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Human Diversity in Killer Cell Inhibitory Receptor Genes

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Summary

The presence and expression of killer inhibitory receptor (KIR) and CD94:NKG2 genes from 68 donors were analyzed using molecular typing techniques. The genes encoding CD94:NKG2 receptors were present in each person, but KIR gene possession varied. Most individuals expressed inhibitory KIR for the three well-defined HLA-B and -C ligands, but noninhibitory KIR genes were more variable. Twenty different KIR phenotypes were defined. Two groups of KIR haplotypes were distinguished and occurred at relatively even frequency. Group A KIR haplotypes consist of six genes: the main inhibitory KIR, one noninhibitory KIR, and a structurally divergent KIR. Allelic polymorphism within five KIR genes was detected. Group B comprises more noninhibitory KIR genes and contains at least one additional gene not represented in group A. The KIR locus therefore appears to be polygenic and polymorphic within the human population.

Introduction

Natural killer (NK) cell function is regulated by the interaction of major histocompatibility (MHC) class I molecules with inhibitory cell surface receptors (Yokoyama, 1993; Lanier and Phillips, 1996). This mechanism is believed to protect healthy cells from lysis by autologous NK cells, while rendering cells for which class I expression is compromised by infection or transformation susceptible to NK cell-mediated lysis (Ljunggren and Kärre, 1990). Unlike cytotoxic CD8$^+$ T cells, which require recognition of a specific MHC class I allotype to lyse a target, NK cells are prevented from lysing a target by recognition of MHC class I (Kärre et al., 1986; Storkus et al., 1987; Ciccone et al., 1992; Karlhofer et al., 1992; Moretta et al., 1993).

Human NK cells use two types of structure as their HLA class I receptors: molecules of the immunoglobulin superfamily (IgSF) called killer cell inhibitory receptors (KIR) are specific for determinants shared by subsets of HLA-B or -C allotypes (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Long et al., 1996), whereas the CD94:NKG2-A heterodimer, which is related to C-type lectins, is specific for a determinant shared by most HLA-A, -B, and -C allotypes (Moretta et al., 1994; Lazetic et al., 1996; Phillips et al., 1996; Brooks et al., 1997; Carretero et al., 1997). Forms of the NKG2 polypeptide determine whether the lectin-like heterodimer mediates inhibition (NKG2-A) or stimulation (NKG2-C) (Houchins et al., 1997). For both kinds of receptor, transduction of an inhibitory signal requires the presence in the cytoplasmic tail of two immune receptor tyrosine-based inhibitory motifs (ITIM) (Burshtyn et al., 1996; Lazetic et al., 1996; Houchins et al., 1997). Several members of the KIR family have truncated cytoplasmic tails and lack such inhibitory motifs (Colonna and Samaridis, 1995; Moretta et al., 1995; Wagtmann et al., 1995; Biassoni et al., 1996); they are designated here as "noninhibitory KIR."

Every person can be expected to express HLA class I molecules that engage the CD94:NKG2-A receptor, whereas the number of KIR ligands depends on a person's HLA type. Three kinds of inhibitory KIR have well-defined HLA ligands, and an individual can have one, two, or three of these: inhibitory KIR with two IgSF domains recognize subsets of HLA-C allotypes determined by alternative amino acid sequence motifs at positions 77 and 80 of the α1 helix (Colonna et al., 1993). A KIR with three IgSF domains recognizes the subset of HLA-B allotypes that share the Bw4 sequence motif at positions 77–83 of the α1 helix (Litwin et al., 1994; Gumperz et al., 1995).

The genes for KIR, CD94:NKG2, and HLA are on different chromosomes (Yabe et al., 1993; Baker et al., 1995; Chang et al., 1995; Colonna and Samaridis, 1995; Wagtmann et al., 1995), so that ligands and receptors segregate independently in human pedigrees. As a consequence, a substantial proportion of the population has genes for KIR for which they have no HLA class I ligand (Gumperz et al., 1996). In the present investigation, we used novel molecular typing techniques for KIR and NKG2 genes to analyze a sample population, revealing an extensive polymorphism in KIR genotypes.

