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Different Selected Mechanisms Attenuated the Inhibitory Interaction of KIR2DL1 with C2$^+$ HLA-C in Two Indigenous Human Populations in Southern Africa

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The functions of human NK cells in defense against pathogens and placental development during reproduction are modulated by interactions of killer cell Ig-like receptors (KIRs) with HLA-A, -B and -C class I ligands. Both receptors and ligands are highly polymorphic and exhibit extensive differences between human populations. Indigenous to southern Africa are the KhoeSan, the most ancient group of modern human populations, who have highest genomic diversity worldwide. We studied two KhoeSan populations, the Nama pastoralists and the ≠Khomani San hunter-gatherers. Comprehensive next-generation sequence analysis of *HLA-A*, *-B*, and *-C* and all *KIR* genes identified 248 different *KIR* and 137 *HLA class I*, which assort into ∼200 haplotypes for each gene family. All 74 Nama and 78 ≠Khomani San studied have different genotypes. Numerous novel *KIR* alleles were identified, including three arising by intergenic recombination. On average, KhoeSan individuals have seven to eight pairs of interacting KIR and HLA class I ligands, the highest diversity and divergence of polymorphic NK cell receptors and ligands observed to date. In this context of high genetic diversity, both the Nama and the ≠Khomani San have an unusually conserved, centromeric *KIR* haplotype that has arisen to high frequency and is different in the two KhoeSan populations. Distinguishing these haplotypes are independent mutations in *KIR2DL1*, which both prevent KIR2DL1 from functioning as an inhibitory receptor for C2$^+$ HLA-C. The relatively high frequency of C2$^+$ HLA-C in the Nama and the ≠Khomani San appears to have led to natural selection against strong inhibitory C2-specific KIR. ***The Journal of Immunology*****, 2018, 200: 2640–2655.**

The greatest diversity of human genomes is present in African populations, from which all others are derived (1–3). Despite this rich and complex genetic history, as well as the large burden of infectious disease, immunogenetic studies of African populations have been limited (4, 5). Within the African continent there exists considerable population substructure, with multiple divergent clades of genetically, geographically, and culturally defined groups (6). In this regard the KhoeSan represent the deepest split among modern humans, having diverged from other populations >100,000 y ago (7, 8). Until the past few hundred years, many groups with broadly defined KhoeSan ancestry still practiced a hunting and gathering lifestyle. Because of their pivotal position in defining human history, anthropological studies of KhoeSan populations have become a major focus for studies of human ancestry and evolution (9, 10). Within this framework we investigated the genetic diversity of

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The sequences presented in this article have been submitted to the Immuno Polymorphism Database (https://www.ebi.ac.uk/ipd/) and GenBank sequence databases (https://www.ncbi.nlm.nih.gov) under accession numbers GQ890694, GQ890695, GQ890697, GQ924778, GQ924779, GQ924780, GQ924781, GU323347, GU323348, GU323349, GU323355, HM235773, JX523630, JX523631, JX523634, JX523635, JX523636, JX523637, JX523638, JX523639, JX523640, JX523641, JX523642, JX523643, JX523644, JX523645, JX523646, JX523647 JX523648 JX523649, JX523650, JX523651, JX523652 JX523653, JX523654, JX523655 LT630459, LT630460, LT630461, LT630462, LT630463, LT630464, LT630465, LT630466, LT630467, LT630468, LT630470, LT630472, LT630473, LT630474, LT630475, LT630476, LT630477, LT630478, LT630479, LT630480, LT630481, LT630482, LT630483, LT630485, MF667961, MF667962, MF678815, MF796632, MF796633, MF796634, MF796635, MF796636, MF796637, MF796638, MF796639, MF796640, MF796641, MF796642, MF796643, MF796644, MF796645, MF796646, MF796647, MG491997, MG491998, and MG491999.

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Abbreviations used in this article: IPD, Immuno Polymorphism Database; KIR, killer cell Ig-like receptor; RDP, Recombination Detection Program; SNP, single nucleotide polymorphism; TB, tuberculosis.

killer cell Ig-like receptors (KIR) and their HLA class I ligands, critical factors in immunity and reproduction that contribute to the health and survival of human populations (11).

"KhoeSan" refers to a broadly dispersed set of human populations indigenous to southern Africa who speak a distinct family of languages characterized by "click" consonants. Studies of Y chromosome, mitochondrial, and autosomal DNA all show that the KhoeSan are the most genetically variable of human populations, and point to an origin for modern humans in the southern part of Africa (12–14). That the KhoeSan made significant contributions to the foundation of modern human populations is supported by archaeological and climatological studies (15–17). The KhoeSan were mostly isolated from other populations until ~2000 y ago when admixture with other groups, including East Africans, Bantu-speakers, and most recently, Europeans, began (9, 18). Nevertheless, significant population substructure remains among the various KhoeSan populations because of major ecological and geographical barriers, including the Kalahari Desert (19–21). The Nama, resident in Namibia and western South Africa, and the ≠Khomani San, from the southern edge of the Kalahari, are linguistically and genetically discrete subpopulations of KhoeSan. During the last ~2000 y, the Nama have practiced a nomadic pastoralism culture, which they acquired from East Africans (22, 23). For the most part, the ≠Khomani San observed a hunter-gathering lifestyle during the 20th century. Both populations have survived until recently with limited access to modern medicine, and little is known of their resistance and susceptibility to disease.

In sub-Saharan Africa, infectious diseases and maternal/infant mortality remain among the highest worldwide and present significant barriers to human survival (24, 25). Relevant to these challenges are peripheral NK cells that respond to microbial infection, and uterine NK cells that control embryo implantation (11, 26, 27). During early stages of infection, peripheral NK cells kill infected cells, release cytokines, and both mobilize and direct the immune response (28, 29). During early pregnancy, uterine NK cells control remodeling of spiral arteries to increase the blood supply to the fetus (30, 31). Modulating these effector functions, as well as NK cell development, are the interactions of KIR with HLA class I molecules expressed on the surface of tissue cells (32). Consequently, the polymorphic *KIR* and *HLA class I* genes help define an individual's NK cell receptor repertoire (33, 34). Because *KIR* and *HLA* segregate on different chromosomes, numerous and varied diseases are associated with particular inherited combinations (35). Accordingly, specific KIR/HLA combinations influence the control of infections that are highly relevant to African populations, including HIV (36), malaria (37, 38), and others (39–42). Specific KIR/HLA combinations also correlate with a spectrum of reproductive syndromes associated with fetuses that are undernourished or overnourished by the placenta (43, 44).

HLA-A, -B, and -C variants that function as KIR ligands are the HLA-A3 and -A11 allotypes, subsets of HLA-A and -B allotypes having the Bw4 or C1 epitope, and HLA-C allotypes, all of which carry either the C1 or C2 epitope (11, 32). HLA-C has thus evolved toward a more intensive interaction with KIR than either HLA-A or -B (45). In addition to the ligand motif, at residues 76–83 in the α_1 helix, natural variation throughout the HLA class I sequence can affect KIR binding and NK cell activity (46, 47). Also having direct impact on NK cell activity is allelic variation of the *KIR*, which modulates expression, binding, and signaling of the receptor (48–53). Genomic structure variation is another factor, with up to 13 *KIR* genes being present, absent, or duplicated on a given *KIR* haplotype (54–58). Eight of the *KIR* genes express receptors with known specificity for HLA class I. Four of these are highly polymorphic and inhibitory in function (KIR2DL1, 2DL2/3, 3DL1, and 3DL2), and four are more conserved (KIR2DS1, 2DS2, 2DS4, and 2DS5) and have activating function. The *KIR* locus partitions into two segments named by their relative centromeric or telomeric orientation on chromosome 19 (57). Each segment has two broad forms, termed *KIR A* and *KIR B*. Among the interactions affecting NK cell activity, recognition of $C2^+$ HLA-C by KIR2DL1 allotypes commonly encoded by European centromeric *KIR A* haplotypes is highly specific and strongly inhibiting (46, 59). In contrast, we recently described in the ≠Khomani San population of KhoeSan people KIR2DL1 allotypes that are not inhibitory C2 receptors (60). To place these unusual receptors in context, we have determined, at high resolution, the complete *KIR* and *HLA class I* repertoires of the Nama and ≠Khomani San populations of KhoeSan.

Materials and Methods

Study population

KIR and *HLA class I* diversity of two southern African populations were determined by analysis of genomic DNA extracted from samples of saliva. These samples were obtained from 81 individuals (74 unrelated) from the Nama population and 94 individuals (78 unrelated) of the ≠Khomani San population (hereafter referred to as Khomani). Familial relationships were determined by demographic interviews, which included all four grandparents. Interviews were coded into multigenerational pedigrees, checked against genome-wide array data when available and unrelated individuals selected using the *pedigree unrelated* function of the *kinship2* R package (61). All samples were collected according to the protocol approved by the Stanford University Institutional Review Board (Protocol 13829) and the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Stellenbosch University (approval number N11/07/210), as described previously (12, 21, 60).

Library preparation and enrichment

For each sample, 500 ng of genomic DNA (as determined by Qubit; Life Technologies, Carlsbad, CA) was sheared into 800-bp fragments using a Covaris E220 instrument (Covaris, Woburn, MA) with the following settings: Duty cycle 5%, Intensity 2, 200 cycles/burst, duration 45 s. Subsequent library preparation was based on the Kapa Hyper Prep protocol (Kapa Biosystems, Wilmington, MA) with the following modifications. A dual size selection was performed after the postligation cleanup. In the first size selection, 35 μl of Ampure beads (Beckman Coulter, Brea, CA) plus 45 μl of H_2O were added to 50 μl of sample. In the second size selection, 15 μl of Ampure beads (Beckman Coulter) were added to 125 μl of sample. In the library amplification step, seven cycles were performed for optimal yield of the library. To label the library obtained from each DNA sample, we used 96 unique dual index Illumina adaptors that were synthesized by Integrated DNA Technologies (Coralville, IA).

Library samples were then subject to enrichment for the *KIR* genomic region, and the complete set of *HLA* genes using a pool of 10,456 oligonucleotide probes manufactured as described previously (62). The enrichment was performed using a modified version of the Nextera Rapid Capture Exome enrichment protocol (Illumina, San Diego, CA) as described previously (62) and then subjected to paired-end sequencing using Illumina's MiSeq instrument and V3 sequencing chemistry (Illumina). The sequencing read length was 2 × 300 bp.

