR [82]. This interaction between C1 and activating and inhibitory KIR of the *KIR A*-like haplotypes in common ancestors of humans and great apes was beneficial for their reproduction and survival and existed for several million years in the absence of C2 and cognate C2-specific KIR. The balance established between *KIR* and *MHC* class I in the common ancestor was probably perturbed as a result of the advantages conferred by bigger-brained babies and the selection this imposed on the invasive mechanisms used by trophoblast to allow for the fetal growth necessary to build a brain. This is where the current clinical picture informs the evolutionary analysis because it fits with the conceptually simplest of complementary functions; namely, the appearance of strong inhibition by C2 helped counterbalance the increased trophoblast invasion driven by C1-activating KIR.

Cen-B evolved soon after the human/chimpanzee split, a change that involved no major rearrangement of the KIR locus [40]. This resulted in weakening of the inhibitory interaction between KIR2DL1 and C2 because cen-B *KIR2DL1* alleles bind less well to C2 than *KIR2DL1* cen-A alleles, allowing more invasion and presumably a modest increase in brain size. It is only in humans that a clear distinction between *KIR A* and *B* haplotypes is seen alongside the weakening of all activating KIR so that, although *KIR2DS1* is still able to bind to C2 allotypes, the strength of the interaction is much weaker than for *KIR2DL1* [83]. The further steady increase in brain size in *Homo sapiens*, which correlates with major reorganization of the *KIR* locus and the evolution of tel-B where activating C2-KIR, *KIR2DS1* is located, provides a further counterbalance to cen-A *KIR2DL1* alleles allowing even more invasion.

A particular situation occurs in SSA where, in contrast to the rest of the world, there are high frequencies of both C2 and *KIR AA* genotypes. The frequency of C2 is higher in Sub-Sahara Africans than in Europeans and although broadly speaking, is detrimental for reproduction, must confer protection probably in responses to pathogens [34]. Unique to SSA, are cen-B1 regions where KIR2DL1 is linked to KIR2DS5 that may also play a role in placentation [44]. In keeping with this, pre-eclampsia and all the GOS are especially common in SSA, but so is obstructed labour [3]. In other words there is more selection in SSA against pregnancies with cephalo-pelvic disproportion with higher frequency of the GOS. This has been somewhat relaxed in European and Asian populations

emerging after 'out of Africa' migrations, possibly due to change in pelvic dimensions [3]. In addition, both the higher frequencies of C1 and tel-B in modern European and Asian populations could be due to adaptive introgression from Denisovans (who had tel-B) or Neandertals (who had Cw7, that bears a C1 epitope) [84].

In summary, because the same two gene families, MHC and KIR have evolved variants beneficial in certain infections and in facilitating reproduction, this is an example of an evolutionary trade-off. Individuals need to survive, particularly from childhood infections, notably measles where the subsequent immunosuppression last 2-3 years predisposing to mortality from other infectious pathogens [85], and successfully reproduce, where a baby is born between the two extremes of the obstetric dilemma.

The LRC and chromosome 19

Reproductive and immune system genes are known to be evolving rapidly and often these involve the same gene families that have been adopted for specific functions in both systems. For example, genes evolving under positive selection in the ancestors of gorillas, chimpanzees and human are *HLA-E* and *KIR2DL4* [86]. It has been speculated that this is driven by their characteristic deep placentation to favour interactions between trophoblast and maternal leukocytes. Chromosome 19 has an unusually high density of genes, with several large clustered gene families and frequent repeat sequences [87]. Genes involved in innate immunity (including *KIR* and *LILRB*) are concentrated in the Leukocyte Receptor Complex (LRC) on human chromosome *19q 13.4*, but many of these have overlapping functions in reproductive biology. In the LRC are *KIR* and *LILR* gene clusters both of which, as discussed, have clearly defined roles in reproduction. In addition, *LAIR2*, encoding a soluble form of LAIR1 that binds to collagen, is located in the centre of the LILR complex and is secreted by EVT [88]. Intriguingly, *LAIR2* is the most down regulated gene in ~10 week placentas taken from women who ultimately develop pre-eclampsia [89]. Other immunoglobulin superfamilies genes are the pregnancy-specific glycoproteins (PSG) that are part of the larger CEA family. PSGs are the most abundant proteins in the blood of pregnant women and are produced by trophoblast only in species with hemochorial placentation. Their receptors and roles in pregnancy are still unclear but they can activate TGF-b and bind to integrins and CD9 [90]. Close to the *KIR* locus on chromosome 19 is the largest microRNA cluster (*C19MC*) in humans, which contains non-coding genes that transcribe 46 pre-micro-RNAs expressed specifically in the placenta and in a range of tumours. Strikingly, this micro-cluster is maternally-imprinted and only found in primates. A number of other genes in the LRC where overlapping

