Co-evolution of KIR2DL3 with HLA-C in a human population retaining minimal essential diversity of KIR and HLA class I ligands

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Edited by Peter Cresswell, Yale University School of Medicine, New Haven, CT, and approved August 31, 2009 (received for review June 1, 2009)

Natural killer (NK) cells contribute to immunity and reproduction. Guiding these functions, and NK cell education, are killer cell Ig-like receptors (KIR), NK cell receptors that recognize HLA class I. In most human populations, these highly polymorphic receptors and ligands combine with extraordinary diversity. To assess how much of this diversity is necessary, we studied KIR and HLA class I at high resolution in the Yucpa, a small South Amerindian population that survived an approximate 15,000-year history of population bottleneck and epidemic infection, including recent viral hepatitis. The Yucpa retain the three major HLA epitopes recognized by KIR. Through balancing selection on a few divergent haplotypes the Yucpa maintain much of the *KIR* variation found worldwide. HLA-C*07, the strongest educator of C1-specific NK cells, has reached unusually high frequency in the Yucpa. Concomitantly, weaker variants of the C1 receptor, KIR2DL3, were selected and have largely replaced the form of KIR2DL3 brought by the original migrants from Asia. HLA-C1 and KIR2DL3 homozygosity has previously been correlated with resistance to viral hepatitis. Selection of weaker forms of KIR2DL3 in the Yucpa can be seen as compensation for the high frequency of the potent HLA-C*07 ligand. This study provides an estimate of the minimal KIR-HLA system essential for long-term survival of a human population. That it contains all functional elements of KIR diversity worldwide, attests to the competitive advantage it provides, not only for surviving epidemic infections, but also for rebuilding populations once infection has passed.

Amerindian | immune diversity | natural selection | NK cells | reproduction

Natural Killer (NK) cells are lymphocytes that make essential contributions to immune defense and placental reproduction. They provide innate immunity against infection, particularly viral infection, by killing infected cells and secreting cytokines that cause inflammation, and recruit adaptive immunity when needed (1). At an early stage of pregnancy, uterine NK cells cooperate with extravillous trophoblast to enlarge the maternal blood vessels that serve to nourish the developing fetus (2). Following hematopoietic stem cell transplantation for leukemia, NK cells can provide alloreactivity that prevents graft-versus-host disease and relapse of leukemia (3). Guiding the NK cell response to infection and pregnancy, and aspects of NK cell development, is a variety of inhibitory receptors that recognize major histocompatibility complex (MHC) class I glycoproteins. CD94:NKG2A is a conserved receptor that recognizes complexes of conserved HLA-E and peptides derived from the leader peptides of HLA-A, -B, and -C (4, 5). In contrast, the killer cell Ig-like receptors (KIR) that recognize determinants of polymorphic HLA-A, -B, and -C are diverse, rapidly evolving, and largely species-specific (6–8).

The *KIR* locus contains a variable number of up to 14 *KIR* genes and pseudogenes (6, 9). Three constant framework genes mark the ends and center of the locus; between them are two regions of variable gene content. Allelic polymorphism gives a further dimension to haplotype variability. Two groups of *KIR* haplotype, *A* and *B*, differ in size, gene content, function, and disease associations (10). Group *A* haplotypes have fixed gene content and comprise mainly of genes for inhibitory KIR that recognize well-defined epitopes of HLA-A, -B, and -C. In addition to these genes, group *B* haplotypes have a variable number of genes for activating KIR with low avidity for HLA class I and uncertain function.

Study of urban populations demonstrated that *KIR* variation, like *HLA*, is sufficient to discriminate unrelated individuals (8). Because the *HLA* and *KIR* loci are on different chromosomes (chr6 and chr19, respectively), their combined potential for diversifying human immune systems is enormous. Urban populations are the result of recent migration and admixture, and the extent of their *KIR* diversity may not always reflect what arises and is maintained by natural selection. In addressing this question, Amerindians have proved particularly informative, as in the study of HLA (11, 12), because of their unique history and geography. Modern Amerindians descend from as few as 80 of the Asian migrants (13) who began to populate the Americas approximately 15,000 years ago (14), expanding their population in the process of settling much of North and South America (15). Following the arrival of Europeans and their diseases in the 16th century, Amerindian populations suffered additional selection by epidemic disease and population bottleneck (16), as has continued into modern times (17).

To see how the interacting system of KIR and HLA factors in Amerindians compares to urban populations, we defined *KIR* and *HLA-A*, *B* and *C* in the Yucpa tribe from the Sierra de Perija, a mountain at the border between Venezuela and Colombia (18). Based on linguistics, the Yucpa were estimated to have been isolated for almost 3,000 years before their discovery by Europeans (18). In the recent past, the Yucpa population expanded from 1,500 in 1960 to 10,000 in 2001. Epidemics of measles and malaria in the 1960s, were followed in 1979–1981 by an epidemic of combined hepatitis B and D that caused considerable mortality of the young (19). The 61 blood samples we studied were donated in 1993 by survivors of this epidemic (20).