Next generation sequence data processing and analysis

Sequence reads specific to the *KIR* region were identified and harvested using Bowtie 2 (63). The 29 sequenced *KIR* haplotypes (57, 64) and all *KIR* alleles from the Immuno Polymorphism Database (IPD)/KIR database 2015 release (65) were concatenated to create a single reference file for this purpose. The equivalent of 70,000 read pairs that passed this filter stage were taken per sample for *KIR* genotyping. *KIR* genotyping was performed using the Pushing Immunogenetics to the Next Generation pipeline (62). This pipeline generates a high-resolution *KIR* gene content and allele level genotype. It can also identify previously unreported single nucleotide polymorphisms (SNPs) and recombinant alleles. Because Pushing Immunogenetics to the Next Generation produces accurate copy number data, for example, it distinguishes haplotypes having zero, one,

two, or more copies of a given *KIR* gene, we consider "gene absence" to be an allele. *HLA class I* allele composition was determined using NGSengine 1.7.0 (GenDX, Utrecht, the Netherlands), with the "IMGT 3.18.0 combined" reference set. There was no prefiltering for *HLA* genes, and the data were analyzed directly by the software.

Novel allele sequences were analyzed by visual inspection: reads specific to the relevant gene were isolated by bioinformatics filtering, aligned to the closest reference allele using MIRA 4.0.2 (66), and inspected using Gap4 of the Staden package (67) or Integrative Genomics Viewer (68). Recombination breakpoints were characterized using Recombination Detection Program (RDP) version 4 (69).

Sanger sequencing and cloning

Novel allele sequences were validated using independent PCR amplification, cloning, and/or Sanger sequencing. PCR amplicons were sequenced in forward and reverse direction using BigDye Terminator Chemistry v. 3.1 and an ABI 3730 Bioanalyzer (MC Lab, San Francisco, CA). Where applicable, the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) was used, with at least three clones having the novel allele sequence being analyzed. When it was not possible to resolve a novel allele's phase by PCR/cloning, the sample was reanalyzed using the NGS protocol, starting from a fresh DNA preparation. Allele sequences were submitted to GenBank and the IPD database (65). They are also available at the Web site: https://github.com/n0rmski/PING. Accession numbers and designated allele names are given in the *Results*. Novel alleles identified in only one heterozygous individual were not submitted to IPD, and they are presented with local allele names. The protein encoded by an allele is referred to as the allotype.

Allele and haplotype frequencies

Among the individuals studied were members of 11 families. Genotypes from these families were used to determine haplotype structures. All the allele and haplotype frequencies we describe are derived from data obtained from the 74 unrelated Nama and 78 unrelated Khomani. In addition to family-based deduction, the composition and frequencies of *KIR* haplotypes were determined at the allelic level using PHASE (70). The following parameters were used: −f1, −x5, and −d1. Similarly, *HLA class I* haplotype composition and frequency were determined using the expectation maximization algorithm of Arlequin (71).

Assessment of receptor/ligand diversity within individuals and populations

The number of KIR/HLA allotype pairs that are known to interact were defined for each individual. For example, an individual expressing KIR2DL1*002, 2DL1*003, HLA-C*02:02, and HLA-C*04:01 (both HLA-C allotypes have the C2 epitope) has four ligand–receptor interactions between KIR2DL1 and HLA-C. In contrast, a KIR2DL1*001 homozygous individual having HLA-C*02:02 and HLA-C*04:01 has only two such ligand–receptor interactions. Experimental data were used to determine the interacting pairs of KIR and HLA class I (46, 72–77). Broadly [with exceptions listed (46, 72–76)], KIR2DL1 binds to $C2^+$ HLA-C allotypes, KIR2DL2 binds to $C1^+$ and $C2^+$ HLA, KIR2DL3 binds to only $C1^+$ HLA, KIR2DS1 and some KIR2DS5 allotypes bind to $C2^+$ HLA-C, KIR2DS2 binds to HLA-C*16, and KIR2DS4 binds to HLA-A*11 and some HLA-C. KIR3DL1 binds to $Bw4^+$ HLA-A and $Bw4^+$ HLA-B. KIR3DL2 binds to HLA-A*03 and HLA-A*11 allotypes. KIR3DS1 was not included in the analysis because it binds to HLA-F (78, 79), and KIR2DS3 was assumed not to bind HLA, based on previous studies (52, 74).

Assessment of KIR2DL1 and $C2^+$ HLA-C functional diversity in populations

To account for differences in the potency of NK cell inhibition mediated by specific combinations of KIR2DL1 and $C2^+$ HLA-C, we generated a table listing the binding affinity for each ligand–receptor combination, as described previously (46). The frequency of each ligand–receptor pair in a population was calculated by multiplying the frequencies of the respective *KIR2DL1* and *$C2^+$ HLA-C* alleles. This number was then multiplied by their binding avidity. For KIR2DL1 allotypes having cysteine at residue 245 the values were halved because these KIRs have attenuated function (48). The final function-adjusted frequency for a population is defined as the sum of frequencies of all the individual receptor–ligand pairs scored in this manner, divided by the sum of frequencies for all the individual receptor–ligand pairs, without weighting for the binding and signaling scores. Absence of *KIR2DL1* was considered an allele. The analysis was repeated for KIR2DL2/3 and $C1^+$ HLA (including results for C1-specific KIR2DL1*022). Populations included in this analysis were Japanese (80), Yucpa Amerindians (81), Polynesians (82), Maori (82), Europeans (83), and Ga-Adangbe West Africans (84). For this analysis we also included *HLA class I* data from 53 Hadza from East Africa (12), who were genotyped for *KIR2DL1* and *KIR2DL2/3* using the method described (84).

Results

Diversity and divergence of Nama and Khomani KIR alleles

KIR diversity was defined for two KhoeSan populations from southern Africa, the Nama and the Khomani. A customized next generation sequencing method (62) was targeted to seven genes encoding inhibitory KIR and five genes encoding activating KIR of 152 individuals: 74 Nama and 78 Khomani. A total of 248 different *KIR* alleles were detected, of which 131 are shared by the two populations (Fig. 1A). The genes encoding inhibitory KIR are far more diverse (17–60 alleles) than the genes encoding activating KIR (2–11 alleles), a bias also observed for other populations (80–84) (Fig. 1A). In comparison with those other populations, for which a cohort of similar size has been analyzed to equivalently high resolution, the Nama and the Khomani exhibit greater numbers of alleles for all the highly polymorphic *KIR* (Fig. 1B).

Consistent with the KhoeSan representing the oldest known divergence of modern humans, 64 of the *KIR* alleles detected (26% of the total) are novel and could be specific to the KhoeSan. Of these alleles, 44 are defined by SNPs, 35 of which cause an amino acid substitution. The other 20 alleles are each distinguished by novel combinations of known SNPs, and 12 of these define novel polypeptide sequences (Fig. 2). Similar analysis of the two *KIR* pseudogenes, *KIR2DP1* and *KIR3DP1*, uncovered 20 novel alleles (Supplemental Fig. 1). With this comprehensive approach we identified novel substitutions in all the protein domains, including those that control ligand specificity (Ig-like domains D0, D1, and D2) and those that mediate intracellular signaling (the transmembrane and cytoplasmic domains) (Fig. 2).

KIR fusion alleles in the KhoeSan

Three of the KhoeSan-specific *KIR* are recombinants comprising a 5′ segment derived from one *KIR* gene and a 3′ segment derived from another. Each of these fusions resulted in a complete open reading frame that combines the ligand-binding properties of one parent with the signaling properties of the other parent (85). These fusion proteins are therefore likely to be expressed as intact and functional KIR on NK cells (Fig. 3).

*KIR3DL2*054*, identified in the Khomani, is the product of a fusion between *3DL1*004* and *3DL2*001*, for which the crossover is in exon 3 encoding the D0 domain. Analysis of the large Khomani family in which *3DL2*054* was identified showed that the haplotype containing *3DL2*054* lacks *3DL1/S1* and *3DL2*, consistent with the asymmetric recombination having deleted the intervening *KIR* genes (Fig. 3A). The leader peptide and much of the D0 domain of KIR3DL2*054 derive from 3DL1*004, whereas the rest of the receptor derives from 3DL2*001. Distinguishing 3DL1*004 from other KIR3DL1 allotypes is leucine 86 in the D1 domain (50). This residue causes almost all the 3DL1*004 protein to misfold and be retained in the endoplasmic reticulum by calreticulin, with only a small amount of properly folded 3DL1*004 reaching the cell surface (86). A key feature of the recombination that formed 3DL2*054 is that leucine 86 was not transferred from 3DL1*004 to 3DL2*054. Thus, 3DL2*054 is predicted to be well expressed at the cell surface and to function there as an inhibitory NK cell receptor.