Results

A System for Typing Patterns of KIR and NKG2 mRNA Expression

To assess variation in the repertoires of KIR and NKG2 HLA class I receptors expressed by individual humans, typing assays based on the reverse transcription polymerase chain reaction (RT-PCR) were developed. The design of the KIR typing system was guided by the

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Table 1. PCR Primers

PCR Group	Template[a]	Sense Primer (5′-3′)	Antisense Primer (5′-3′)	Location[b]	Length[c]
KIR2DL1	RNA	GCA GCA CCA TGT CGC TCT	GTC ACT GGG AGC TGA CAC	−8–348	356
KIR2DL1	DNA	ACT CAC TCC CCC TAT CAG G	AGG GCC CAG AGG AAA GTC A	315–567	∼1750
KIR2DL2	RNA	CCA CTG CTT GTT TCT GTC AT	CAG CAT TTG GAA GTT CCG C	645–1015	370
KIR2DL2	DNA	CCA TGA TGG GGT CTC CAA A	GCC CTG CAG AGA ACC TAC A	228–523	∼1800
KIR2DL3	RNA	CCA CTG AAC CAA GCT CCG	CAG GAG ACA ACT TTG GAT CA	692–1044	352
KIR2DL3	DNA	CCT TCA TCG CTG GTG CTG	CAG GAG ACA ACT TTG GAT CA	792–1043	798
KIR2DL4	RNA	CTG TCC CTG AGC TCT ACA A	CAC TGA GTA CCT AAT CAC AG	206–747	541
KIR3DL1	RNA	ACA TCG TGG TCA CAG GTC C	TGC GTA TGT CAC CTC CTC	641–1197	556
KIR3DL1	DNA	CCA TCG GTC CCA TGA TGC T	AGA GAG AAG GTT TCT CAT ATG	542–679	∼1600
KIR3DL2	RNA;DNA	CGG TCC CTT GAT GCC TGT	GAC CAC ACG CAG GGC AG	546–914	368; ∼1900
KIR2DS1	RNA;DNA	TCT CCA TCA GTC GCA TGA A/G	AGG GCC CAG AGG AAA GTT	254–567	313; ∼1800
KIR2DS2	RNA;DNA	TGC ACA GAG AGG GGA AGT A	CAC GCT CTC TCC TGC CAA	179–435	256; ∼1750
KIR2DS3	RNA;DNA	TCA CTC CCC CTA TCA GTT T	GCA TCT GTA GGT TCC TCC T	315–594	279; ∼1800
KIR2DS4	RNA;DNA	CTG GCC CTC CCA GGT CA	GGA ATG TTC CGT TGA TGC	94–544	450; ∼2000
KIR2DS5	RNA;DNA	AGA GAG GGG ACG TTT AAC C	GCC GAA GCA TCT GTA GGC	184–600	416; ∼1950
KIR3DS1	RNA	GGC ACC CAG CAA CCC CA	AAG GGC ACG CAT CAT GGA	321–567	246; ∼1750
KIR3DS1	DNA	GGC AGA ATA TTC CAG GAG G	AGG GGT CCT TAG AGA TCC A	217–493	∼1800
CD94	RNA	GCA GTG TTT AAG ACC ACT CT	CTG TTG CTT ACA GAT ATA ACG	4–531	527
NKG2-A	RNA	CCA GAG AAG CTC ATT GTT GG	CCA ATC CAT GAG GAT GGT G	202–527	325
NKG2-A	DNA	AGG AGT AAT CTA CTC AGA CC	AGG GAA TAA CAA CTA TCG TTA C	12–283	661
NKG2-C	RNA	GGA AAT ATT CCA AGT AGA ATT AAA T	CTG ATG CAC TGT AAA CGC AAA T	108–727	619
NKG2-C	DNA	TTT CTG GCC AGC ATT TTA CCT	CTG ATG CAC TGT AAA CGC AAA T	477–727	∼1100
NKG2-D	RNA	CTG GGA GAT GAG TGA ATT TCA TA	GAC TTC ACC AGT TTA AGT AAA TC	35–451	416
NKG2-E	RNA	CTG TGC TTC AAA GAA CTC TTC T	CTG GTC TGA TAT AAG TCC ACG T	432–657	225