Author contributions: K.G., P.J.N., and P.P. designed research; K.G., P.J.N., T.G., and A.K.M. performed research; Z.L. contributed new reagents/analytic tools; K.G., P.J.N., and L.A.-R. analyzed data; and P.J.N. and P.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession no. EU482059 (*2DL3*008N*), EU482060 (*2DL3*009*), EU482061 (*2DS4*010*), EU482062 (*3DP1*007*), FJ178097 (*3DL3*01002*)].

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This article contains supporting information online at www.pnas.org/cgi/content/full/0906051106/DCSupplemental.

A

HLA-A			HLA-B			HLA-C		
Allele	KIR Ligand	Frequency %	Allele	KIR Ligand	Frequency %	Allele	KIR Ligand	Frequency %
*6801		46.7	*3909		41.8	*0702	C1	76.2
*0204		27.8	*3905		34.4	*1503	C2	9.8
*0212		0.8	*5201	Bw4	9.8	*0401	C2	7.4
*0213		0.8	*3512		7.4	*0302	C1	4.1
*2402	Bw4	17.2	*3543		1.6	*0304	C1	0.8
*3101		6.5	*4002		4.1	*0102	C1	1.6
			*4004		0.8			
Total	Bw4	17.2	Total	Bw4	9.8	Total	C1	82.8 (97)
Phenotype frequency of Bw4+ = 44.3%							C2	17.2 (33)

B

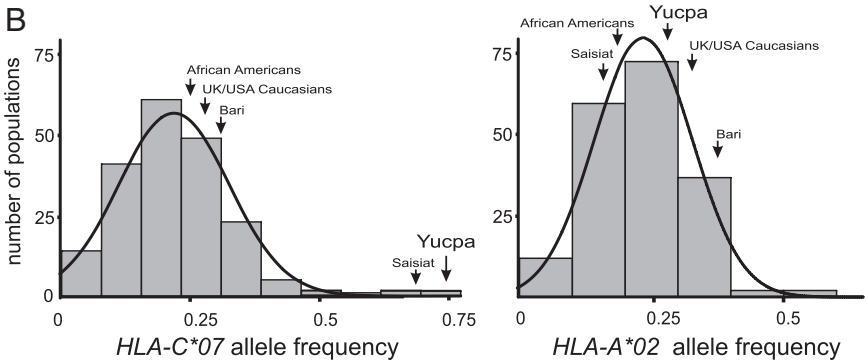

Fig. 1. Genetic diversity of HLA class I in the Yucpa. (*A*) Shows Yucpa frequencies for *HLA-A*, *-B*, and *-C* alleles and the Bw4, C1, and C2 epitopes recognized by KIR, called KIR ligands. C1 and C2, carried by HLA-C, are defined by asparagine (C1) and lysine (C2) at position 80; Bw4, carried by HLA-A and -B, is defined by arginine at position 83 (7, 10). Phenotype frequencies are shown in parentheses for C1 and C2, and below for Bw4. Gray shading indicates Amerindian-specific alleles, defined as being present in the Yucpa but in no more than two non-Amerindian human populations (where their frequencies were 0.6% or less) of 197 populations compared (31, 46, 49). (*B*) Compares the frequency distribution of *HLA-C*07* (mean 0.21; SD 0.1) with *HLA-A*02* (mean 0.23; SD 0.09) in 197 populations, both being consistent with a normal distribution. The Yucpa and Taiwanese Saisiat populations have high *HLA-C*07* frequencies outside the normal distribution ($P < 0.0001$). The Bari, geographical neighbors of the Yucpa but linguistically distant, is well within the normal distribution. The Shapiro-Wilk test was performed using SAS software (SAS Inc.).

Results

Yucpa Have the Highest Frequency Worldwide of HLA-C*07, a Strong NK Cell Educator. The Yucpa exhibit a typical Amerindian HLA class I distribution, in which a fraction of the major allele groups worldwide is represented (11, 20). One *HLA-A* and three *HLA-B* alleles are "new" Amerindian-specific variants, the other 15 alleles, including all six *HLA-C* alleles, are shared with Asian populations and represent "founder" alleles brought by the Asian migrants who populated the Americas (Fig. 1*A*). This set of HLA class I allotypes retains the C1, C2, and Bw4 epitopes, the major ligands for inhibitory KIR (7), but lacks the A3/A11 epitope recognized by KIR3DL2 (21). During NK cell development, interactions between C1, C2, and Bw4 and cognate KIR determine the strength with which mature NK cells respond to cells whose HLA class I expression is perturbed by disease, a developmental process termed NK cell education (22). The A3/A11 epitope contributes little to NK cell education (23, 24), a distinction that correlates with the exceptionally high sensitivity of KIR3DL2 to the peptide bound by HLA-A*03 or A*11 (21).