A

KIR	KhoeSan total alleles (152 ind.)	Nama (74 individuals) alleles N	alleles H	alleles novel	allotypes N	Khomani (78 individuals) alleles N	alleles H	alleles novel	allotypes N	Shared alleles N	Shared alleles %
2DL1	22	16	0.84	3	11	17	0.86	5	11	11	50.0
2DL2/3	27	21	0.84	4	18	18	0.84	3	15	12	44.4
2DL4	17	14	0.84	2	9	15	0.86	3	8	12	70.6
2DL5	18	16	0.60	2	10	15	0.76	3	9	11	61.1
3DL1/S1	33	26	0.93	6	23	26	0.92	5	24	19	57.6
3DL2	41	30	0.92	6	25	32	0.91	6	23	21	51.2
3DL3	60	43	0.94	13	32	41	0.92	11	33	24	40.0
2DS1	2	2	0.24		1	2	0.25		1	2	100
2DS2	4	3	0.50	2	2	3	0.49	2	2	4	100
2DS3	4	4	0.60	1	2	2	0.77	1	1	2	50.0
2DS4	9	8	0.71		1	8	0.64	1	1	7	77.8
2DS5	11	8	0.60	4	6	9	0.77	4	7	6	54.5

B

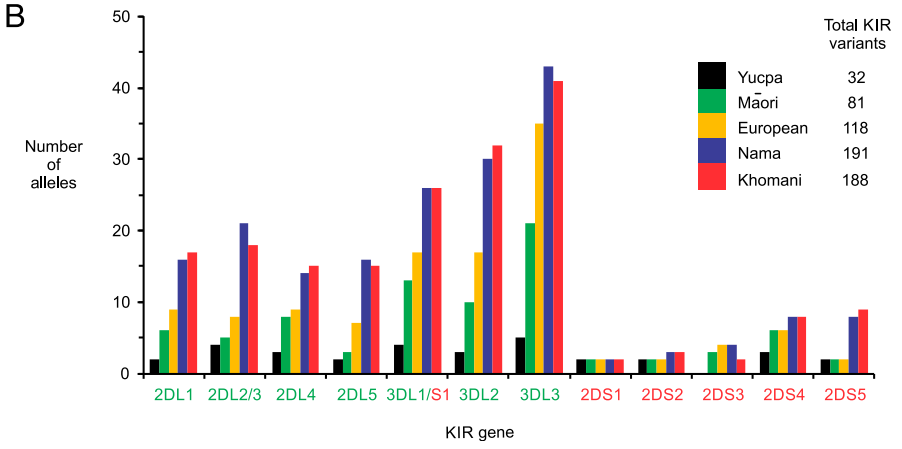


FIGURE 1. High genetic diversity of KhoeSan KIR. (**A**) Shown for each *KIR* gene is the number of alleles (N) and the heterozygosity (H), the number of newly identified (novel) *KIR* alleles, and the number of KIR allotypes detected in the Nama and Khomani. Names for genes encoding inhibitory KIR are given in green text, and those encoding activating KIR in red text. *KIR* gene absence is considered an allele. At the left is shown the total number of different alleles in the KhoeSan, and the right is the number of those observed in both Nama and Khomani (shared alleles). (**B**) Shown is the number of alleles observed for each *KIR* gene in the Nama (blue, $n = 74$) and Khomani (red, $n = 78$), and three populations are analyzed to the same level of resolution: Yucpa Amerindians (black, $n = 61$), Māori (green, $n = 73$), and Europeans (gold, $n = 74$) (62, 81–83).

The *KIR3DL1*1501/3DL2* fusion allele, which was identified in two unrelated Nama individuals, is also a product of recombination between *KIR3DL1* and *KIR3DL2* alleles. It combines the leader peptide and Ig-like domains of 3DL1*01501 with the stem, transmembrane, and cytoplasmic domains of 3DL2*001 (Fig. 3B). The crossover occurred in a LINE element of intron 5, a site implicated in the origin of other *KIR3DL1/2* fusion alleles (56, 85). That the same 30-bp segment identifies the crossover point in different variants suggests that independent crossovers occurred in exactly the same place, or that other *KIR3DL1/2* fusion alleles were generated from one founder *KIR3DL1/2* fusion allele by further recombination. The haplotype containing *3DL1*015/3DL2* has the same gene content as that containing *3DL2*054* (Fig. 3B).

The third fusion allele, *KIR2DL3*002x*, observed in the Nama, was formed by recombination between *2DL3* and *2DL1* genes (Fig. 3C). Because the encoded protein combines the extracellular domains of 2DL3*002 with the stem, transmembrane, and cytoplasmic domains of 2DL1*012, KIR2DL3*002x is predicted to be an inhibitory C1-specific receptor. *KIR2DL3*002x* is part of a haplotype containing either four or six *KIR* genes. These alternatives could not be distinguished because *KIR2DL3*002x* was identified in only one individual (Fig. 3C).

At high frequency in the Nama and Khomani are novel KIR2DL1 allotypes that do not function as inhibitory C2 receptors

The distribution of *KIR* allele frequencies (Fig. 4) shows that for both populations, each inhibitory *KIR* gene is represented by multiple alleles. One to three alleles are present at intermediate frequencies (i.e., alleles observed in ≥10 individuals), and the majority of alleles have low frequencies (Fig. 4A–G). In contrast, all but one of the activating KIRs are represented by one high-frequency allotype (Fig. 4H, 4I). The exception, KIR2DS5, is represented by three to four allotypes of intermediate frequency (Fig. 4J). On average, each KhoeSan individual has 3.5 novel *KIR* alleles, and 81.5% of the KhoeSan has ≥1 novel alleles. In terms of frequency, KhoeSan-specific alleles comprise 21% of the Nama *KIR* alleles and 13% of the Khomani *KIR* alleles (Fig. 4K). The genes contributing most of the novel alleles are *KIR2DL1* (22% Nama and 26% Khomani), *2DL5B* (20 and 28%), *2DS5* (18 and 30%), and *3DL3* (30 and 40%). All of these genes are in the centromeric region of the *KIR* locus (57, 64) and they potentially represent a single *KIR* gene-content haplotype. This haplotype is commonly found in other sub-Saharan African populations, but is rarely found outside Africa (84). In this context we examined the *KIR* haplotype diversity of the KhoeSan.

In the KhoeSan we identified 159 centromeric, 108 telomeric, and 221 combined centromeric and telomeric *KIR* haplotypes distributed between the two populations (Supplemental Fig. 2). Most of these have low frequency, with only 16 (~10%) of the centromeric and 21 (~20%) of the telomeric *KIR* haplotypes being detected in more than three individuals (Fig. 5A, 5B). Dominating the centromeric *KIR* region are *B* haplotypes (64% in the Nama and 71% in the Khomani; Fig. 5C), whereas the telomeric *KIR* region is dominated by *A* haplotypes (81 and 77%, respectively). Only 15 centromeric, 27 telomeric, and 7 combined *KIR* haplotypes are common to the Nama and Khomani populations (Supplemental Fig. 2). A majority of the *KIR* haplotypes (67% centromeric, 55% telomeric, 80% complete *KIR*) were seen in single individuals (Supplemental Fig. 2).

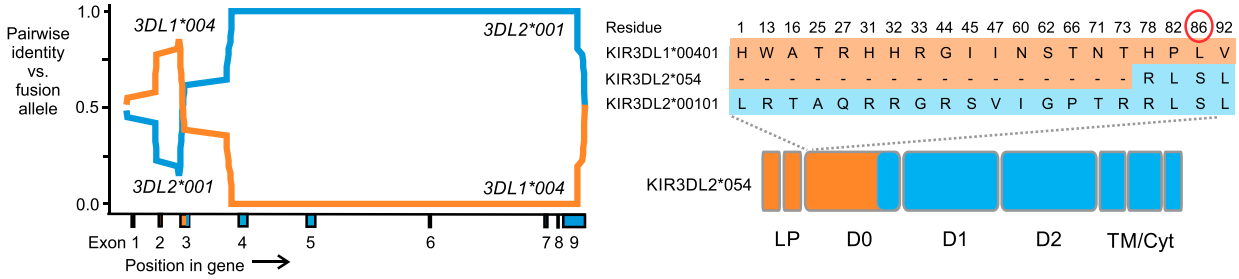
KIR gene	GenBank ID	Allele name	Closest allele	Nucleotide change	Coding change	Domain
2DL1	GU323355	*02201	*001	194 T-A	44 M-K	D1
	JX523630	*026	*012	801 G-A	246 W - stop	TM
	LT630466	*035	*00401	709 G-A	216 E-K	
	LT630467	*036	*010	523 A-C, 596 T-G	154 T-P, 178 F-C	D2
	LT630479	*02202	*022	213 C-A	syn	
	LT630480	*00306	*00303	813 G-A	syn	
2DL2	JX523631	*012	*003	71 G-A	3 G-E	D1
	MF796632	L2_001syn	*00101	333 G-A	syn	
2DL3	LT630463	*035	*00501	95 T-A	11 L-Q	D1
	LT630464	L3_012syn	*01202	1034 T-A	syn	
	MF796633	L3_002x	*00201	fusion	TM/Cyt = 2DL1	
2DL4	JX523634	*024	*01201	1046 G-C	326 S-T	Cyt
	JX523635	*025	*005	1046 G-C	326 S-T	Cyt
	JX523636	*026	*001	1046 G-C	326 S-T	Cyt
	JX523637	*027	*013	821 A-C	251 stop-S	TM
	MF796634	001syn	*00103	343 C-T	syn	
2DL5	JX523638	*00803	*00801	1098 T-C	syn	
	JX523639	*018	B*00801	119 C-A, 286 C-T	19 P-H, 75 R-W	D0
	LT630465	*00805	B*00803	693C-G	syn	
2DS2	MF667961	004b	*004	36 G-C	syn	
	MF678815	*00105	*001	735 T-C	syn	
2DS3	JX523640	*007	*001	709 A-G	216 K-E	TM
2DS4	LT630485	*00106	*00101	339 T-G	syn	
2DS5	JX523641	*00502	*005	399 C-A	syn	
	JX523642	*012	*006	23 A-T, 843 G-T	-14 K-M, syn	LP
	LT630481	*017	5*012	670 T-A, 709 G-A	203 S-T, 216 E-K	TM
	MF667962	5*01202	*012	843 T-G	syn	LP
3DL1	GQ890694	*069	*00401	320 T-C	86 L-S	
	GQ890695	*070	*01701	337 G-C	92 V-L	D0
	GQ890697	*071	*035	337 G-C	92 V-L	D0
	LT630468	*110	*00401	393 T-G, 400 G-C	syn, 113 V-L	D1
	LT630470	*111	*022	202 G-A	syn	
	MF796635	015_356V	*01501	1129 C-T	356 A-V	Cyt
	MF796636	*113	*035	982 G-C	307 E-Q	TM
	MF796637	01501/2v	*01501	fusion	TM/Cyt = 3DL2	
3DL2	GU323347	*052	*040	84 A-G, 139 C-T	syn, 26 L-F	D0
	GU323348	*053	*055	791 G-A	243 R-H	
	GU323349	*054	*001	fusion	LP/D0 = 3DL1, D1-CYT = 3DL2	
	JX523649	*063	*002	1291 C-T	410 L-F	Cyt
	JX523650	*00103	*001	12 G-T	syn	
	LT630472	*11001	*00201	791 G-A	243 R-H	D2
	LT630473	*11002	*00202	791 G-A	243 R-H	D2
	LT630474	*088	*007	1211 G-A	383 C-Y	Cyt
	MF796638	010_LP	*010	16 G-A	-15 V-I	LP
3DL3	GQ924778	*037	*01403	496 C-T	145 R-C	
	GQ924779	*038	*020	647 T-C, 648 G-A	195 V-A, syn	D1
	GQ924780	*039	*01403	319 T-C	86 S-P	D0
	GQ924781	*040	*027	937 G-A	292 V-I	D2
	HM235773	*041	*02701	502 G-A	147 V-I	
	JX523651	*057	*00801	869 A-C	269 N-T	
	LT630459	*066	*00102	155 G-A	31 R-H	
	LT630460	*067	*01101	565 C-T	168 L-F	D1
	LT630461	*068	*01101	230 G-A, 541 T-A, 565 C-T	56 R-Q, 160 S-T, 168 L-F	D1
	LT630462	*069	*020	319 T-C	86 S-P	D0
	LT630483	*070	*01403	106 G-A	15 G-S	D0
	MF796639	049R	*049	869 A-C	269 N-T	
	MF796640	01402_168F	*01402	230 G-A, 565 C-T, 869 A-C	56 R-Q, 168 L-F, 269 N-T	D1
	MF796641	*073	*01403	565 C-T, 869 A-C	168 L-F, 269 N-T	D1
	MF796642	012R	*012	1042 G-C, 1119 A-T	327 A-P, 352 E-D	
	MF796643	11syn1	*00202	6 G-A	syn	
	MF796645	020syn	*020	438 A-C	syn	
	MF796644	*01103	*01102	36 G-T	syn	
	MF796646	13_277H	*01303	893 G-A	277 R-H	D2
	MF796647	017R	*017	69A-G, 230 A-G	syn, 56 Q-R	