[a] Primer pairs were used for RNA-based typing (RNA), genomic typing (DNA), or both (RNA; DNA).
[b] Nucleotide positions of amplified fragments were calculated from the start codon according to the sequences previously reported (Houchins et al., 1991; Adamkiewicz et al., 1994; Biassoni et al., 1995; Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Döhring et al., 1996a; and Selvakumar et al., 1996).
[c] In cases where primer pairs were used for RNA- and DNA-based typing, the length (in base pairs) of the RT-PCR fragment is given first.

topology of a tree of KIR sequences. The 12 groups of KIR chosen as the types to be defined in the assay represent the smallest clades of the tree (Valiante et al., 1997a). Oligonucleotide primer pairs (Table 1) were designed to match polymorphic positions unique to each KIR group, as determined from an alignment of 36 different KIR sequences. The resulting assay accounts for all reported KIR sequences (except certain alternative splice variants) and permits comparison of the five groups of inhibitory KIR (KIR2DL1–3 and KIR3DL1–2), the six groups of noninhibitory KIR (KIR2DS1–5 and KIR3DS1), and the unusual KIR2DL4, for which a function has yet to be assigned (Table 2). Each KIR group comprises one to six members, which differ by 1–9 nucleotide substitutions and may represent alleles. By contrast, members of different groups differ by 20 nucleotides or more. Expression of the four NKG2 genes was monitored with specific amplifications covering the inhibitory NKG2-A gene (NKG2-B is a mRNA splice variant of NKG2-A) (Plougastel et al., 1996); the noninhibitory NKG2-C; and the NKG2-D and NKG2-E genes, of unknown function.

The validity of the PCR typing system was demonstrated in several ways. First, it was demonstrated by direct sequencing of the PCR products obtained from two "control" individuals (donors PP and NV) for whom the expressed KIR and NKG2 genes had been determined by cloning and sequencing of cDNA (Valiante et al., 1997b [this issue of *Immunity*]). Second, its validity was demonstrated by application of the typing system to a panel of seven transfected cell lines, each expressing single KIR or the lectin-like NKG2-A. When the PCR typing system was applied to RNA samples from donors PP and NV, the types obtained correlated precisely with those determined by cDNA cloning (data not shown). Similar analysis of individual NK cell clones obtained from PP and NV showed that the KIR and NKG2 type obtained with the PCR assay corresponded to that determined using a panel of KIR- and NKG2-specific antibodies and flow cytometry (Figure 1).

The Combination of KIR Genes Expressed Is Highly Polymorphic in the Human Population

Using the RT-PCR–based typing system, peripheral blood mononuclear cells (PBMC) from 52 blood donors were compared for their expression of KIR and NKG2. Considerable variability in KIR expression was observed. Within this panel, which consisted mostly of caucasoid donors, 18 different phenotypes were detected (Table 3). The most common phenotype was present in 33% of the donors and was composed of four major inhibitory KIR (KIR2DL1, KIR2DL3, and KIR3DL1–2), the noninhibitory KIR2DS4, and the divergent KIR2DL4. The other KIR phenotypes were less common; they were found in 2%–8% of the panel. Whereas the common phenotype consisted of KIR from six different amplification groups, the less common phenotypes could include KIR from as many as ten of the amplification groups (Figure 2A).

Three KIR groups were represented in all the individuals typed: KIR2DL1 and KIR3DL2 of the inhibitory type and KIR2DL4. The majority of individuals expressed four different inhibitory KIR (Figure 2B). KIR with the two different HLA-C inhibitory specificities were present in