Distinguishing the Yucpa is the frequency of *HLA-C*07*, the highest observed worldwide and an outlier from the distribution formed by other populations. In contrast, the Yucpa frequency of *HLA-A*02*, as common and widespread as *HLA-C*07*, is well within the distribution (Fig. 1*B*). Furthermore, the neighboring but linguistically separated Bari Amerindians have a *C*07* frequency within the range of other populations. Some 97% of Yucpa carry C*0702 and 57% are homozygotes. HLA-C*07 carries the C1 epitope, the ligand for the inhibitory KIR2DL3 receptor. Comparison of the capacity of different C1-bearing HLA-C allotypes to educate KIR2DL3-expressing NK cells, has shown that HLA-C*07 is the most potent (23).

Natural Selection Has Maintained a Balance of *KIR* Haplotypes and Alleles in the Yucpa. Although only three (*KIR2DL4*, *3DL2*, and *3DL3*) of the 14 *KIR* genes are fixed, all but *KIR2DS3* are retained in the Yucpa (25). To define the underlying polymorphism in these genes, we determined complete allele-level *KIR* haplotypes and their frequencies (Fig. 2). Five *A* haplotypes are evenly distributed with a cumulative frequency of 46%. In contrast there is a dominant *B* haplotype, *B1*, with a frequency of 47.5%, and three low-frequency haplotypes, all representing recombinants between *B1* and one of the *A* haplotypes (Fig. 2*A*). The even balance between *A* and *B* haplotypes was preserved in each of three villages contributing samples to this study (Table S1). Striking is that *B1* shares no *KIR* allele with any *A* haplotype; even the *3DP1* pseudogene is represented by a distinctive allele. *A1*/*B1* heterozygotes (21% of the Yucpa population) have 19 different *KIR*, thus they possess two-thirds of the 29 *KIR* variants present in the population.

Simulations show that maintenance of gene-content diversity of the magnitude observed between Yucpa *KIR* haplotypes, which is close to the maximum possible, was highly improbable under neutral evolution or under positive or negative selection, which would lead to loss or fixation of haplotypes, respectively (Table 1). Thus, the most likely cause of the haplotype diversity of the Yucpa is balancing selection. To further assess this possibility, we analyzed the haplotype sequences for Tajima's D in a sliding window. Statistically significant evidence for balancing selection was obtained for all of the polymorphic *KIR* except the *3DP1* pseudogene (Fig. 2*B*). Supporting evidence for this mode of selection on KIR is the numerous functional differences caused by the polymorphism (8, 26–28). For example, the four Yucpa *KIR3DL1*/*S1* alleles represent the breadth of functional variation defined at this well-studied locus: the three main

A

KIR Haplo-type	*KIR* gene													Haplotype Frequency %
	3DL3 (5)	*2DS2* (1)	*2DL2/3* (4)	*2DP1* (1)	*2DL1* (1)	*3DP1* (2)	*2DL4* (3)	*3DL1/S1* (4)	*2DL5* (1)	*2DS5* (1)	*2DS1* (1)	*2DS4* (2)	*3DL2* (3)	
A1	**01002*		*3*009*	**00201*	**003*	**00302*	**001*	**029*				**00101*	**002*	22.1 (5)
A2	**00901*		*3*009*	**00201*	**003*	**00302*	**001*	**01502*				**00101*	**002*	9.0 (1)
A3	**00802*		*3*008N*	**00201*	**003*	**00302*	**001*	**01502*				**00101*	**002*	7.4 (1)
A4	**01002*		*3*001*	**00201*	**003*	**00302*	**001*	**01502*				**00101*	**002*	4.1 (0)
A5	**00901*		*3*009*	**00201*	**003*	**00302*	**011*	**005*				**010*	**010*	3.3 (0)
B1	**00301*	**001*	*2*003*			**007*	**005*	**013*	**001*	**002*	**002*		**007*	47.5 (17)
B2	**00901*		*3*001*	**00201*	**003*	**00302*	**005*	**013*	**001*	**002*	**002*		**007*	4.1 (0)
B3	**00301*	**001*	*2*003*			**007*	**011*	**005*				**010*	**010*	1.6 (0)
B4	**00402*	**001*	*2*003*			**007*	**001*	**01502*				**00101*	**002*	0.8 (0)