FIGURE 2. Novel *KIR* alleles are observed in the Nama and Khomani. Shown are the novel *KIR* alleles detected. From left to right: the *KIR* gene, GenBank ID, new allele name (asterisk indicates official name), the closest matched of the previously established alleles, nucleotide changes compared with closest match, corresponding amino acid substitutions, and the domains where the latter occur (leader peptide [LP], Ig-like domains [D0–D2], transmembrane [TM], and cytoplasmic [Cyt]). Red text indicates novel substitutions. Blue text indicates allele detected in Khomani, violet text detected in Nama, and black text detected in both populations. syn, synonymous nucleotide substitution.

In the context of a highly variable genetic background, it is striking that two centromeric *KIR* haplotypes carrying novel *KIR* alleles are present at high frequency, one in the Nama and the other in the Khomani. With 8.9% frequency, haplotype 1 is the most common centromeric *KIR* haplotype in the Nama, whereas haplotype 2 is the most common in the Khomani and has a frequency of 11.7% (Fig. 5A). To investigate whether these distinctive haplotypes could have derived from admixture with other sub-Saharan Africans or Europeans (9, 18), we examined whole-genome data (21). Strong correlation between KhoeSan ancestry of the *KIR* haplotype, as independently derived from the whole genome data, and the number of novel *KIR* alleles carried by that

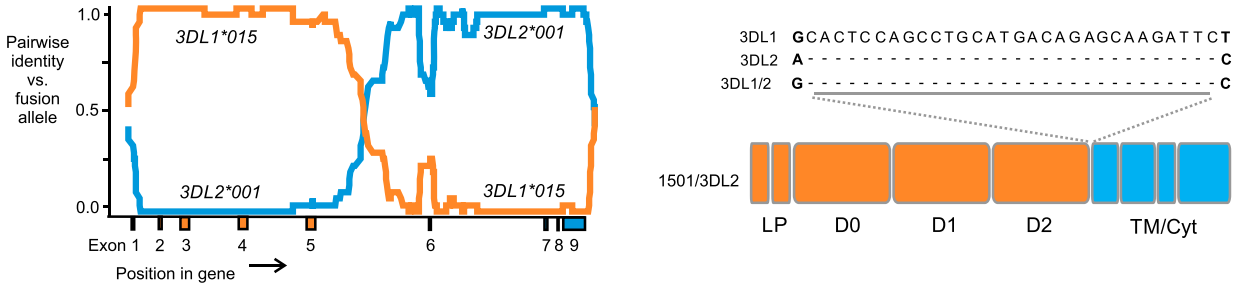
A

	3DL3	2DS2	2DL2/3	2DL5B	2DS3	2DP1	2DL1	2DL4	3DL1	2DL5A	2DS5	2DS1	2DS4	3DL2	
Parent 1	*01403	*001	2*003					*00802	3DL2*054						a
	*040		3*005			*009	*01202	*00103	*01502				*006	*00103	b
Parent 2	*01405	*001	2*001	B*00201	3*00103	*012	*00401	*00602	*007				*004	*008	c
	*038	*001	2*003	B*00201	3*007	*00102	*00401	*00801	*00501				*010	*010	d
Child 1,2,3	*01403	*001	2*003					*00802	3DL2*054						a,c
	*01405	*001	2*001	B*00201	3*00103	*012	*00401	*00602	*007				*004	*008	
Child 4	*01403	*001	2*003					*00802	3DL2*054						a,d
	*038	*001	2*003	B*00201	3*007	*00102	*00401	*00801	*00501				*010	*010	
Child 5	*040		3*005			*009	*01202	*00103	*01502				*006	*00103	b,d
	*038	*001	2*003	B*00201	3*007	*00102	*00401	*00801	*00501				*010	*010	



B

	3DL3	2DS2	2DL2/3	2DL5B	2DS3	2DP1	2DL1	2DL4	3DL1	2DL5A	2DS5	2DS1	2DS4	3DL2
Ind. 1	11syn1		3*00101			*00301	*00302	*00103	1501/3DL2					
Ind. 2	11syn1		3*00101			*00301	*00302	*00103	1501/3DL2					



C

	3DL3	2DS2	2DL2/3	2DL5B	2DS3	2DP1	2DL1	2DL4	3DL1	2DL5A	2DS5	2DS1	2DS4	3DL2
Ind. 1	*01801		3*002x					*00103	*01501				*00101	*11001
(genotype)	*038	*001	2*00301	B*00805	5*012	*023	*026	*00802	*059					

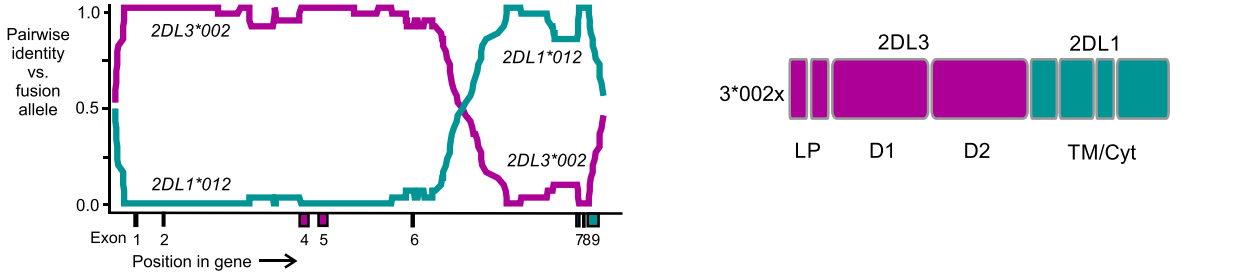


FIGURE 3. Three novel *KIR* gene fusion alleles are present in the KhoeSan. (**A**) Segregation in a family of the *KIR* haplotype carrying *KIR3DL2*054*, a fusion formed from *3DL1*004* and *3DL2*001*, is shown. The parental haplotypes are shown at the right (a, b, c, d), and the haplotype containing the fusion allele is shaded red. Lower left: a comparison of each parental sequence with the fusion allele shows the crossover occurred in exon 3, encoding the D0 domain. The plot was generated using the RDP4 (69). Lower right: a cartoon of the molecular structure (orange, 3DL1; blue, 3DL2) and an alignment of the amino acid residues distinguishing D0 of 3DL1*004 from 3DL2*001. Leucine 80 that reduces expression of 3DL1*004 is circled red. (**B**) A *KIR* haplotype, observed in two unrelated individuals, that has a fusion allele formed from *3DL1*01501* and *3DL2*001* (provisionally named *1501/3DL2*) is shown. Lower left: comparison with the parental sequences shows the crossover occurred in intron 5. Lower right: a cartoon of the molecular structure (orange, 3DL1; blue, 3DL2) and an alignment of the nucleotide sequence at the crossover point, which is common to other *3DL1/2* variants: *3DL1*059*, *060*, *063* (85). (**C**) The *KIR* genotype of an individual having a fusion of *2DL3*002* with *2DL1*012* (provisionally named 3*002x) is shown. RDP analysis (lower left) shows the crossover likely occurred in intron 5, resulting in a KIR (lower right) combining the ligand specificity of 2DL3 (violet) with the signaling properties of 2DL1 (green).

haplotype indicate the alleles we discovered here are KhoeSan-specific (Fig. 5D). Thus, haplotypes 1 and 2 contain distinctive *KIR2DL1*, *2DL5*, *2DS5*, and *3DL3* alleles that are characteristic of the KhoeSan. In the Khomani, haplotype 2 encodes KIR2DL1*022, which is C1-specific and not C2-specific like other KIR2DL1 allotypes. In the Nama, haplotype 1 encodes 2DL1*026, which is C2-specific but lacks the cytoplasmic domain and so cannot transmit inhibitory signals (60). Thus, the common and defining feature of haplotypes 1 and 2 is that their KIR2DL1 alleles do not encode proteins that function as inhibitory C2-specific receptors.

The KhoeSan favored KIR2DL1 allotypes that are not strong inhibitory receptors

In the Khomani, KIR2DL1*022 is encoded on one high-frequency and five low-frequency haplotypes (Fig. 6A). This distribution suggests this unusual form of KIR2DL1, an inhibitory C1-specific receptor, has functional benefit. Similarly, in the Nama, KIR2DL1*026, which lacks inhibitory signaling function, is encoded by one high-frequency and four low-frequency haplotypes (Fig. 6B). For all other *2DL1* alleles, there are no high-frequency haplotypes, but instead a variety of low-frequency haplotypes (Supplemental Fig. 2) (60). For example, *KIR2DL1*004* has frequencies similar to *2DL1*022* in the Khomani and *2DL1*026* in the Nama, but is associated with 15 different low-frequency haplotypes (Fig. 6A, 6B). Thus, *2DL1*004* likely rose to high frequency over an extended period during which it recombined onto a range of *KIR* haplotypes. In contrast, *2DL1*022* and *2DL1*026* rapidly increased in frequency over a time frame that precluded their haplotypic background being diversified by recombination. This latter distinction is the hallmark of haplotypes that have been subject to recent, positive natural selection (87, 88). That the *2DL1*022* and **026* haplotypes are predominantly distinguished by alleles of *KIR3DL3*, which is the most distal centromeric *KIR* locus from *KIR2DL1* (Fig. 6C), and contains a recombination hotspot (89), reinforces this model for the evolution of *KIR2DL1*022* and *KIR2DL1*026*. That both parental haplotypes are present in both populations, yet only one of them dominates in each KhoeSan population, argues against a role for population bottleneck as the cause of the elevated frequency of KIR2DL1*026 in the Nama and KIR2DL1*022 in the Khomani. Instead, the data argue strongly for positive natural selection being the causative mechanism.