Table 2. PCR-Defined Groups of KIR Sequences

Group[a]	Structure	Specificity[b]	cDNA Name[c]	Amino Acid Substitutions[d]
KIR2DL1	2Ig, inhibitory	C2	P58cl47-11	5V, 132R, 230L
			NKAT1	5V, 132P, 230P
			P58cl42	5F, 132P, 230P
KIR2DL2	2Ig, inhibitory	C1	NKAT6	385R, 386Q
			p58cl43	385S, 386E
KIR2DL3	2Ig, inhibitory	C1	NKAT2/p58cl6	9V, 127L, 151Q, 166H, 324P, 338H 399A, 413R
			NKAT2A	9V, 127L, 151Q, 166H, 324L, 338H, 399A, 413H
			NKAT2B	9V, 127L, 151Q, 166H, 324P, 338H, 399T, 413R
			KIR-023GB	9A, 127R, 151E, 166R, 324P, 338I, 399A, 413R
KIR2DL4	2Ig		KIR-103AS	138T, 209A, 271D
			KIR-103LP	138A, 209P, 271N
KIR3DL1	3Ig, inhibitory	Bw4	NKAT3/AMB11/cl11	2S, 13L, 23M, 68I, 75I, 259G
			NKB1/cl2	2L, 13F, 23V, 68V, 75L, 259R
KIR3DL2	3Ig, inhibitory	A?	cl5/AMC5	40P, 113L, 158D, 166R, 228A, 252I
			NKAT4	40P, 113L, 158E, 166R, 228A, 252I
			NKAT4A/cl1-1	40P, 113V, 158E, 166R, 228A, 252I
			NKAT4B	40P, 113V, 158E, 166H, 228A, 252I
			17-1c	40A, 113V, 158E, 166H, 228P, 252T
			8-11c	40A, 113V, 158E, 166H, 228A, 252I
KIR2DS1	2Ig, noninhibitory	C2	EB6act1	4T, 186R
			EB6act2	4M, 186K
KIR2DS2	2Ig, noninhibitory	C1	NKAT5/p58cl49	20G
			GL183act1	20W
KIR2DS3	2Ig, noninhibitory		NKAT7	
KIR2DS4	2Ig, noninhibitory		NKAT8/cl39	
KIR2DS5	2Ig, noninhibitory		NKAT9	
KIR3DS1	3Ig, noninhibitory		NKAT10	166R
			123FM	166H

[a] KIR genes were designated according to nomenclature agreed upon by E. Long (National Institutes of Health, Bethesda, MD) L. Lanier (DNAX, Palo Alto, CA), and M. Colonna (Basel Institute, Basel, Switzerland). KIR2D and KIR3D refer to receptors with two or three IgSF domains, respectively. L stands for receptors having long and S for those having short cytoplasmic tails, consistent with the presence or absence of ITIM motifs, respectively. Each KIR subfamily is designated by an individual number, for example KIR2DS1. In tables and figures the receptor subfamilies are sometimes abbreviated (e.g., 2DS1).

[b] The C1 and C2 groups of HLA-C alleles are distinguished by the dimorphic positions Ser 77–Asn 80 (C1) and Asn 77–Lys 80 (C2) (Colonna et al., 1993).

[c] Sequences with identical coding regions are listed together and separated by a slash. No alternative splicing forms are listed.

[d] Amino acid substitutions between members of each group and their positions as calculated from the start codon are shown.

all individuals; KIR2DL1 represented one specificity and either KIR2DL2 or KIR2DL3 represented the other (Table 3). By contrast, the HLA-B–specific inhibitory receptor, KIR3DL1, was not expressed by four members of the panel. These appear to be true negative results, since antibodies specific for KIR3DL1 failed to bind these individuals' NK cells and since amplification with alternative sets of primers specific for KIR3DL1 gave similarly negative results (data not shown).

The majority of the polymorphism in the expressed KIR phenotypes is due to the noninhibitory receptors. Every individual expresses between one and five noninhibitory KIR (Figure 2B). None of these receptor groups is shared by all individuals, the most common, KIR2DS4, being represented in 80% of the panel (Figure 3). With one exception, the other noninhibitory KIR were represented in 27% or more of the panel. KIR2DS5 was not represented in the donor panel, although it could be amplified from the original cDNA clone, suggesting that this KIR is relatively uncommon.

