KIR2DS5 allotypes that recognize the C2 epitope of HLA-C are common among Africans and absent from Europeans

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Abstract

Introduction: KIR2DS5 is an activating human NK cell receptor of lineage III KIR. These include both inhibitory KIR2DL1, 2 and 3 and activating KIR2DS1 that recognize either the C1 or C2 epitope of HLA-C. In Europeans KIR2DS5 is essentially monomorphic, with KIR2DS5*002 being predominant. Pioneering investigations showed that KIR2DS5*002 has activating potential, but cannot recognize HLA-A, -B, or -C. Subsequent studies have shown that KIR2DS5 is highly polymorphic in Africans, and that KIR2DS5*006 protects pregnant Ugandan women from preeclampsia. Because inhibitory C2-specific KIR2DL1 correlates with preeclampsia, whereas activating C2-specific KIR2DS1 protects, this association pointed to KIR2DS5*006 being an activating C2-specific receptor. To test this hypothesis we made KIR-Fc fusion proteins from all ten KIR2DS5 allotypes and tested their binding to a representative set of HLA-A, -B and -C allotypes.

Results: Six African-specific KIR2DS5 bound to C2+HLA-C but not to other HLA class I. Their avidity for C2 is ~20% that of C2-specific KIR2DL1 and ~40% that of C2-specific KIR2DS1. Among the African C2 receptors is KIR2DS5*006, which protected a cohort of pregnant Ugandans from preeclampsia. Three African KIR2DS5 allotypes and KIR2DS5*002, bound no HLA-A, -B or -C. As a group the C2-binding KIR2DS5 allotypes protect against pre-eclampsia compared to the non-binding KIR2DS5 allotypes. Natural substitutions that contribute to loss or reduction of C2 receptor function are at positions 127, 158, and 176 in the D2 domain.

Conclusions: KIR2DS5*005 has the KIR2DS5 consensus sequence, is the only allele found at both centromeric and telomeric locations of KIR2DS5, and is likely the common ancestor of all KIR2DS5 alleles. That KIR2DS5*005 has C2 receptor activity, points to KIR2DS5*002, and other allotypes lacking C2 receptor function, being products of attenuation, a characteristic feature of most KIR B haplotype genes. Alleles encoding attenuated and active KIR2DS5 are present in both centromeric and telomeric locations.

Introduction

Killer cell immunoglobulin-like receptors (KIR) are expressed by subsets of human NK cells, a subpopulation of lymphocytes that contributes to innate immunity, adaptive immunity and reproduction [1, 2]. The target cell ligands recognized by KIR are epitopes of HLA-A, -B and -C. These epitopes are defined by alternative sequence motifs at residues 76–83 of the α1 domain. The human KIR family comprises four phylogenetic lineages, of which lineage III includes all KIR that recognize the C1 and C2 epitopes of HLA-C. These epitopes are defined by dimorphism at
Functional variation of KIR2DS5

J. H. Blokhuis et al.

position 80 of HLA-C, where asparagine confers C1 specificity and lysine confers C2 specificity [3].

KIR2DL1 is an inhibitory receptor that is highly specific for C2 whereas KIR2DL2 and KIR2DL3 are inhibitory receptors that principally recognize C1 [4]. Moreover, KIR2DL2, and to lesser extent KIR2DL3, cross-react with some C2+HLA-C [5, 6]. In addition to these much-studied inhibitory lineage III receptors, there are five activating lineage III receptors: KIR2DS1, 2DS2, 2DS3, 2DS4, and 2DS5. In comparison to the inhibitory receptors, these activating receptors are not as well characterized [7, 8]. Only KIR2DS1 has been shown to recognize HLA-C with specificity like that of an inhibitory KIR. Like inhibitory KIR2DL1, KIR2DS1 has methionine 44 and is specific for the C2 epitope. But the avidity of KIR2DS1 for C2 is about half that of KIR2DL1 [7, 8].