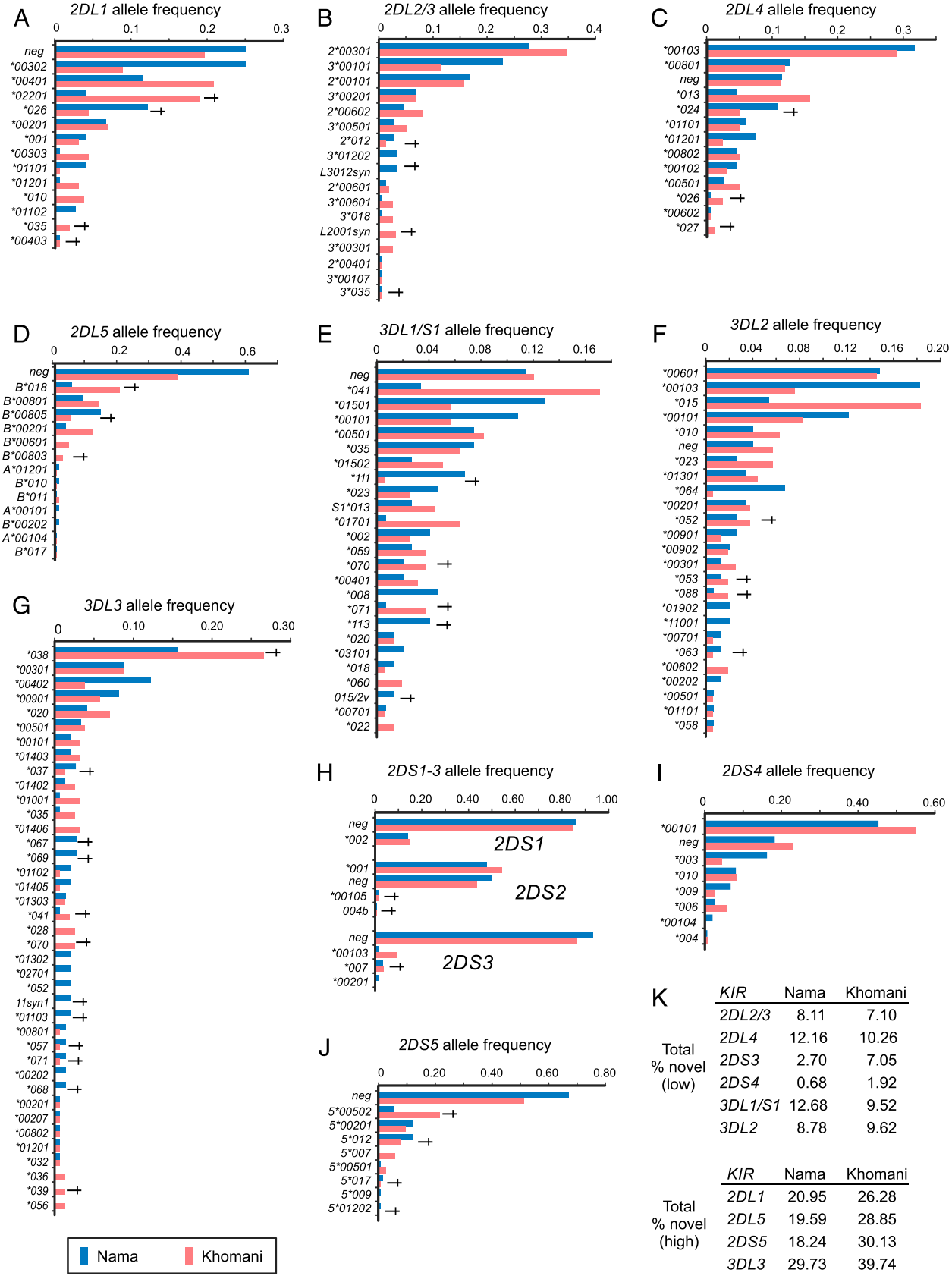
KIR2DL1*004 is frequent in both KhoeSan populations (11% Nama, 21% Khomani; Supplemental Fig. 2) and has a reduced binding affinity and signaling capability compared with KIR2DL1*003, the common European allotype (46, 48). Thus, the haplotypes carrying *2DL1*004* also make a significant contribution to the overall reduction in frequency of strong C2-specific inhibitory receptors, as do haplotypes lacking the *KIR2DL1* gene. The combined frequency of haplotypes lacking the *KIR2DL1* gene is 25% in Nama and 19.6% in Khomani (Fig. 4, Supplemental Fig. 2). Other allotypes with reduced KIR2DL1 function in the KhoeSan are KIR2DL1*006, *007, *010, *011, and *035 (which share the binding and/or signaling domains of 2DL1*004; Fig. 6D), 2DL1*032 (likely not expressed because of early termination), and the 2DL3/2DL1 fusion allotype (predicted C1 specificity; Fig. 3). Although each of these KIR2DL1 allotypes is present at low frequency, their combined frequency is 8.3% in the Nama and 6.5% in the Khomani (Supplemental Fig. 2). Thus, there is a considerably reduced capacity for C2-specific inhibition mediated by KIR2DL1 in the KhoeSan compared with European populations (Fig. 6E). In Europeans, KIR2DS1 is the one activating KIR specific for $C2^+$ HLA-C. In addition to KIR2DS1, the KhoeSan have several allotypes of KIR2DS5 that are activating C2-specific receptors (Supplemental Fig. 2) (72). In conclusion, the KhoeSan are seen to have a reduction in the frequency of C2-specific inhibitory KIRs and an increase in the frequency of C2-specific activating KIRs, compared to Europeans (Fig. 6E).

KhoeSan have high diversity of interacting KIR and HLA allotypes

We detected 41 *HLA-A*, 53 *HLA-B*, and 44 *HLA-C* alleles in the KhoeSan (Fig. 7A); these encode 37 HLA-A, 53 HLA-B, and 34 HLA-C allotypes, respectively (Fig. 7B). As seen for the *KIR*, both population groups have a distribution of *HLA class I* alleles at intermediate and low frequencies (Fig. 7A). *HLA-C*015:152* and *HLA-C*17:39* are the only novel *HLA class I* alleles we identified in the KhoeSan (Supplemental Fig. 3). Of these, *HLA-C*015:152* is a component of a common KhoeSan *HLA class I* haplotype (Supplemental Fig. 2). Of 186 *HLA class I* haplotypes detected, only 11 are present in both groups and together they account for 15–20% of the combined haplotype frequencies. Observed in fewer than three individuals were 155 of the 186 *HLA class I* haplotypes (Supplemental Fig. 2). Thus, a large majority of *HLA class I* haplotypes in the KhoeSan are present at very low frequency (Supplemental Fig. 2). Despite being small and relatively isolated populations, this analysis uncovered striking amounts of HLA class I diversity in the Nama and Khomani.

In the KhoeSan, many of the HLA-A, -B, and -C allotypes are KIR ligands (Fig. 7A) including the most common HLA-A, -B, and -C allotypes: A*23:17, B*44:03, and C*06:02 (Supplemental Fig. 2). The nine HLA-A allotypes that are KIR ligands have a combined frequency of 24.7% in the Nama and 33.5% in the Khomani (Fig. 7B). The 22 $Bw4^+$ HLA-B allotypes have combined frequencies of 43.2% in the Nama and 36.1% in the Khomani, and all 34 HLA-C allotypes have either the C1 or C2 KIR ligand. Ten and 15 $C2^+$ HLA-C allotypes are present in the Khomani and the Nama, respectively (Fig. 7B). At 61.4% in Khomani and 65.4% Nama, the KhoeSan have higher $C2^+$ HLA-C frequencies than most other populations worldwide (60, 90). Because variation at HLA-C residues outside of the KIR binding motif can affect the KIR/HLA interaction (59, 75, 91–93), the numerous HLA class I allotypes of the KhoeSan provide a rich diversity of KIR ligands. In quantifying this combinatorial diversity, we observed a mean of 7.9 pairs (minimum 2, maximum 14) of interacting KIR and HLA class I ligands in the Khomani and 7.1 pairs (minimum 3, maximum 12) in the Nama (Fig. 7C). For the Khomani, these interactions form a normal distribution, whereas the distribution is skewed slightly downward for the Nama (Fig. 7C). From this analysis we see that KhoeSan individuals have a greater diversity of KIR/HLA allotype interactions than in any other population studied thus far (Fig. 7D).

In separating the analysis into its major components, there is worldwide a relatively stable number of ~2 interactions of KIR with HLA-A and HLA-B per individual (Fig. 7D). In contrast, a wider range of interactions between KIR and HLA-C is observed. For example, 1.95 interactions in Yucpa Amerindians and 4.49 in the Khomani. This effect accounts for the main difference between the Nama and Khomani (Fig. 7D). The Nama are similar to European and West African populations (mean: 3.56 Europe, 3.67 Ghana, 3.74 Nama), whereas Khomani individuals have more functional interactions (mean 4.49, $p < 0.001$ by t test). Although both KhoeSan populations have reduced their number of strong inhibitory interactions between KIR2DL1 and $C2^+$ HLA-C (Fig. 6E), the Nama have achieved this while losing



K

Total % novel (low)

KIR	Nama	Khomani
2DL2/3	8.11	7.10
2DL4	12.16	10.26
2DS3	2.70	7.05
2DS4	0.68	1.92
3DL1/S1	12.68	9.52
3DL2	8.78	9.62

Total % novel (high)

KIR	Nama	Khomani
2DL1	20.95	26.28
2DL5	19.59	28.85
2DS5	18.24	30.13
3DL3	29.73	39.74

FIGURE 4. *KIR* allelic diversity in the KhoeSan. (**A**–**J**) The frequency distribution of alleles for each *KIR* gene in the Nama (blue) and Khomani (pink) is shown. The alleles are ordered from high to low frequency as observed across both populations. Only alleles observed in more than one individual are shown. The frequencies for all alleles are given in Supplemental Fig. 2. Horizontal crosses (†) indicate the novel alleles. (**K**) The combined frequency of the novel alleles identified for each *KIR* gene in the Nama and Khomani is shown. These are divided into two groups: those with a low contribution from the newly identified alleles (<13%, top) and those with a higher contribution (>18%, bottom).