roles in reproduction and the immune system are found are: *FcRn* (an MHC molecule mediating transplacental transport of immunoglobulins and half life in the serum [91]; 6 *CGb* genes in tandem, (the beta subunit of hCG, the hormone of pregnancy produced by syncytio-trophoblast); women with recessive mutations in *NALP7* (a member of the NLR inflammasome family) have diploid biparental and not androgenetic familial hydatifdiform moles [92]; the kallikrein family, unique to mammals, is the largest protease cluster in humans and is implicated in the development of the uteroplacental blood supply [93].

Future Directions

In all countries where delivery of obstetric care is adequate, maternal and neonatal mortality rates have dropped, whilst at the same time caesarian sections rates have increased to 25-30% in Europe and USA. Timely intervention by caesarian sections for pre-eclampsia and obstructed labour has certainly had a major impact on lowering mortality despite the obvious risks associated to any surgical intervention. This increased survival of mothers and babies who would previously have died might affect the KIR/HLA balance in populations in the future. However, of note is that placenta percreta and uterine rupture are now more common due to implantation of the blastocyst in the subsequent pregnancy at the site of a uterine scar.

Also of note are the large numbers of pregnancies delivered as a result of assisted reproduction (~2% of all births) where there is a higher risk of preterm birth, low birth weight and pre-eclampsia [94,95]. About 1 in 4 women will develop pre-eclampsia after oocyte donation, even higher than the increased risk already seen with conventional IVF [96]. In these cases the chance of an allogeneic C2 comes not just from the father but also from the egg donor and the embryo/placenta share no 'self' with the mother. Recipient mothers who have a KIR AA genotype are indeed at greater risk of poor outcomes in this situation [97]. If these findings are confirmed in larger cohorts with good clinical information it may be possible to avoid the risky maternal KIR/fetal HLA-C combinations by typing the donors and recipients as happens already in HCT. It might also be envisaged that, in mothers who have had pre-eclampsia and the father is C1C2 heterozygote, sperm selection for C1 sperm would avoid the risk of recurrence. Clearly, these ideas need careful clinical research and discussion before they can be introduced.

Concluding remarks

It seems clear that the *KIR/HLA-C* molecular system does provide a mechanism for maternal immune recognition of the fetus. The C2 and C2-specific KIR and the fixation of the *MHC-C* locus evolved in the context of the pre-existing C1

and C1-specific KIR interaction. That KIR binding to C1 and C2 epitopes not only have differences in binding strengths and specificities but arose separately during primate evolution is worth high lighting. Both systems now co-exist in all viable human populations and appear to be necessary for their survival with complementary functions maintained by balancing selection [98]. Reproduction is now balanced on *KIR A* and *B* haplotypes binding to C2 epitopes to keep birth weight between two extremes. Both very low and high birth weights will be selected against, a situation that has only arisen in last 50-100,000 years when brains grew so much [99]. This occurred long after the anatomical changes to the pelvis and birth canal as a result of bipedalism that emerged ~4 million years ago. The size of the birth canal set the upper limit to which the human brain/head can grow in utero and at some time during the history of *Homo* began to provide an additional selective force through morbidity and mortality caused by obstructed labour. At this point the human system began to reach a balance between KIR A, KIR B, C1 and C2 that reflects a functional compromise between the competing demands of immunity and reproduction.

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Figure Legends

Figure 1.
A poster displayed in the ante-natal clinic at Mulago Hospital, Kampala vividly demonstrating an eclamptic fit.

Figure 2.
Stages in the Pathogenesis of pre-eclampsia

Figure 3.
The obstetric dilemma. The normal distribution of birth weight is shown (black) from 800,000 babies born in Norway. The frequency of transfer to neonatal units, a proxy for morbidity, is shown in green.
Conditions affecting the mother and fetus at the extremes of the birth weight spectrum are in red and blue respectively.

Figure 4.
Receptors on uterine NK cells and macrophages that bind to trophoblast HLA class I molecules

Figure 5.
Potential functional impact of different maternal KIR binding to fetal HLA-C2 epitopes.

Figure 6.
Mouse implantation site at E9.5 showing maternal NK cells (brown) and fetal trophoblast cells (pink).

Figure 7.
Correlation of KIR and MHC genes, placental anatomy and decidualisation in primates

Table 1.
HLA class I genes showing number of alleles, sites of expression and cognate receptors. The HLA class I genes expressed by EVT are shown in red.

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