Epidemiological studies have correlated the presence or absence of the KIR2DS1 gene with several pregnancy syndromes [9–11]. In these and other disease association studies, it has proved useful to divide the numerous KIR haplotypes into two groups, KIR A and KIR B, based upon their content of activating KIR genes. KIR A haplotypes can have either no activating KIR genes, a functional form of the KIR2DS4 gene or a non-functional form of the KIR2DS4 gene. In contrast, KIR B haplotypes can have many different combinations of the five KIR2DS, as well as KIR3DS1. Pregnant women who have a KIR A/A genotype are at greater risk for pre-eclampsia than pregnant women having an A/B or B/B genotype. This correlation indicates that B haplotypes protect against pre-eclampsia. For pregnancy of A/A mothers, the risk increases further if the fetus has C2+HLA-C, especially if the mother lacks C2+HLA-C. This correlation strongly implies that interaction of fetal C2+HLA-C with KIR2DL1 is the cause of the increased risk [9]. Study of European cohorts has shown that the protective effect of a maternal B haplotype is mediated by KIR2DS1, the activating C2 receptor [10]. Thus the interaction between fetal C2 and activating KIR2DS1 can counter that of fetal C2 with inhibitory KIR2DL1, and in this way reduce the likelihood of pre-eclampsia [12].

Similar study of a Ugandan cohort of pregnant women correlated a different activating receptor, KIR2DS5, with protection from pre-eclampsia. Of 11 KIR2DS5 alleles present in this cohort of sub-Saharan Africans, KIR2DS5*006 was associated with significant protection [11]. That KIR2DS5*006 appears to counter the interaction between C2 and KIR2DL1, implies that KIR2DS5*006 is an activating receptor that recognizes the C2 epitope of HLA-C.

Whereas KIR2DS5 is highly polymorphic in Africans [13] and African–Americans [14] that is not the case for Europeans and other populations outside Africa. In these populations the KIR2DS5*002 allotype dominates [15]. Also present in the cohort of Ugandan women studied by Nakimuli et al. [11], the KIR2DS5*002 allotype provided no protection against pre-eclampsia, consistent with previous cellular and molecular analyses that failed to detect any functional or molecular interaction of KIR2DS5*002 with C1+HLA-C, C2+HLA-C, or any HLA-A or HLA-B variant [8, 16]. With this background, the aim of our study was to test the hypothesis that KIR2DS5*006 differs from KIR2DS5*002, and is an activating receptor for the C2 epitope of HLA-C.

Results and Discussion

KIR2DS5*006 is a C2-specific receptor

Fc-fusion proteins corresponding to the D1 and D2 domains of KIR2DS5*002 and KIR2DS5*006 were tested for binding to a panel of 97 Luminex beads [17], each coated with a different HLA-A, -B, or -C allotype (Fig. 1). Serving as controls were Fc-fusion proteins corresponding to C2-specific KIR2DL1*003 and C1-specific KIR2DL3*001. None of the KIR-Fc bound to any HLA-A allotype. The only observed binding to HLA-B was of KIR2DL3*001-Fc with HLA-B*13:01 and -15:01, allotypes that have the C1 epitope [5, 18]. As expected, KIR2DL3*001 bound strongly to C1+HLA-C but weakly to C2+HLA-C, and KIR2DL1*003 bound strongly to C2+HLA-C but weakly to C1+HLA-C.

KIR2DS5*006-Fc gave no significant binding to either C1+HLA-C or C2+HLA-C. In contrast, KIR2DS5*006 exhibited little interaction with C1+HLA-C, but bound C2+HLA-C to a higher level that was statistically significant (Fig. 1A). The mean binding of KIR2DS5*006 to the seven C2+HLA-C allotypes was 19% that of C2-specific KIR2DL1*003. These results demonstrate that KIR2DS5*006 is a C2-specific receptor, with an avidity around one fifth that of KIR2DL1*003. In contrast, KIRDS5*002 has no significant interaction with C2+HLA-C, consistent with previous analyses [8, 16].

KIR2DS5*006 bound the seven C2+HLA-C allotypes with variable avidity. For six of the C2+HLA-C allotypes, the binding to KIR2DS5*006 exceeds the binding to KIR2DS5*002 or KIR2DL3*001 (Fig. 1B). The exception is HLA-C*04:01, a common and widespread C2+HLA-C allotype, that bound KIR2DL3*001, KIR2DS5*002 and KIR2DS5*006 to a similarly low extent. The strongest binding to KIR2DS5*006 was with HLA-C*17:01, a common allotype of sub-Saharan African populations (Fig. 1B), as is C2+HLA-C*18:02. The range of KIR2DS5*006 binding to C2+HLA-C allotypes is 9–35% that of KIR2DL1*003. This compares to 30–65% for KIR2DS5*002, the most common KIR2DS1 allotype and a well-characterized C2-specific activating KIR [4, 6, 19–21]. KIR2DS5*006 is thus seen to be a weaker activating C2 receptor than KIR2DS1*002. In
part, the lower avidity of KIR2DS5*006 is likely to be caused by threonine at position 44, because mutation of lysine 44 to threonine in KIR2DL3*001 reduced its avidity by ~50%, as well as changing its specificity from C1 to C2 [22].