The frequency with which certain combinations of KIR are expressed by individuals exceeded that predicted by random association, whereas one combination (KIR2DS3/2DS4) was found at less than the expected value (Table 4). The former phenomenon could be due to linkage disequilibrium, indicating that these combinations of KIR represent haplotypes. One putative haplotype comprises the inhibitory KIR2DL2 and the noninhibitory KIR2DS2 and KIR2DS3. Linkage was also detected between the two noninhibitory KIR2DS1 and KIR3DS1, which are expressed together in 19 of the 21 samples expressing either KIR2DS1 or KIR3DS1. Segregation of these two linkage groups was observed in a family, where donor NV inherited KIR2DL2, KIR2DS2, and KIR2DS3 from his mother and KIR2DS1 and KIR3DS1 from his father. KIR2DL2 was present in all samples negative for KIR2DL3, a finding suggesting that these two functionally similar inhibitory KIR segregate on different haplotypes. The four samples, which were negative for the inhibitory KIR3DL1, were positive for the noninhibitory KIR3DS1, which is the most similar KIR. Again, this finding argues that these two receptors segregate independently and that they are related to each other as alleles.

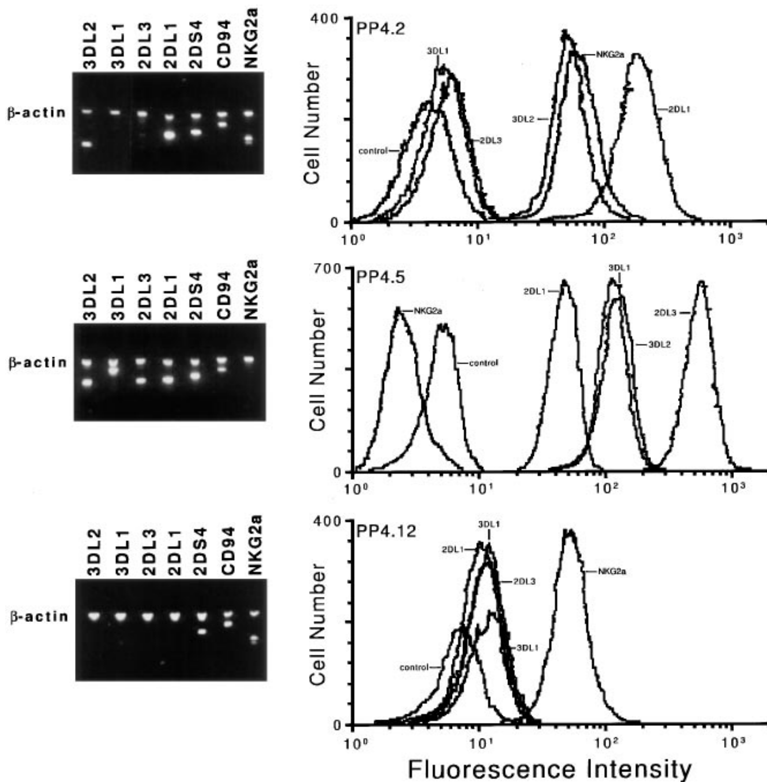

Figure 1. Detection of KIR and NKG2-A Transcripts in NK Clones by RT-PCR Correlates with Surface Expression

Three representative NK clones from donor PP were analyzed by RT-PCR for expression of KIR2DL1, KIR2DL3, KIR3DL1–2, and KIR2DS4 as well as the lectin-like receptors CD94 and NKG2-A (left). Detection of KIR or NKG2-A transcripts by RT-PCR correlated with receptor surface expression as determined by flow cytometry analysis using MAbs against KIR2DL1 (HP-3E4), KIR2DL3 (DX27), KIR3DL1 (DX9), and KIR3DL2 (DX31) as well as an CD94:NKG2-A–specific antiserum (right). Donor PP does not express the noninhibitory KIR2DS1 and KIR2DS2, which are also recognized by MAbs HP-3E4 and DX27, respectively (Lanier et al., 1997). Second-step antibodies alone served as negative controls.