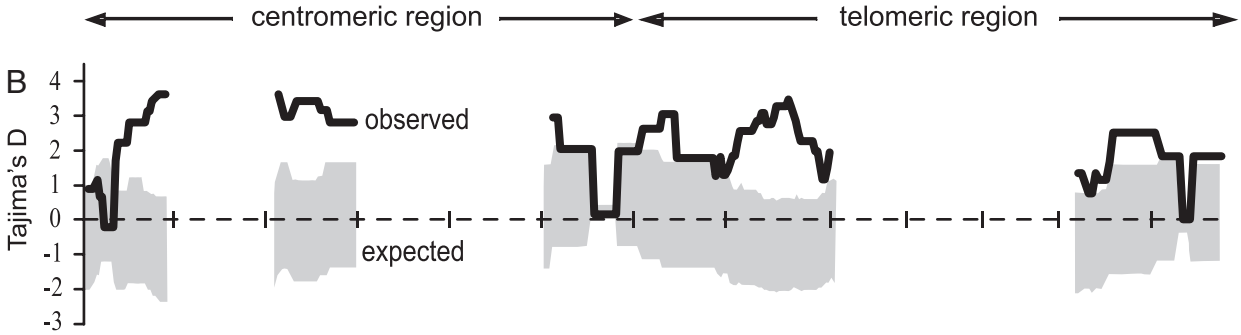

Fig. 2. Genetic diversity of KIR in the Yucpa. (*A*) Shows the Yucpa *KIR* haplotypes and their frequencies. Each *KIR* gene on a haplotype is represented by a box containing the allele name. Gray shading indicates previously undiscovered alleles. Under each *KIR* gene the number of alleles present in the Yucpa is given in parentheses. Under haplotype frequency the number of homozygotes in the panel of 61 individuals is given in parentheses. (*B*) Shows the results of a test for the role of balancing selection in Yucpa *KIR* diversity. A 600- × 30-bp sliding window of Tajima's D calculated from the 122 Yucpa *KIR* haplotypes. The area shaded gray gives the 99% range expected from neutral evolution, which was determined using *ms* (47); a separate simulation was performed for each window according to the number of segregating sites present in each window and with recombination set at 0. The black line shows the observed D. Values of observed D above the gray area are evidence for balancing selection (at $P < 0.01$). The position of genes in the plot corresponds to those in 'A.' This analysis is dependant on polymorphism, so there is no contribution from the non-polymorphic *KIR* genes.

lineages (29), both the activating and inhibitory receptors, and inhibitory receptors having high and low levels of expression on NK cell surfaces (30). The polymorphic *HLA class I* genes provide a classical example of balancing selection seen in all populations (31). For the Yucpa, the evidence for balancing selection on the KIR is as strong as that on their HLA class I ligands (Table S2). This contrasts with the ABO system of blood group antigens, which has a strong signature of balancing selection in many populations (32) but no variation at all in the Yucpa (18). Furthermore the dominance of group O in Amerindians existed before European contact as it is observed in 2,000-year-old skeletal remains (33). Such dominance of O is not typical of Asians and Siberians, who retain the ABO polymorphism (34) and were likely the source of Amerindian founders (35).

KIR2DL3 Variants with Lower Avidity for C1 Have Largely Replaced the 2DL3*001 Founder. Of the 29 *KIR* in the Yucpa, 25 are founder alleles and four are variants not seen in other populations. Of these variants (gray-shaded boxes in Fig. 2*A*), the substitutions in the *3DP1* pseudogene and the synonymous substitution that

Table 1. The gene-content diversity of Yucpa *KIR* haplotypes unlikely arose from neutral evolution

Founder population			Final population	
Modern equivalent	Haplotype number	Gene-content difference (mean)	Gene-content difference (mean)	"*p*"
Yucpa	4	5.5	2.5	<0.05
Japanese	5	2.0	0.95	<0.001
Han	9	2.1	1.1	<0.001
European	12	4.3	2.0	<0.01

We tested if the Yucpa's high *KIR* haplotype gene-content diversity is consistent with neutral evolution (genetic drift). Four founder populations with an effective population size of 100 were based upon the modern Yucpa, Chinese Han (50), Japanese (23), and European Caucasoid (37) populations. Shown is the mean gene-content difference between pairs of *KIR* haplotypes in these populations, before and after simulation of 600 generations of neutral evolution in which the effective population size increased to 1,000 (13). The demographic model was conservative because it did not include additional population bottlenecks. The mean gene-content difference was calculated assuming Hardy–Weinberg equilibrium. The values for the final populations are the means of 10,000 simulations. "*p*" indicates the proportion of simulations in which the final gene content difference exceeds that observed in the Yucpa (5.2, 5.1 and 5.4 for the three villages, 5.5 for the combined panel). Further models are shown in Table S3. Maximum mismatch diversity remained an improbable outcome when other models were tested. These included increasing the effective size of the founder population and applying a range of different demographic models Table S3. Forward simulations were performed using simuPOP 0.8 (ref. 48).