**KIR2DS5 polymorphism modulates receptor avidity for C2+HLA-C**

Ten of the eleven KIR2DS5 allotypes present in the Ugandan cohort studied by Nakimuli et al. [11] are distinguished by substitutions in the extracellular part of the receptor. Of the eight dimorphisms that distinguish the 10 allotypes, six are in the D2 domain, one is in the D1 domain and one is in the stem (Fig. 2). KIR-Fc corresponding to the ten KIR2DS5 allotypes were made and compared for binding to HLA-A, -B, and -C. Positive reactions were observed only for KIR2DS5-Fc binding to C2+HLA-C. The 10 KIR2DS5 allotypes divide into two groups: one group of six allotypes (2DS5*003, *004, *005, *006, *007, and *008), that bound C2+HLA-C to a level that is 15–20% of the KIR2DL1*003 binding and a second group of four allotypes (2DS5*002, *009, *010, and *011) that bound C2+HLA-C to an extent that was less than 8% of the KIR2DL1*003 binding (Fig. 2).

In the Ugandan cohort we see that 66.5% of the KIR2DS5 allotypes recognize C2+HLA-C and 33.5% of them do not (Fig. 3). Thus in Africans a majority of the KIR2DS5 allotypes are active C2+HLA-C receptors, whereas in Europeans almost none of them are.

We repeated the analysis described by Nakimuli et al. [11], but with the KIR2DS5 allotypes clustered according to their binding avidities (Table 1). The C2-binding KIR2DS5 allotypes are seen to protect against pre-eclampsia compared to the non-binding allotypes (p = 0.01801, OR 0.68). Thus, our functional analysis helps resolve which particular KIR gene in the African-specific cB01 region protects Ugandan women against pre-eclampsia.

![Figure 1](image-url)
Functional variation of KIR2DS5

KIR2DS5+ Nakimuli et al. [11], Frequencies are calculated from allotypes observed in the cohort of pre-eclampsia patients studied by the same allotypes. Shown on the right is the mean cohort, is set as the consensus sequence with blank boxes indicating allotypes that do or do not, bind C2* HLA-C. Shown are the relative frequencies of the KIR2DS5 allotypes that do, or bind C2* HLA-C, whereas only three of the six KIR2DS5 allotypes

Figure 2. KIR2DS5 allotypes differ in their capacity to recognize C2* HLA-C. Shown are the relative frequencies of the ten KIR2DS5 allotypes in the cohort of pre-eclampsia patients studied by Nakimuli et al. [11]. Larger cohorts will address this question as well as the contributions to pre-eclampsia of different C2* HLA-C ligands in the fetus.

KIR2DS5*005 has the consensus sequence of all KIR2DS5 alleles. It is also the only allele found at both the centromeric and telomeric locations of the KIR2DS5 gene within the KIR locus. These properties point strongly to KIR2DS5*005 being the common ancestor of all other KIR2DS5 alleles. That KIR2DS5*005 is a high-binding allotype implies that KIR2DS5*002 and the other low-binding receptors are derived forms, having acquired substitutions that reduced their avidity for C2* HLA-C. Phenylalanine 127 and arginine 158 correlate with conversion from high to low binding. Comparison of KIR2DS5*002 and KIR2DS5*009 shows how threonine 176 augments the reduction caused by arginine 158 (Fig. 2). In addition to this affect on C2 binding, Steiner et al. [23] showed that arginine 158, the only difference between KIR2DS5*002 and KIR2DS5*005, causes intracellular retention of KIR2DS5*002, as well as KIR2DS5*009. They attribute this effect to arginine 158 interfering with glycosylation of asparagine 157 [23]. Despite these deleterious properties, cells expressing KIR2DS5*002 can transmit activating signals when incubated with an antibody that binds KIR2DS5 [16].