Variability in the expression of the NKG2 receptor family was also observed. The inhibitory receptor NKG2-A was found to be expressed by all individuals, whereas in six samples no expression of the noninhibitory NKG2-C receptor was found (data not shown). The presence or absence of NKG2-C expression was not correlated with the expression of particular KIR haplotypes. The NKG2-D and NKG2-E genes, for which functions are unknown, are expressed by all the individuals we analyzed. Although the variation in NKG2 gene expression is more limited than that for KIR, it is similarly focused on a noninhibitory receptor, NKG2-C.

Distinct Patterns of KIR Expression Are Due to Differences in KIR Genes

The variation of KIR gene expression in the human population raised the question of whether it is due primarily to polymorphism of the genes or to differential regulation of gene expression. To address this issue, we developed a method for PCR typing of KIR and NKG2 genes using

Table 3. KIR Expression Patterns in the Population Survey

	KIR												
Phenotype	2DL1	2DL3	3DL1	3DL2	2DL2	2DS1	2DS2	2DS3	2DS4	3DS1	2DL4	Number[a]	Frequency (%)
1 (PP)	+	+	+	+	−	−	−	−	+	−	+	17	33
2	+	+	+	+	−	−	+	−	+	−	+	4	7.70
3	+	+	+	+	+	−	+	+	+	−	+	4	7.70
4	+	+	+	+	−	+	−	−	+	+	+	4	7.70
5 (NV)	+	−	+	+	+	+	+	+	+	+	+	3	5.80
6	+	+	+	+	+	+	+	+	−	+	+	3	5.80
7	+	+	+	+	+	−	+	−	+	−	+	3	5.80
8	+	+	+	+	−	+	+	−	+	+	+	2	3.80
9	+	+	+	+	+	+	+	−	+	+	+	2	3.80
10	+	+	+	+	+	−	+	+	−	−	+	2	3.80
11	+	+	+	+	+	−	−	−	+	−	+	1	1.90
12	+	−	−	+	+	+	+	+	−	+	+	1	1.90
13	+	+	+	+	+	−	+	−	+	+	+	1	1.90
14	+	+	−	+	−	+	−	−	−	+	+	1	1.90
15	+	+	−	+	+	+	+	+	−	+	+	1	1.90
16	+	+	−	+	−	+	−	−	+	+	+	1	1.90
17	+	+	+	+	−	+	−	−	−	+	+	1	1.90
18	+	+	+	+	−	+	+	−	−	−	+	1	1.90

[a] A total of 52 individuals were analyzed.

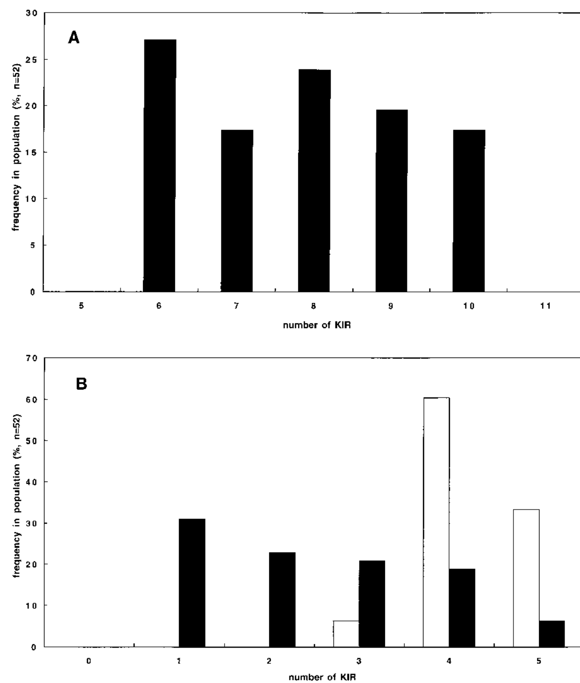

Figure 2. Variation in the Number and Type of KIR Genes Expressed by Different Individuals

KIR expression by PBMC was analyzed by KIR-specific RT-PCR.
(A) The total number of KIR expressed by individuals is plotted as a frequency distribution within the panel (n = 52).
(B) The number of inhibitory (open bars) and noninhibitory (filled bars) KIR is compared. The divergent KIR2DL4 of unknown function that is expressed by all individuals is included in the analysis shown in (A) but not that in (B).