A

	Centromeric *KIR* region							KhoeSan	
	3DL3	*2DS2*	*2DL2/3*	*2DL5B*	*2DS3/5*	*2DP1*	*2DL1*	Nama	Khomani
1	*038	*001	2*00301	B*00805	5*012	*023	*026	14	7
2	*038	*001	2*00301	B*018	5*00502	*00103	*022	1	19
3	*00402	*001	2*00101	B*00801	5*00201			10	4
4	*00301		2*00602					6	3
5	*00901		3*00101			*00301	*00401	2	6
6	*00101		3*00201			*00301	*00201	2	5
7	*020	*001	2*00301	B*018	5*00502	*00103	*022		7
8	*00402	*001	2*012					4	2
9	*00501		3*00101			*00201	*00302	4	1
10	*00901		3*00101			*00201	*00302	5	
11	*067		3*00101			*00301	*00302	5	
12	*00301	*001	L2001syn	B*00201	3*00103	*00102	*00401		5
13	*00901		3*00101			*00203	*00302	4	
14	*069	*001	2*00301	B*00201	3*007	*011	*00401	4	
15	*00501		3*00601			*007	*00303		4
16	*070	*001	2*00101	B*00201	3*007	*00102	*00401		4

B

	Telomeric *KIR* region							KhoeSan	
	2DL4	*3DL1/S1*	*2DL5*	*2DS5*	*2DS1*	*2DS4*	*3DL2*	Nama	Khomani
1					*002		*00601	15	12
2	*013	*041				*00101	*015	4	23
3	*00801	*00101				*003	*00101	14	2
4	*01201	*111				*009	*064	12	1
5	*00103	*01501				*00101	*01301	5	7
6	*00103	*023				*00101	*00103	7	4
7	*00801	*00501				*010	*010	5	6
8	*01101	*00501				*010	*00103	7	1
9	*024	*113				*00101	*00601	7	
10	*00103	*01501				*00101	*00103	7	
11	*024	*035				*00101	*052	3	4
12	*024	*035				*00101	*00103	3	3
13	*00801	*059						1	5
14	*00802	*059						4	1
15	*00103	*01501				*00101	*053	4	1
16	*00102	*002				*00101	*00201	3	2
17	*00103	*070				*00101	*023	1	4
18	*00103	*008				*003	*00901	4	
19	*00103	*008				*003	*01902	4	
20	*00501	S1*01301	A*00101	5*00201	*002		*088	1	3
21	*00103	*01701				*00101	*00101		4

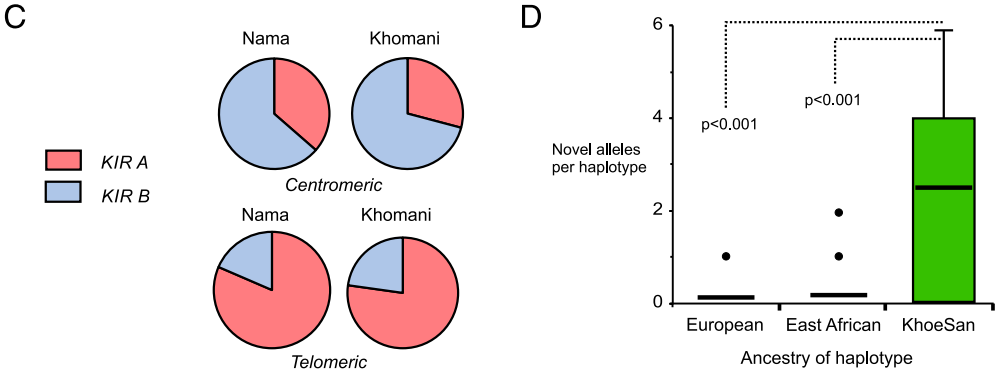
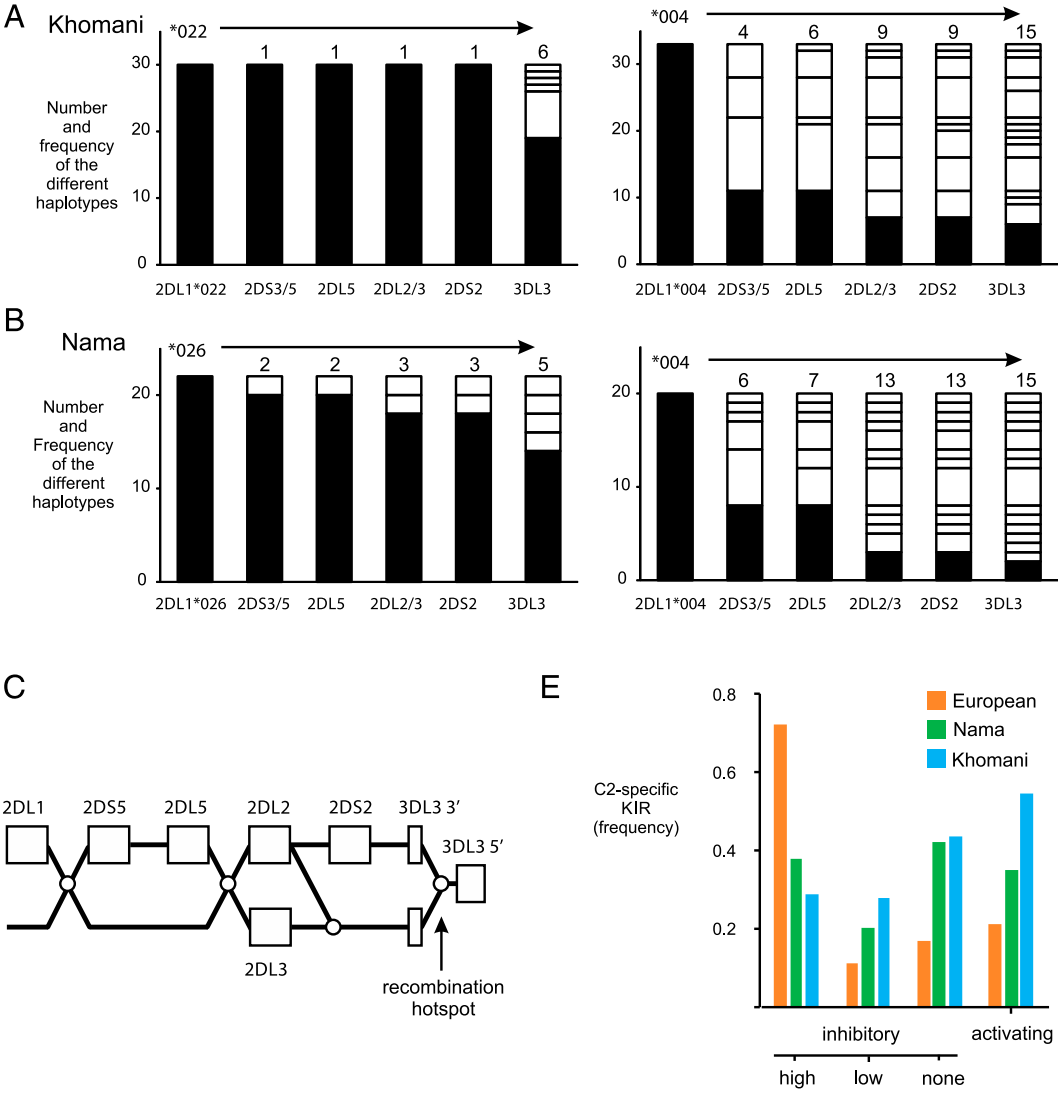


FIGURE 5. *KIR* haplotype diversity in the KhoeSan. Shown are the centromeric (**A**) and telomeric (**B**) *KIR* haplotypes that were identified in four or more unrelated KhoeSan individuals. *KIR A* haplotypes are shaded in red, and *KIR B* haplotypes are shaded in blue; yellow shading indicates novel alleles identified in this study. (**C**) Overall frequencies of centromeric and telomeric *KIR A* (red) and *KIR B* (blue) haplotype segments. (**D**) Compared is the local ancestry estimate for each *KIR* region haplotype (chr19: 55,200,000–55,600,000) against the number of novel alleles present on that haplotype. Three possible ancestral origins were derived from whole genome SNP data (21): European, East African, and KhoeSan. Horizontal black bar indicates the mean. Indicated p values are from Mann–Whitney U tests.

diversity in their NK cell repertoire (Fig. 7C, 7D), whereas the Khomani have increased diversity in their NK cell repertoire. This difference is because the Nama selected for a KIR2DL1 allotype exhibiting loss of receptor function, whereas the Khomani selected an allotype with a change in epitope specificity: from C2 to C1.