A

KIR2DL2/3	Frequency (%)	Haplotypes	Amino Acid Position 86	124	148
*2DL3*009*	34.4	*A1, A2, A5*	S	V	P
*2DL3*001*	8.2	*A4, B2*	S	V	R
*2DL3*008N*	7.4	*A3*	Δ 1nt	Ter	
*2DL2*003*	49.9	*B1, B3, B4*	S	V	C

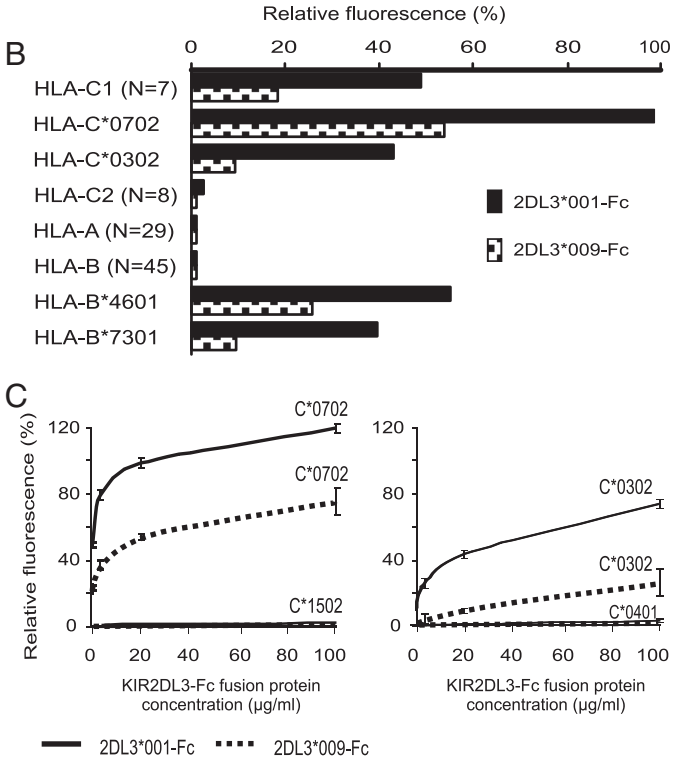

Fig. 3. 2DL3*001 and 2DL3*009 have similar specificity for the C1 epitope but differ in the extent of their binding. (*A*) Shown are the amino acid substitutions that distinguish the three Yucpa KIR2DL3 allotypes, and the residues at these positions in KIR2DL2*003, also present in the Yucpa. The frequencies of the *KIR2DL2/3* alleles and the haplotypes on which they are found are also shown. *KIR2DL3*008N* has a one nucleotide deletion (Δ 1nt) in codon 86, leading to premature termination (Ter) in codon 124. (*B*) Compares the binding of 2DL3*001-Fc and 2DL3*009-Fc fusion proteins (20 μg/ml) to a panel of Luminex beads, each coated with one of 95 different HLA-A, -B, and -C allotypes. Shown are data from selected individual allotypes and averaged data from groups of allotypes. HLA-B*4601 and -B*7301 that have the C1 epitope were not included in the group of 45 HLA-B. (*C*) Compares 2DL3*001-Fc and 2DL3*009-Fc for binding to Luminex beads coated with individual HLA-C allotypes, representing the major allotypes present in Yucpa. The data are shown in two graphs for clarity.

distinguishes *3DL3*01002* are unlikely to have had any functional effect. However, the single non-synonymous substitutions that distinguish the *2DL3*008N* and *2DL3*009* variants from *2DL3*001*, the founder allele for this inhibitory C1 receptor, are likely to have altered its function.

*KIR2DL3*008N* differs from *2DL3*001* by deletion of one nucleotide from codon 86, causing premature termination at codon 124 (Fig. 3*A*). As the encoded protein lacks half the ligand-binding site and the signaling domain, *2DL3*008N* is almost certainly non-functional. KIR2DL3*009 differs from 2DL3*001 by substitution of proline for arginine at position 148 in domain D2 (Fig. 3*A*). Previous comparison of 2DL3 to 2DL2, showed that substitution of arginine 148 for cysteine in 2DL2 (Fig. 3*A*) increased its avidity for C1 and its cross-reactivity with C2 (28). Further pointing to the functional importance of variation at position 148, phylogenetic analysis showed position 148 was a site for positive natural selection during hominoid evolution (29). Although this residue does not directly contact bound HLA-C (36), it is proposed to modulate binding avidity by altering the angle of the hinge between D1 and D2 (28).