Comparison of the organization of the KIR locus in humans and other hominoids shows that lineage III KIR genes originated in the centromeric region. During human evolution, about 1.7 million years ago, a duplication of the centromeric progenitor of the KIR2DS5 and KIR2DS3 genes introduced a copy of the KIR2DS3/5 progenitor into the telomeric region [24]. Subsequent divergence resulted in the distinctive KIR2DS3 and KIR2DS5 allelic lineages that have an average of 18 amino acid differences compared to an average of two differences between pairs of sequences in the same lineage. The substitutions that distinguish KIR2DS3 and KIR2DS5 are spread throughout the sequence, suggesting that the two KIR2DS3/5 genes diverged independently. Having the consensus sequence points to KIR2DS5*005 as the ancestor of all extant KIR2DS5 alleles. The location of KIR2DS5*005 in both the centromeric and telomeric intervals could be interpreted as evidence for KIR2DS5*005 being the ancestor of all KIR2DS3 and KIR2DS5. However, the large number of differences distinguishing these allelic lineages indicates that the dual position of KIR2DS5*005 is more likely the product of a subsequent unequal recombination event that occurred after the divergence of the KIR2DS3 and KIR2DS5 allelic lineages. Of the five KIR2DS5 alleles encoded in the centromeric region, four recognize C2* HLA-C, whereas only three of the six KIR2DS5 alleles

As a group the six KIR2DS5* allotypes that bind C2 are associated with protection from pre-eclampsia, but in testing the allotypes individually only KIR2DS5*006 is significantly protective [11]. A key question is why the other C2-binding KIR2DS5 allotypes differ in their capacity to recognize C2* HLA-C? Phenylalanine 127 and arginine 158 correlate with conversion from high to low binding. Comparison of KIR2DS5*002 and KIR2DS5*009 shows how threonine 176 augments the reduction caused by arginine 158 (Fig. 2). In addition to this affect on C2 binding, Steiner et al. [23] showed that arginine 158, the only difference between KIR2DS5*002 and KIR2DS5*005, causes intracellular retention of KIR2DS5*002, as well as KIR2DS5*009. They attribute this effect to arginine 158 interfering with glycosylation of asparagine 157 [23]. Despite these deleterious properties, cells expressing KIR2DS5*002 can transmit activating signals when incubated with an antibody that binds KIR2DS5 [16].

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Figure 3. The majority of African KIR2DS5 allotypes recognize C2* HLA-C. Shown are the relative frequencies of the KIR2DS5 allotypes that do, or do not, bind C2* HLA-C. The frequencies are from the cohort studied by Nakimuli et al. [11] and represent relative frequencies of KIR2DS5 allotypes in KIR2DS5* individuals in this population. The KIR2DS5 alleles distribute between locations in centromeric and telomeric regions of the KIR locus. Shown is the distribution between the centromeric and telomeric regions of the KIR locus of the alleles encoding KIR2DS5 allotypes that do, or do not, bind C2* HLA-C. KIR2DS5*005 has the highest frequency and is the only allele found in both genomic regions.
Table 1. Risk of pre-eclampsia associated with the presence of KIR2DS5 alleles capable of binding to C2⁺HLA-C (‘003, ‘004; ‘005; ‘006; ‘007; ‘008) from non-binding alleles (‘002; ‘009; ‘010; ‘011).

| KIR2DS5 alleles | Pre-eclampsia cases (N = 251) n (%) | Controls (N = 483) n (%) | p-valuea | OR (CI) |
|-----------------|-------------------------------------|--------------------------|----------|---------|
| ‘003; ‘004; ‘005; ‘006; ‘007; ‘008 | 82 (32.7) | 201 (41.6) | 0.01801 | 0.68 (0.49–0.94) |
| ‘002; ‘009; ‘010; ‘011 | 44 (17.5) | 101 (20.9) | NS | 0.80 (0.54–1.19) |

Fisher’s exact test with mid-p adjustment.

encoded in the telomeric region recognize C2⁺HLA-C (Fig. 3). Thus there has been increased attenuation of telomeric KIR2DS5 alleles than centromeric KIR2DS5 alleles. Of the eight substitutions that distinguish other KIR2DS5 allotypes from KIR2DS5*005 (the ancestral allele), three (F127, R158, and T176) caused major loss of ligand binding, two had little effect (R1 and H182) and three gave modest (F127, R158, and T176) caused major loss of ligand binding, asparagine at position 123, to the carbohydrate attached to KIR2DS5*005. This residue has had two effects by interfering with the glycosylation at asparagine 157. Asparagine 123, which distinguishes KIR2DS5*003, ‘004, ‘005, ‘006, ‘007, and ‘008 have higher cell-surface expression. This difference correlates with the presence of arginine 123 in KIR2DS5*002 and KIR2DS5*009 (Fig. 2). Acquisition of this residue has had two critical effects, reducing cell-surface expression and reducing avidity for C2. It has been proposed that arginine 123 exerts these effects by interfering with the glycosylation at asparagine 157. Asparagine 123, which distinguishes KIR2DS5*003 from KIR2DS5*005, creates a glycosylation site and increases the level of cell-surface expression of KIR2DS5*003. Thus the increased expression could be due to the substitution of serine for asparagine at position 123, to the carbohydrate attached to asparagine 123 or combination of these two factors. Cell-surface expression of KIR2DS5*002 is also increased by having asparagine at position 123 instead of serine [25].