D

KIR2DL1 allotype	Domain	D1	D2					Tm		C2-specific inhibition
	Residue	44	114	154	163	182	186	245	246	
*001/*002/*003/*005 /*012/*036		M	L	P	D	H	Y	R	W	high
*004/*007/*011/*035		M	P	T	N	R	Y	C	W	low
*006		M	L	P	D	H	Y	C	W	low
*010		M	P	T	N	R	Y	R	W	low
*022		K	L	P	D	H	Y	R	W	none
*026		M	L	P	D	H	Y	R	X	none
*032		M	L	P	D	H	X	-	-	none
absent		-	-	-	-	-	-	-	-	none

Effect: Avidity Signaling Specificity Expression

FIGURE 6. KhoeSan have low capacity for mediating NK inhibition through KIR2DL1 interaction with $C2^+$ HLA. (**A**) The number and distribution of centromeric *KIR* haplotypes associated with *2DL1*022* (left) and *2DL1*004* (right), which are at similar frequency, in the Nama. The bars are divided to show the number of different haplotypes (given above each bar) and the number of copies of each haplotype when the respective gene is included (from left to right in each panel). Black shading shows the assumed parental haplotype. (**B**) The number and distribution of centromeric *KIR* haplotypes associated with *2DL1*026* (left) and *2DL1*004* (right), which are at similar frequency, in the Khomani. The bars are divided to show the number of different haplotypes (given above each bar) and the number of copies of each haplotype when the respective gene is included (from left to right in each panel). Black shading shows the assumed parental haplotype. (**C**) Map of the centromeric *KIR* haplotype structures that are common in the KhoeSan. Boxes indicate genes, circles depict nodes of the alternative paths through the map, and the arrow shows a recombination hotspot near the 3′ end of the *KIR3DL3* gene (89). (**D**) An alignment of the known functional residues and motifs for the KIR2DL1 allotypes observed in the KhoeSan is shown. *(Figure legend continues)*

Coevolution evident in the correlation of 2DL1 function with HLA-C2 frequency

Our analysis of NK cell receptor and ligand diversity in the Nama and Khomani suggests that the independent attenuation of KIR2DL1 in both of these KhoeSan populations is a direct consequence of their high frequencies of $C2^+$ HLA-C. To test this hypothesis, we assessed the effect of variation in the functional interaction of KIR2DL1 with $C2^+$ HLA-C across populations worldwide. As observed previously (94), there was no correlation between the *2DL1* gene frequency and the frequency of *HLA-C2* (Fig. 8A). To incorporate the functional differences that distinguish pairs of interacting KIR and HLA allotypes, we adjusted the interaction score according to the signaling strength (48) and binding strength (46) of each ligand–receptor pair present in a population. With this adjustment, there was a strong ($r = -0.82$) and statistically significant ($p = 0.023$) inverse correlation between the frequencies of KIR2DL1 and $C2^+$ HLA-C (Fig. 8B). At one end of the spectrum is the Japanese population, which combines a low frequency of C2 with a high frequency of strong KIR2DL1. At the other end of the spectrum is the KhoeSan population, combining a high frequency of C2 with a low frequency of strong KIR2DL1 (Fig. 8B).

We extended this analysis to the Hadza and Ga-Adangbe populations, which are distantly related to the KhoeSan (6). Of note, the Hadza have a uniquely high frequency of HLA-C*06:11 (12.5%) (12), which has a C1 epitope instead of the C2 epitope expressed by other HLA-C*06 allotypes. Thus, change in HLA-C, not KIR2DL1, has reduced the interaction of KIR2DL1 with $C2^+$ HLA-C in the Hadza. The Ghanaian Ga-Adangbe population has a similar ratio of C2 to strong KIR2DL1 to that of the Hadza (Fig. 8), but this is obtained through a different set of interacting HLA and KIR allotypes (84, 95). When the interaction of KIR2DL2/3 with C1 was analyzed in the same manner as for KIR2DL1, a similar inverse correlation was observed, but it is weak and not statistically significant (Fig. 8C, 8D). Taken together these findings show that the previously observed inverse correlation of *KIR A* haplotypes with *HLA-C2* frequency (43) is a consequence of the *KIR2DL1* alleles present on *KIR A* haplotypes and not the *KIR2DL3* alleles. In humans there is a general trend to reduce the incidence that $C2^+$ HLA-C interacts with strong inhibitory KIR2DL1 allotypes. Natural selection is clearly implicated in driving coevolution of the interactions between KIR2DL1 and HLA-C.

Discussion

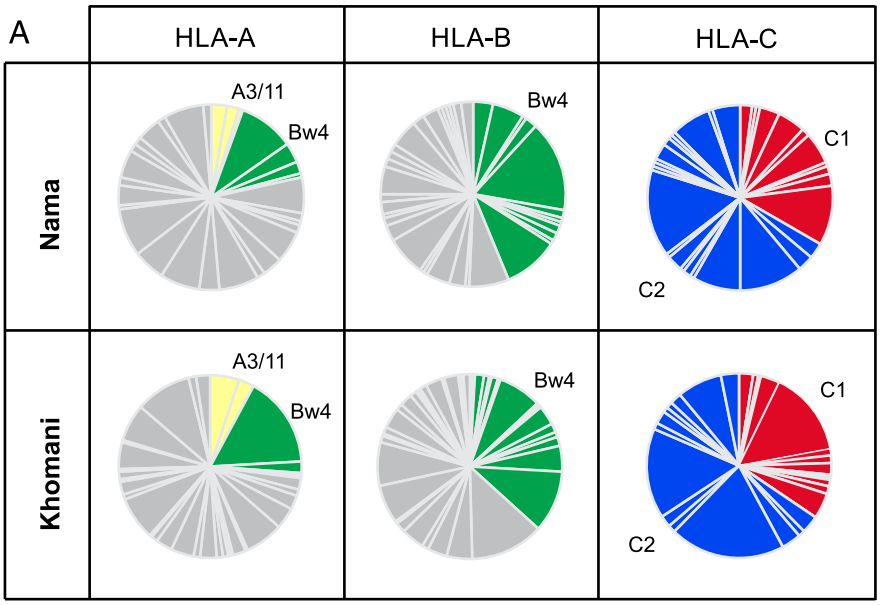
The inheritance of combinations of variable and independently segregating KIR and HLA class I ligands has major implications for the physiological functions mediated by NK cells and T cells. Our study investigated the immunogenetics of KIR and HLA class I in the KhoeSan populations of southern Africa. They comprise the longest surviving group of human populations and the most genetically diverse (6, 12). We studied two KhoeSan groups, the Nama and the Khomani, which at the level of whole genomes represent only part of the KhoeSan gene pool (20–22). Despite this limitation we observed exceptional genetic diversity of *KIR* (248 variants) and *HLA class I* (137 variants) in our analysis of 152 individuals (74 Nama and 78 Khomani). Of the 248 *KIR*, 64 were novel alleles, not seen in previous studies. Moreover, >80% of individuals carried one or more of the novel alleles. In striking contrast, only two novel *HLA class I* alleles were identified in the KhoeSan. This difference in the numbers of novel alleles reflects the fact that *HLA class I* polymorphism is well studied worldwide at high resolution (96), whereas almost all studies of *KIR* diversity have been at low resolution that detects few allelic differences. Our results vividly demonstrate the limitations in current knowledge of *KIR* diversity.

One consequence of their extensive *KIR* and *HLA class I* diversity is that each KhoeSan individual studied has a different *KIR/HLA* genotype, and thus a unique repertoire of NK cell receptor and ligand interactions. Similar individuality of KIR/HLA interactions is observed in other populations that were studied to the same resolution (62, 82–84). The only exception is the Yucpa Amerindian population, who have very low genome-wide diversity (81). Even in that population 93% of the individuals studied had unique KIR/HLA genotypes. Because the genotypic differences have clear implications for NK cell function, these results indicate that individuals, and to a greater extent populations, have the potential for considerable diversity in their NK cell–mediated immune responses. Given the established roles for NK cells in human health and reproduction, these differences are likely to be essential for the survival of individuals and populations. In this sense, diversity can be considered a necessary component of NK cell–mediated immunity (97).

A general feature of KhoeSan populations is the low linkage disequilibrium of their genomes, which are therefore characterized by shorter haplotypes (9, 12, 21). In contrast with this general rule, we find that one *KIR* haplotype in the Nama and one other in the Khomani are characterized by extended linkage disequilibrium and high frequency. These two haplotypes, which clearly have KhoeSan ancestry, are absent from Europeans, Bantu speakers, and East Africans, as are the highly distinctive *KIR* alleles they carry (60, 90). Thus, these unusual haplotypes were not acquired by the KhoeSan through admixture with other Africans or Europeans (9, 18). Their properties are consistent with the two *KIR* haplotypes having been independently subject to positive natural selection (87, 88): one in the Nama population, the other in the Khomani population. The likely target for selection was the *KIR2DL1* gene that encodes inhibitory receptors for $C2^+$ HLA-C. The effect of that selection was to favor *KIR2DL1* alleles that encode less effective inhibitory C2 receptors. The allotype selected in the Nama, KIR2DL1*026, is a receptor that cannot transduce inhibitory signals. Consequently, it cannot contribute significantly to either NK cell education or an effector response. The allotype selected in the Khomani, KIR2DL1*022, is a receptor that cannot recognize $C2^+$ HLA-C, but instead recognizes $C1^+$ HLA-C. Thus, in mechanistically different ways, Nama KIR2DL1*026 and Khomani KIR2DL1*022 are not inhibitory C2 receptors.

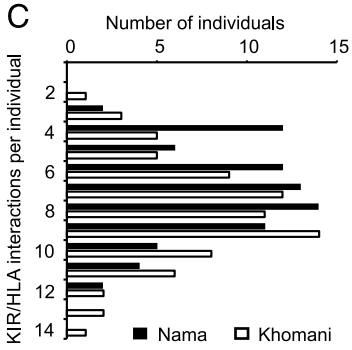
By comparison with C2-specific KIRs, C1-specific KIRs are conserved in the KhoeSan. The novel *KIR2DL2/3* and *KIR2DS2*

Residue 245 (shaded red) affects signaling (48), the motif of 4 aa residues in D2 (shaded green) affects binding avidity (46), residue 44 (shaded blue) affects specificity, and mutations that abrogate expression are shaded purple (X represents the termination codon). At the right is shown the inhibition potential of each allotype: high, low, or none. The allotypes are grouped according to the residues shown and are further defined by additional residues of unknown or no function that are not shown. (**E**) A summary of the frequency of KIR2DL1 allotypes in the KhoeSan defined by their functional characteristics (from C, rightmost column) and compared with a European population (62). Shown at the right is the total frequency of activating receptors specific for HLA-C2 in the KhoeSan compared with Europeans.