To determine the effect of the proline 148 substitution, we made Fc-fusion proteins from 2DL3*001 and 2DL3*009 and compared their binding to beads individually coated with one of 95 different HLA-A, -B, and -C allotypes (Fig. 3*B*). Although 2DL3*001-Fc and 2DL3*009-Fc exhibited similar selectivity for HLA-C, and two exceptional HLA-B allotypes (B*4601 and B*7301), that carry the C1 epitope (28), 2DL3*001-Fc consistently bound to higher level than 2DL3*009 (Fig. 3*B*). This was not because of difference in quality of the fusion proteins, both bound equivalently to the conformation-dependent anti-KIR2DL2/3 antibody, DX27 (Fig. S1). Moreover, the difference was reproduced in titrations against HLA class I-coated beads, as illustrated for HLA-C*0702 and C*0302 that account for 92% and 5% of Yucpa C1 epitopes, respectively (Fig. 3*C*). One possible cause of the difference is that 2DL3*009 and 2DL3*001 have different affinities for C1, another is that they bind to different subsets of the target HLA-C molecules. Distinguishing such subsets are the variable peptides that form an integral component of MHC class I, and which are known to influence HLA-C interaction with KIR2D (36). Thus, 2DL3*009 could bind to a subset of the HLA-C*0702 molecules bound by 2DL3*001. Whichever interpretation is correct, the net effect is the same, namely 2DL3*009 has less avidity for C1 than 2DL3*001. For 2DL3*008N, which is nonfunctional, this trend to lower avidity is taken to the limit.

When *KIR2DL2* and *KIR2DL3* were first identified, they were considered (and named) as separate genes encoding C1 receptors because of the extent of their sequence differences (9), but subsequent population and family analyses showed they segregate as alleles (37). In general, *KIR2DL3* is fixed on *A* haplotypes and *KIR2DL2* is present only on *B* haplotypes. In the Yucpa, the haplotype segregation is particularly strong: 92% of the B haplotypes having *2DL2*, only 8% *2DL3* (Fig. 2*A*). Of note, *KIR2DL2* is represented only by the *2DL2*003* founder, and the only form of *KIR2DL3* on *B* haplotypes is also the founder, *2DL3*001*. In contrast, *KIR2DL3* on the *A* haplotypes is represented by three forms: the founder, *2DL3*001*, representing 16.4% of total Yucpa *2DL3*; the low avidity *2DL3*009*, representing 68.8%; and the non-functional *2DL3*008N* representing 14.8% (Fig. 3*A*). Thus, the *KIR2DL3* founder has been largely replaced by the two new variants. We could not find *2DL3*008N* or *2DL3*009* in other populations, including the neighboring but linguistically separated Bari and the more distant Warao Amerindians ($n = 41$), and Venezuelan Mestizos ($n = 21$). The new variants appear to have restricted distribution, or to be at very low frequencies in other populations, and it is possible that *2DL3*008N* and *2DL3*009* are Yucpa-specific, having evolved in that population by point mutation from *2DL3*001*. Because *2DL3*009* is present on haplotypes *A1*, *A2*, and *A5*, at least two of them acquired the new variant by recombination (Fig. 2*A*). The substantial changes in the structure and function of KIR2DL3, but in none of the other functional KIR in the Yucpa, points to these changes being a consequence of natural selection, as does the sliding-window analysis shown in Fig. 2*B*.

Discussion

The Yucpa Retain the Worldwide Range of *KIR* Haplotypes, Genes, and Alleles. The Yucpa descend from Asian migrants who arrived in Alaska approximately 15,000 years ago and peopled the Americas through population expansion and southward migration (14). During the approximately 750 generations following the initial bottleneck (13), Yucpa ancestors likely experienced cycles

of population contraction and expansion caused by epidemics of infectious disease, most recently an epidemic of combined hepatitis B and D (19). We have defined the system of KIR and HLA class I ligands in the surviving Yucpa population.

Despite the population bottlenecks, we find that with 29 forms of *KIR* and 19 forms of *HLA-A*, *-B*, and *-C*, the Yucpa retain almost all major elements of the KIR-HLA class I system present worldwide, including all components for which immunological functions are well defined. The one *KIR* gene missing from the Yucpa, *KIR2DS3* is of questionable function, because its protein product is not cell-surface expressed (27). And in the Yucpa, and other Amerindian groups, the absence of *KIR2DS3* is compensated by increased frequency of the closely related *KIR2DS5* (25, 38–40). The polymorphic *KIR* genes of the Yucpa retain the breadth of the polymorphism worldwide with three to four alleles, and similar numbers of HLA class I allotypes provide the three major epitopes recognized by KIR: C2 carried by HLA-C, C1 carried by HLA-C and HLA-B, and Bw4 carried by HLA-A and HLA-B. Absent from the Yucpa, and generally rare in Amerindians, is the A3/A11 epitope recognized by KIR3DL2.