KIR2DS5*002 is in strong linkage disequilibrium with KIR2DS1*002

In non-African populations, KIR2DS5*002 is part of a conserved, telomeric KIR haplotype that consists of KIR2DL4*00501, 3DS1*013, 2DL5A*001, 2DS5*002, 2DS1*002, and 3DL2*00701. This conservation is well illustrated by the IHWG panel of HLA homozygous cells lines that are predominantly of European origin. Although homozygous for HLA, these cells are almost all heterozygous for the KIR locus. Their KIR haplotypes have been determined at allele-level resolution by complete nucleotide sequencing [26]. Among the 194 KIR haplotypes of the 97 cell lines, are 31 that contain KIR2DS5*002. Of these, 28 have the conserved telomeric haplotype. The other three haplotypes differ only from the conserved haplotype by their KIR3DL2 allele. One additional telomeric haplotype contains a KIR2DS5 gene. This haplotype is the same as the conserved haplotype with the exception of the KIR2DS5 allele which is KIR2DS5*015. This allele differs by a single substitution that results in substitution of aspartate by asparagine at position 271. Thus all haplotypes with KIR2DS5*002 have KIR2DS1*002 as the neighboring gene, which encodes an activating C2 receptor that is stronger than any of the KIR2DS5 allotypes. This raises the possibility that emergence of KIR2DS1 directly led to the attenuation of KIR2DS5, as is most clearly manifest for KIR2DS5*002.

In a variant of the conserved telomeric haplotype, KIR2DS5*002 is replaced by KIR2DS3*002 and KIR2DL5A*001 is replaced by KIR2DL5A*005. KIR2DS3 has sequence similarity with KIR2DS5 and also encodes KIR with threonine 44. There is considerable evidence for the attenuation of KIR2DS3 [27] which is poorly expressed at the cell surface. This too could have been caused by emergence of KIR2DS1. The third human KIR that encodes threonine 44 is the inactivated KIR2DP1 gene, which once encoded two lineages of inhibitory KIR allotypes, one having lysine 44 and specificity for the C1 epitope, the other having threonine 44 and specificity for the C2 epitope [28]. The complete or partial demise of the human KIR with threonine 44, could have been driven by the emergence of the stronger KIR2DL1 and KIR2DS1 C2-specific receptors with methionine 44. KIR with threonine 44 have been found only in the human species and may represent an evolutionary intermediate that allowed the human KIR system to recover from the loss of KIR diversity that accompanied human speciation [28].

The rarity of the conserved telomeric KIR2DS5*002 haplotype in anthropologically well characterized African populations (Nemati-Gorgani et al., unpublished data and [13]) and its prevalence in European populations, raises the possibility that modern Europeans acquired this haplotype from archaic Europeans, such as Neandertals, a phenomenon that we previously considered in the context of the KIR3DS1*013 component of the haplotype [29]. A major benefit of the haplotype was likely conferred by KIR2DS1 in reducing the incidence of pre-eclampsia and related pregnancy syndromes [9]. On the other hand, study of European pregnancies has shown that excessive activation caused by paternal C2⁺HLA-C interacting with maternal
KIR2DS1 can lead to babies with high birth-weight and the potential complication of obstructed labor, which in the absence of surgical intervention can cause death of both mother and child [30]. Because KIR2DS5*006 has a lower avidity for C2 than KIR2DS1, this activating C2 receptor of Africans is predicted to have a reduced propensity for excessive activation than KIR2DS1 in Europeans. Indeed, as has recently been reviewed [12], Africans have fewer high birth-weight babies than Europeans with the result that pre-eclampsia and other conditions associated with low birth weight, are more prevalent than obstructed labor.

Material and Methods

Ethics statement
The experiments reported here involved no animal or human subjects. Results presented in Table 1 regarding the Ugandan cohort are a reanalysis of previously published data [11].