B

		HLA-A			HLA-B			HLA-C		
		Total	A3/11	Bw4	Total	Bw4	-21M	Total	C1	C2
Nama	Number of allotypes	26	3	4	35	12	9	26	13	15
	Frequency (%)	-	4.9	19.8	-	43.2	22.8	-	34.6	65.4
Khomani	Number of allotypes	33	2	5	40	17	9	24	14	10
	Frequency (%)	-	8.2	25.3	-	36.1	32.3	-	38.6	61.4
Number of common allotypes		22	2	3	24	7	7	16	8	8



D

	Ligand-receptor pairs per person				
	HLA-A and B		HLA-C		
Population	Inhibit	Activate	Inhibit	Activate	Sum
Yucpa	0.52	0.00	1.95	0.39	2.87
Japanese	2.37	0.12	2.19	0.56	5.24
Maori	1.55	0.30	3.00	1.13	5.98
European	2.04	0.30	3.56	0.57	6.47
Ghanaian	2.12	0.00	3.67	1.17	6.96
Nama	1.96	0.02	3.74	1.49	7.22
Khomani	1.84	0.02	4.49	1.41	7.76

FIGURE 7. KIR2DL1*022 maintains high diversity of KIR interactions with HLA-C. (**A**) The allele-frequency spectra of *HLA class I* in the KhoeSan are shown. Each segment of the pie corresponds to a distinct allotype: (yellow) allotype has A3/11 epitope, (green) Bw4 epitope, (red) C1 epitope, or (blue) C2 epitope, or (gray) the allotype does not carry a KIR ligand. The allele names and frequencies are given in Supplemental Fig. 2. (**B**) The number of HLA-A, HLA-B, or HLA-C allotypes present within each category of KIR ligand and their frequencies in the Nama (upper) and Khomani (lower) populations are shown. Also indicated are the number and frequency of allotypes having methionine at position -21 in the leader peptide of HLA-B (-21M). Presence of -21M increases HLA-E expression, allowing HLA-B allotypes that are not KIR ligands to educate NK cells through CD94:NKG2A (109). The alleles that carry -21M are given in Supplemental Fig. 2. "Common" indicates the alleles that were observed in both populations. (**C**) Plot of the total number of different KIR/HLA allotype pairs per individual in the Nama (black bars) and Khomani (white bars). (**D**) The mean number of different ligand–receptor allotype pairs per individual in each population for inhibitory and activating KIR interactions with HLA-A and -B combined (left), and with HLA-C (right).

alleles identified in the KhoeSan are either rare or characterized by synonymous substitutions. Only one KhoeSan-specific allele, *2DL2*012*, was identified as potentially common, being detected in six individuals (Fig. 4B). Neither KIR2DL2*012 nor any KhoeSan specific KIR2DL2/3 or KIR2DS2 allotype is predicted to change receptor specificity or signaling function. Emphasizing this point, the common KIR2DL2 allotypes of the KhoeSan, 2DL2*001, *003, and *006, bind $C1^+$ HLA-C with similar avidity, but differ significantly in their avidity for $C2^+$ HLA-C (60). Striking is that both common KhoeSan-specific haplotypes encode distinctive KIR2DL1, 2DP1, 2DL5, 2DS5, and 3DL3 allotypes, whereas their KIR2DL2/3 and 2DS2 allotypes are identical to ones present in every other population studied (45). These findings, together with the stronger correlation in human populations between KIR2DL1 and C2 frequencies than between KIR2DL2/3 and C1 frequencies, indicate that KIR2DL2/3 and KIR2DS2 are conserved in function across human populations, whereas KIR2DL1 is not (Fig. 8).

That the frequency of $C2^+$ HLA-C in the KhoeSan is at the high end of the human spectrum has suggested that selection against inhibitory C2-specific receptors was caused by the high C2 frequency (60). Our observations on the KhoeSan are consistent with the general and worldwide trend for $C2^+$ HLA-C frequency to be inversely correlated with the frequency of *KIR A* haplotypes and the strong inhibitory C2-specific KIR2DL1 they encode (43). Another mechanism for attenuating the interaction of KIR2DL1 with $C2^+$ HLA-C occurred in the Hadza, an East African population that diverged from the KhoeSan >40,000 y ago (12, 13). At high frequency (12.5%) in the Hadza, HLA-C*06:11 carries the C1 epitope, whereas other common C*06 allotypes of sub-Saharan Africans carry the C2 epitope (95). Thus, in the Hadza, the effect has been to reduce the frequency of the C2 ligand, not that of its receptor: KIR2DL1.

NK cells and other mechanisms of innate immunity enable humans to respond rapidly to a first infection with a pathogen. The

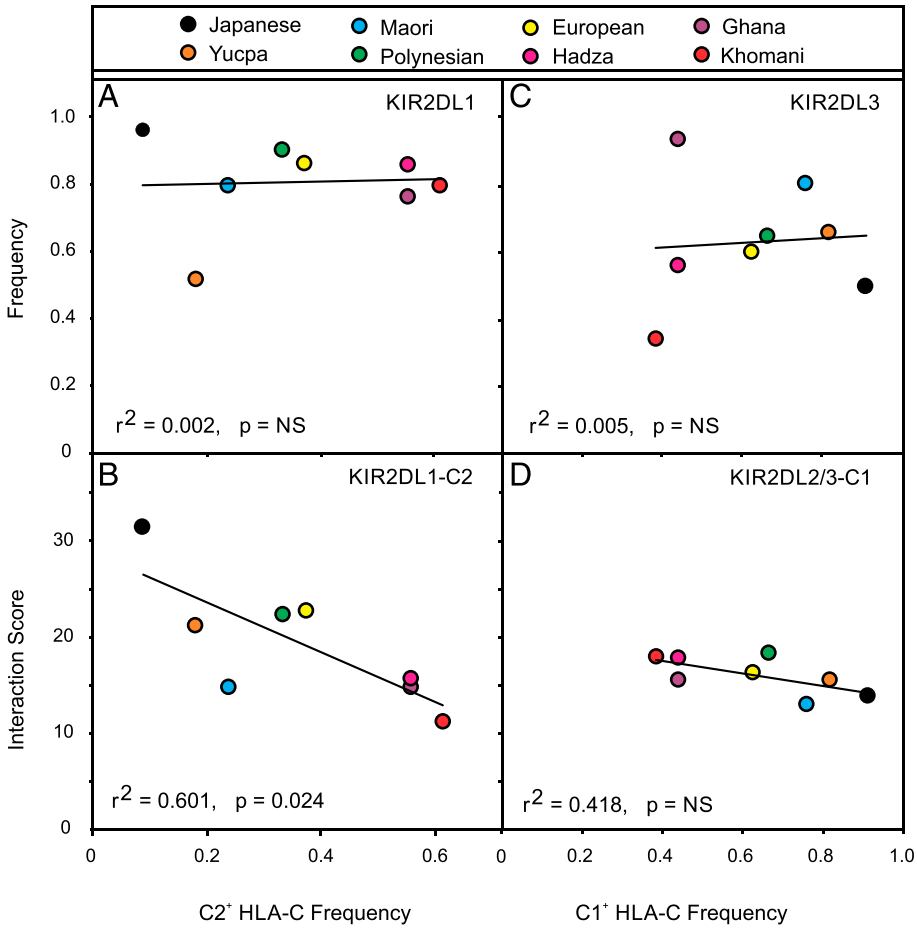


FIGURE 8. Inverse correlation of HLA-C2 with strength of inhibition mediated by KIR2DL1. (**A**) The frequency of KIR2DL1 against the frequency of HLA-C2 across worldwide populations is plotted. (**B**) The interaction score (which incorporates the strength of binding and signaling, as well as frequency, for each receptor–ligand allotype pair) against the frequency of C2 in the same populations is plotted. (**C**) The frequency of KIR2DL3 against the frequency of HLA-C1 is plotted. (**D**) The interaction score of KIR2DL2/3 against the frequency of C1 is plotted. The populations are Japanese (80), Yucpa Amerindian (81), Polynesian (82), Maori (82), European (110), Hadza East Africans (12), Ga-Adangbe West Africans (84), and the Khomani.

role of inhibitory KIR in NK cell education enables NK cells to kill infected cells, which exerts pressure on pathogens to evade the NK cell response (28, 98, 99). Activating KIRs, which can be specific for particular combinations of HLA class I and a virus-derived peptide (100), are also implicated in the control of infection. Of importance to sub-Saharan African populations is the control of Ebola, Chikungunya, Dengue, HIV, EBV, *Chlamydia*, and malaria infections (36–38, 40–42). $C2^+$ HLA-C is associated with hepatitis B susceptibility (101). Moreover, the KIR2DL1/C2 ligand combination is associated with susceptibility to Chikungunya specifically in sub-Saharan Africans (40). In addition, candidates for diseases that could have affected KhoeSan KIR diversity are measles, smallpox, and tuberculosis (TB), which were introduced to the KhoeSan during the last 250 y by Europeans (102). A case for the role of KIR in NK cell–mediated control of TB is being made (103, 104). Supporting this hypothesis, KhoeSan ancestry is associated with increased risk for development of active TB (102) and with the role of activating KIR in preventing that development (105).

Pre-eclampsia has historically been a strong selective force in much of Africa and remains so today (24). A major risk factor for pre-eclampsia is a homozygous *KIR A* mother lacking $C2^+$ HLA-C and carrying a $C2^+$ HLA-C child (11, 43). This combination implicates the interaction of centromeric *KIR A KIR2DL1* with $C2^+$ HLA-C in the incomplete placentation that causes pre-eclampsia (11). Selection by pre-eclampsia and other pregnancy syndromes associated with poor placentation would have the effect of creating the observed inverse correlation between the $C2^+$ HLA-C ligand and the KIR2DL1 receptor in human populations. Consistent with this interpretation is the involvement of activating C2-specific receptors that counteract the inhibitory C2-specific receptors (106). The *KIR* haplotypes selected in the Nama and Khomani encode KIR2DS5 allotypes that are activating C2-specific receptors. These allotypes likely facilitate placentation, as indicated by the protection KIR2DS5 affords against pre-eclampsia in African women (107).

Selection in the Nama and Khomani of KIR haplotypes encoding attenuated KIR2DL1 allotypes was likely driven by their capacity to give increased resistance to infectious disease and/or improved success in reproduction. In one scenario, resistance to a sporadic pathogen led to increased frequency of $C2^+$ HLA-C, but when exposure or risk from the pathogen subsided it then became critical to reduce KIR2DL1 function, facilitating reproduction and thus regrowth of the population. This explanation would be consistent with the geographically discrete and temporally cyclic nature of the balancing selection that creates and maintains *KIR* diversity worldwide (11, 45, 81, 108). Analysis of further sub-Saharan African populations, such as KhoeSan from the Northern Kalahari, and other isolated populations in other parts of the world will contribute toward understanding the timing and causes of the selection pressures.

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Disclosures

C.R.G. owns stock in 23 and Me, Inc. and is an advisor to Encompass Bioscience, Inc. J.M.G. is currently an employee of AncestryDNA. The other authors have no financial conflicts of interest.

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