The Yucpa maintained high *KIR* diversity through population bottlenecks as a consequence of balancing selection. At the time of sampling in 1993, the population had an even frequency of *A* and *B* haplotypes that are maximally divergent in gene content and share no single *KIR* allele. The *B* haplotype is essentially invariant, whereas five *A* haplotypes, give breadth to the polymorphism of *KIR2DL3*, *2DL4*, *2DS4*, *3DL1*, *3DL2*, and *3DL3*. Contrasting with their diverse *KIR*, the Yucpa retain only one of three to five allele lineages of the *ABO* blood group locus, which in other populations have been subject to balancing selection (41). Because of the intensity of selection and small population size, the Yucpa system of six *KIR* haplotypes and three HLA class I epitopes has become streamlined, and emerges as a candidate for having the minimal essential diversity needed for long-term survival of a human population.

Selective Evolution of the Interaction between KIR2DL3 and C1 in the Yucpa. All Yucpa *HLA class I* alleles encoding KIR ligands, and 25 of the 29 Yucpa *KIR*, are founder alleles; they came from Asia with the original migrants and have remained unchanged since then. In this general context of stability, the functional changes in KIR2DL3 and its cognate C1 ligand are striking. The founder allotype, 2DL3*001 has severely decreased in frequency, being replaced by two variants, mainly by 2DL3*009, that has reduced avidity C1, but also by the non-functional 2DL3*008N. Such changes are evidence for selection to reduce the strength of the KIR2DL3-C1 interaction. These changes in KIR2DL3 are associated with an unusually high frequency of HLA-C*07, the dominant C1-bearing allotype and the one most potent at educating NK cells to attack and kill cells deficient in MHC class I (23). In the Japanese, who share many KIR factors with the Yucpa, homozygosity of C1 results in the education of fewer 2DL3*001-expressing NK cells than C1 heterozygosity (23), suggesting that increasing the avidity of 2DL3-C1 interactions beyond a certain point is disadvantageous. Emergence of weaker KIR2DL3 variants in the Yucpa can thus be interpreted as a compensatory response to the elevated frequencies of C*0702 and of C*0702 homozygotes, one that reduces the avidity of the 2DL3-C1 interaction and increases the abundance of functional NK cells expressing KIR2DL3.

A further distinguishing characteristic of C*0702 is that its leader peptide forms complexes with HLA-E that prevent interaction with the conserved inhibitory receptor CD94:NKG2A (5). Thus, C*0702 not only favors NK cell regulation through interaction with KIR2DL3, but it may also act to disfavor education and regulation through interaction of CD94:NKG2A with HLA-E. Contrasting with C*0702, the second most frequent C1-bearing allotype in the Yucpa, C*0302, combines with HLA-E to form a high avidity ligand for CD94:NKG2A (5).

Homozygosity for C1 and KIR2DL3 has been correlated with successful termination of acute hepatitis C virus (HCV) infections in U.K./U.S. Caucasians and African Americans (42). The observed co-evolution of C1 and KIR2DL3 in the Yucpa, suggests that the combination of KIR2DL3 with C1 could also be beneficial for terminating other types of infection, including the combined hepatitis B and D that affected the Yucpa in 1979–1981. Although KIR2DL2 is allelic to KIR2DL3, it was not associated with resistance to HCV (42) and unlike KIR2DL3, it has undergone no change in the Yucpa. *KIR2DL3* is a fixed locus of the *A* haplotype and the *2DL3*008N* and *2DL3*009* variants are only present on *A* haplotypes, whereas *KIR2DL2* is only present on *B* haplotypes. These qualitative differences point to the group *A* and *B* *KIR* haplotypes having been subject to different types of selection pressure.

A Model in Which *A* and *B* *KIR* Haplotypes Diversified under Selection for Immunity and Reproduction, Respectively. Although group *A* *KIR* haplotypes protect against HCV infection, they are risk factors for the pregnancy syndromes preeclampsia (43) and recurrent miscarriage (44). Preeclampsia and eclampsia are leading causes of death for women of child-bearing age, especially in undeveloped countries (45). At risk are pregnancies in which C2-expressing fetuses are carried by group *A* *KIR* homozygous mothers; conversely, maternal group *B* *KIR* haplotypes and fetal C1 homozygosity are protective (43, 44). The inverse correlation between C2 and group *A* *KIR* haplotype frequencies in human populations worldwide, argues for the importance of selection by diseases of pregnancy (43). Although the evidence is correlative and the studies are few in number, they raise the intriguing possibility that *A* *KIR* haplotypes are principally selected for their role in immune defense, whereas *B* *KIR* haplotypes are selected for their role in placental reproduction. In this model the balancing selection that has maintained *A* and *B* haplotypes in all human populations would come from distinctive pressures upon the immune and reproductive systems. Thus, an episode of viral infection is predicted to select for *A* *KIR* haplotypes, which will be enriched in the survivors, but in subsequent expansion of the surviving population there will be selection for *B* haplotypes and against *A*.