Sequences of KIR2DS5 alleles and proteins

The amino acid sequences of 12 KIR2DS5 allotypes are deposited in the Immunoreceptor Polymorphism Database (IPD) [31]. These sequences were first aligned automatically in Seaview 4.4.0 [32] and at positions where the alignment could not be resolved it was manually aligned within the same program. KIR-Fc fusion proteins, corresponding to 10 of the KIR2DS5 allotypes, were made. One KIR-Fc represented both KIR2DS5*006 and KIR2DS5*012, because they have identical D1 and D2 domains, differing only by a single amino acid substitution in the signal sequence. The KIR2DS5*001 sequence was not used to make a KIR-Fc because it is unlikely to represent a natural KIR2DS5 allotype, does not fold properly and is retained inside cells [23, 25]. Although it was the first KIR2DS5 sequence determined [33] subsequent analyses failed to identify any individual or cell line that types for the KIR2DS5*001 allele [34, 35]. That no confirmation of KIR2DS5*001 has occurred in the 20 years since it was first reported [33], argues strongly for the four coding nucleotide substitutions (in codons -20, 111, 164, and 174) that distinguish the coding region of KIR2DS5*001 from those of all other KIR2DS5 alleles, being artifacts of sequencing error. It is time for KIR2DS5*001 to be retired, in the same way that HLA-A*24:01 and HLA-B*07:01 were retired [36].

KIR-Fc fusion proteins and assay of their binding to HLA class I

A sequence encoding residues 1–224 of the mature KIR2DS5*005 protein was synthesized by Genscript (Piscataway, NJ). This KIR2DS5*005 construct was used as the template for mutagenesis, from which we made equivalent constructs corresponding to nine other KIR2DS5 alleles. Site-directed mutagenesis of KIR2DS5*005 used the Quick-Change kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. The KIR2DS5 constructs were cloned into the baculovirus transfection vector, pVL1393 (Expression Systems, Davis, CA). The sequences of these constructs were determined and verified to be correct. Together with linearized baculovirus DNA (Expression Systems) the constructs were transfected into Sf9 insect cells to generate baculoviruses. The baculoviruses were used to transduce Hi5 insect cells and express KIR-Fc fusion proteins, as is fully detailed in our methods paper [17]. The integrity of KIR-Fc fusion protein folding was assessed by flow cytometry as described [17]. The Sf9 and Hi5 cells were kindly provided by Chris Garcia, Stanford University.

KIR-Fc fusion proteins corresponding to KIR2DS5 allotypes and site-directed mutants, as well as the KIR2DL1*003 and KIR2DL3*001 controls, were tested for binding to a panel of 97 microbeads, each coated with one of 31 HLA-A, 50 HLA-B, and 16 HLA-C allotypes (LabScreen Single-Antigen beads lot #8, One Lambda, Kittridge, CA). To account for differences in the amount of HLA class I protein coating each of the 97 beads, the binding of KIR-Fc fusion proteins was normalized to that of W6/32, a monoclonal antibody detecting an epitope shared by all HLA class I. Normalized values were calculated using the formula: (specific binding-bead background)/(W6/32 binding-bead background). As a comparison for KIR2DS5 binding, we used our published binding data for KIR2DS1*002, the known activating receptor that is specific for C2 [4, 8].

Genetic analysis

Samples of DNA from a case-control study of pre-eclampsia involved 738 pregnant women at Mulago Hospital, Kampala in Uganda were typed for KIR and HLA-C variants including for presence/absence of KIR2DS5 alleles [11, 37]. Categorical data was analyzed with chi-square and Fisher’s exact test with two-tailed mid-p adjustment. A p-value of ≤0.05 was considered to be statistically significant. The magnitude of the effect was estimated by conditional maximum likelihood estimate of Odds Ratio (OR) for the mid-p exact test and their 95% confidence intervals (CI).

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Conflicts of Interest

The authors declare no commercial or financial conflict of interest.

References

1. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. Science 331:44–49.

2. Parham, P., and A. Moffett. 2013. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. Nat. Rev. Immunol. 13:133–144.

3. Winter, C. C., and E. O. Long. 1997. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. J. Immunol. 158:4026–4028.

4. Hilton, H. G., L. A. Guethlein, A. Goyos, N. Nemat-Gorgani, D. A. Bushnell, P. J. Norman, and P. Parham. 2015. Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. J. Immunol. 195:3160–3170.

5. Moesta, A. K., P. J. Norman, M.Yawata, N. Yawata, M. Gleimer, and P. Parham. 2008. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J. Immunol. 180:3969–3979.

6. Pende, D., S. Marcenaro, M. Falco, S. Martini, M. E. Bernardo, D. Montagna, E. Romeo, C. Cognet, M. Martinetti, R. Maccario, et al. 2009. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. Blood 113:3119–3129.

7. Saulquin, X., L. N. Gastinel, and E. Vivier. 2003. Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158). J. Exp. Med. 197:933–938.

8. Moesta, A. K., T. Graef, L. Abi-Rached, A. M. Older Aguilar, L. A. Guethlein, and P. Parham. 2010. Humans differ from other hominids in lacking an activating NK cell receptor that recognizes the C1 epitope of MHC class I. J. Immunol. 185:4233–4237.

9. Hiby, S. E., R. Apps, A. M. Sharkey, L. E. Farrell, L. Gardner, A. Mulder, F. H. Claas, J. J. Walker, C. W. Redman, L. Morgan, et al. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. J. Clin. Invest. 120:4102–4110.

10. Hibi, S. E., J. J. Walker, K. M. O’Shaughnessy, C. W. G. Redman, M. Carrington, J. Trowsdale, and A. Moffett. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. J. Exp. Med. 200:957–965.

11. Nakimuli, A., O. Chazara, S. E. Hiby, L. Farrell, S. Tukwasibwe, J. Jayaraman, J. A. Traherne, J. Trowsdale, F. Colucci, E. Lougee, et al. 2015. A KIR B centromeric region present in Africans but not Europeans protects pregnant women from pre-eclampsia. Proc. Natl. Acad. Sci. U. S. A 112:845–850.

12. Moffett, A., and F. Colucci. 2015. Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. Immunol. Rev. 267:283–297.

13. Norman, P. J., J. A. Hollenbach, N. Nemat-Gorgani, L. A. Guethlein, H. G. Hilton, M. J. Pando, K. A. Koram, E. M. Riley, L. Abi-Rached, and P. Parham. 2013. Co-evolution of human leukocyte antigen (HLA) class I ligands with killer-cell immunoglobulin-like receptors (KIR) in a genetically diverse population of sub-Saharan Africans. PLoS Genet. 9:e1003938.

14. Hou, L., M. Chen, B. Jiang, K. Kariyawasam, J. Ng, and C. K. Hurley. 2009. In contrast to other stimulatory natural killer cell immunoglobulin-like receptor loci, several KIR2DS5 alleles predominate in African Americans. Hum. Immunol. 70:733–737.

15. Hou, L., N. K. Steiner, M. Chen, I. Belle, A. L. Kubit, J. Ng, and C. K. Hurley. 2008. Limited allelic diversity of stimulatory two-domain killer cell immunoglobulin-like receptors. Hum. Immunol. 69:174–178.

16. Della Chiesa, M., E. Romeo, M. Falco, M. Balsamo, R. Augugliaro, L. Moretta, C. Bottino, and M. Vitale. 2008. Evidence that the KIR2DS5 gene codes for a surface receptor triggering natural killer cell function. Eur. J. Immunol. 38:2284–2289.

17. Hilton, H. G., A. K. Moesta, L. A. Guethlein, J. Blokhuis, P. Parham, and P. J. Norman. 2015. The production of KIR-Fc fusion proteins and their use in a multiplex HLA class I binding assay. J. Immunol. Methods. 425:79–87.

18. Barber, L. D., L. Percival, N. M. Valiante, L. Chen, C. Lee, J. E. Gumperz, J. H. Phillips, L. L. Lanier, J. C. Bigge, B. R. Parekh, et al. 1996. The inter-locus recombinant HLA-B*4601 has high selectivity in peptide binding and functions characteristic of HLA-C. J. Exp. Med. 184:735–740.

19. Moretta, A., S. Sivori, M. Vitale, D. Pende, L. Morelli, R. Augugliaro, C. Bottino, and L. Moretta. 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J. Exp. Med. 182:875–884.

20. Pittari, G., X. R. Liu, A. Selvakumar, Z. Zhao, E. Merino, M. Huse, J. H. Chewing, K. C. Hsu, and B. Dupont. 2013. NK cell tolerance of self-specific activating receptor KIR2DS1 in individuals with cognate HLA-C2 ligand. J. Immunol. 190:4650–4660.

21. Stewart, C. A., F. Laugier-Anfossi, F. Vely, X. Saulquin, J. Riedmuller, A. Tisserant, L. Gauthier, W. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. Proc. Natl. Acad. Sci. U. S. A 102:13224–13229.
22. Hilton, H. G., L. Vago, A. M. Older Aguilar, A. K. Moesta, T. Graef, L. Abi-Rached, P. J. Norman, L. A. Guethlein, K. Fleischhauer, and P. Parham. 2012. Mutation at positively selected positions in the binding site for HLA-C shows that KIR2DL1 is a more refined but less adaptable NK cell receptor than KIR2DL3. J. Immunol. 189:1418–1430.