Although individuals homozygous for *A* or *B* haplotypes are numerous, healthy and able to reproduce, none of the >140 human populations examined for *KIR* gene content lacks either *A* or *B* haplotypes (8, 10, 46). Thus, long-term survival of human populations appears to have selected for retention of both *A* and *B* haplotypes, a corollary being that populations losing either *A* or *B* haplotypes were out-competed by those retaining both haplotype groups. Because viruses and other pathogens can evolve rapidly in response to the human immunity, the pressure on the immune functions of the KIR is likely to be more variable and changing than the pressure on the reproductive functions. Consistent with this thesis, the group *A* *KIR* haplotype genes are highly polymorphic, whereas the group *B* *KIR* haplotype genes are generally conserved (10). Although observed in all populations studied, these properties are most vividly illustrated by the Yucpa, for whom we speculate that the exigencies of selection have retained what is both minimal and essential for long term survival in the struggle against infectious disease and other human populations.

Materials and Methods

DNA samples were obtained from 61 individuals from three villages (Aroy, Marewa, and Peraya) of the Yucpa Amerindian tribe (Table S1) (20). DNA samples from two other Amerindian Venezuelan tribes, Bari (n = 19) and Warao (n = 22), and one Mestizo mixed population from Caracas (n = 21) were sequenced for selected *KIR* genes as indicated. Ethical approval was granted

by the Stanford University Administrative Panel on Human Subjects in Medical Research.

Defining Yucpa *KIR* Haplotypes. To define allele-level *KIR* haplotypes for the panel of Yucpa donors, we first sequenced coding regions for all *KIR* genes from the 23 individuals who were homozygous for *KIR* gene-content haplotypes (Fig. S2), then extended the analysis to selected heterozygotes and finally genotyped the remaining individuals. In 15 *A/A* homozygotes, *KIR2DL4*, *2DS4*, *3DL1*, *3DL2*, and *3DL3* were represented by two to five alleles; *KIR2DL1* and the *2DP1* and *3DP1* pseudogenes were monomorphic. Seven of these individuals were homozygous for one of three common allele-level *A* haplotypes: *A1*, *A2*, and *A3*. Reasoning that the eight *A/A* heterozygotes each carried a common haplotype, we defined rarer haplotypes *A4* and *A5*. All eight *B/B* homozygotes were homozygous for *B1* (Fig. 2*A*). In analyzing heterozygotes suspected to harbor additional *B* haplotypes, we reasoned that one *KIR* haplotype was already defined (either *A1*, *A2*, *A3*, *A4*, *A5*, or *B1*), thus permitting identification of *B2*, *B3*, and *B4*. Based on the gene sequences, allele-typing methods were devised and applied to the panel (Fig. S2). Within the panel studied were 35 members of 13 families. Segregation analysis for these family members gave results consistent with the assigned haplotypes (Table S1). The *KIR* haplotype distribution within the panel complied with Hardy-Weinberg equilibrium, providing further evidence for the validity of the defined haplotypes.

Statistical Analysis. Statistical significance of Tajima's D was assessed by comparing the observed values to those expected under neutral evolution. Expected values were generated by coalescence simulations using the program ms (47). ms was used to generate 10,000 independent replicate samples under user-defined models. The simulations were performed with a founder of 100 effective population size, followed by growth to 1,000 over 600 generations; similar to the model proposed by Hey (13). The forward-simulations (Table 1 and Table S3) were performed using simuPOP 0.8 (ref. 48).

KIR/HLA-Binding Assay. Soluble Fc-fusion proteins were produced using methods described (28). The purified fusion proteins were tested for binding to a panel of 29 HLA-A, 47 HLA-B, and 16 HLA-C allotypes using the LABScreen single-antigen beads (One Lambda) and a Luminex 100 reader (Luminex Corp.). Relative fluorescence ratios were calculated using the formula (specific binding-control bead binding)/(positive binding-control bead binding). W6/32, an antibody specific for HLA class I was used as the positive control (28). The nucleotide substitution that distinguishes *2DL3*009* was introduced to *2DL3*001* cDNA using site-directed mutagenesis (Invitrogen).

ACKNOWLEDGMENTS. We thank the Yucpa community and the Fundacion Zumaque for their support of the field work. This work was supported by a grant from the Lymphoma and Leukemia Society of the USA (to P.J.N.) and National Institutes of Health Grant AI017892 (to P.P.).

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