23. Steiner, N. K., S. Dakshanamurthy, C. J. VandenBussche, and C. K. Hurley. 2008. Extracellular domain alterations impact surface expression of stimulatory natural killer cell receptor KIR2DS5. Immunogenetics. 60:655–667.

24. Pyo, C. W., L. A. Guethlein, Q. Vu, R. Wang, L. Abi-Rached, P. J. Norman, S. G. Marsh, J. S. Miller, P. Parham, and D. E. Geraghty. 2010. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. PLoS ONE. 5:15115.

25. Steiner, N. K., S. Dakshanamurthy, N. Nguyen, and C. K. Hurley. 2014. Allelic variation of killer cell immunoglobulin-like receptor 2DS5 impacts glycosylation altering cell surface expression levels. Hum. Immunol. 75:124–128.

26. Norman, P. J., J. A. Hollenbach, N. Nemat-Gorgani, W. M. Marin, S. J. Norberg, E. Ashouri, J. Jayaraman, E. E. Wroblewski, J. Trowsdale, R. Rajalingam, et al. 2016. Defining KIR and HLA Class I genotypes at highest resolution via high-throughput sequencing. Am. J. Hum. Genet. 99:375–391.

27. VandenBussche, C. J., T. J. Mulrooney, W. R. Frazier, S. Dakshanamurthy, and C. K. Hurley. 2009. Dramatically reduced surface expression of NK cell receptor KIR2DS5 is attributed to multiple residues throughout the molecule. Genes Immun. 10:162–173.

28. Hilton, H. G., J. H. Blokhuis, L. A. Guethlein, P. J. Norman, and P. Parham. 2017. Resurrecting KIR2DP1: a key intermediate in the evolution of human inhibitory NK cell receptors that recognize HLA-C. J. Immunol. 198:1961–1973.

29. Abi-Rached, L., M. J. Jobin, S. Kulkarni, A. McWhinnie, K. Dalva, L. Gragert, F. Babrzadeh, B. Gharizadeh, M. Luo, F. A. Plummer, et al. 2011. The shaping of modern human immune systems by multiregional admixture with archaic humans. Science. 334:89–94.

30. Hibi, S. E., R. Apps, O. Chazara, L. E. Farrell, P. Magnus, L. Trogstad, H. K. Gjessing, M. Carrington, and A. Moffett. 2014. Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. J. Immunol. 192:5069–5073.

31. Robinson, J., J. A. Halliwell, J. D. Hayhurst, P. Flicek, P. Parham, and S. G. Marsh. 2015. The IPD and IMGT/HLA database: allele variant databases. Nucleic Acids Res. 43: D423–D431.

32. Gouy, M., S. Guindon, and O. Gascuel. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27:221–224.

33. Dohring, C., J. Samaridis, and M. Colonna. 1996. Alternatively spliced forms of human killer inhibitory receptors. Immunogenetics. 44:227–230.

34. Vilches, C., M. J. Pando, R. Rajalingam, C. M. Gardiner, and P. Parham. 2000. Discovery of two novel variants of KIR2DS5 reveals this gene to be a common component of human KIR 'B' haplotypes. Tissue Antigens. 56:453–456.

35. Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. Immunity. 7:753–763.

36. Bodmer, J. G., S. G. Marsh, E. D. Albert, W. F. Bodmer, R. E. Bontrop, D. Charron, B. Dupont, H. A. Erlich, B. Mach, and W. R. Mayr. 1995. Nomenclature for factors of the HLA system. Tissue Antigens. 46:1–18.

37. Nakimuli, A., O. Chazara, L. Farrell, S. E. Hiby, S. Tukwasibwe, O. Knee, J. Jayaraman, J. A. Traherne, A. M. Elliott, P. Kaleebu, et al. 2013. Killer cell immunoglobulin-like receptor (KIR) genes and their HLA-C ligands in a Ugandan population. Immunogenetics. 65:765–775.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site

Figure S1. Binding of KIR2DS5-Fc to HLA-C allotypes is specific to C2 epitopes. (A-B) Individual KIR2DS5-Fc were incubated with a panel of 97 microbeads, each coated with one of 31 HLA-A, 50 HLA-B and 16 HLA-C allotypes. KIR2DS5-Fc binding normalized to W6/32 binding (described in Materials and Methods) is shown in (A) C2+ HLA-C and (B) C1+ HLA-C and C1+ HLA-B. Data is displayed as mean ± SD and comes from a minimum of two independent binding assays for each KIR2DS5-Fc. No binding was observed to any HLA-A or the remaining 48 C1+ HLA-B allotypes (data not shown).