Table 4. Association of KIR in a Survey of 52 Individuals

KIR Combination	Observed[a]	Expected[b]	Factor[c]
2DL2/2DS2/2DS3	26.9	5.64	4.77
2DL2/2DS3	26.9	10.9	2.47
2DS1/3DS1	36.5	14.9	2.44
2DS2/2DS3	26.9	13.9	1.94
2DL2/2DS2	38.5	20.9	1.84
2DS1/2DS3	15.4	10.4	1.48
2DS3/3DS1	15.4	10.4	1.48
2DL2/3DS1	21.2	15.6	1.36
2DS1/2DS2	25	19.9	1.26
2DS2/3DS1	25	19.9	1.26
2DL2/2DS1	19.2	15.6	1.23
2DL2/2DS4	32.6	26.9	1.21
2DS2/2DS4	36.5	41.9	0.87
2DS4/3DS1	25	30.9	0.81
2DS1/2DS4	23.1	31.1	0.74
2DS3/2DS4	13.5	21.7	0.62

[a] Observed frequencies of KIR combinations in a survey of 52 samples. KIR combinations shown underlined are associated more frequently ($P < 0.0001$) than expected by random association, as determined by a test of statistical independence (G test). The results for KIR2DL1, KIR2DL3–4, and KIR3DL1–2 are not shown because they are expressed at frequencies greater than 0.9.

[b] Expected frequencies for KIR combinations are the product of each receptor's individual frequency.

[c] The fold increase of the observed frequencies over the expected frequencies.

genomic DNA as the template. We modified the primers used in the RT-PCR assay in order to limit the length of the amplification products to no more than 2 kb (Table 1). The DNA-based typing system was applied to samples of genomic DNA obtained from PBMC and the results compared to those obtained by RT-PCR typing of the same preparations of PBMC. For donors PP and NV, for whom the expressed KIR are known, identical KIR types were obtained in the two assays, a result that extended to all of ten other individuals analyzed (Figure 4). In no instance did an individual possess a KIR gene that was not found to be expressed by some cells within the population of PBMC. This correlation demonstrates that differences in the KIR expressed by individuals within the human population result from structural polymorphisms of the KIR gene family rather than from polymorphisms in the regulation of their expression.

DNA typing of KIR genes was next performed for 18 individuals of different ethnicity (Table 5). Within this panel the commonest genotype corresponded to the common phenotype seen at the RNA level in the panel of 52 mostly caucasoid individuals. This genotype, which carries a single noninhibitory KIR gene (KIR2DS4), was

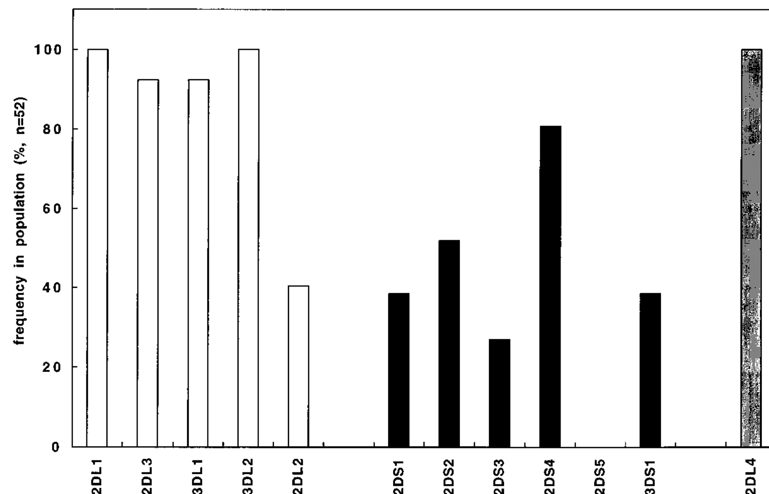

Figure 3. The Frequencies with Which Individual KIR Groups Are Expressed

Shown are the percentages of individuals within the panel (n = 52) who express each inhibitory KIR group (open bars), noninhibitory KIR group (solid bars), and the divergent KIR2DL4 group (hatched bars).

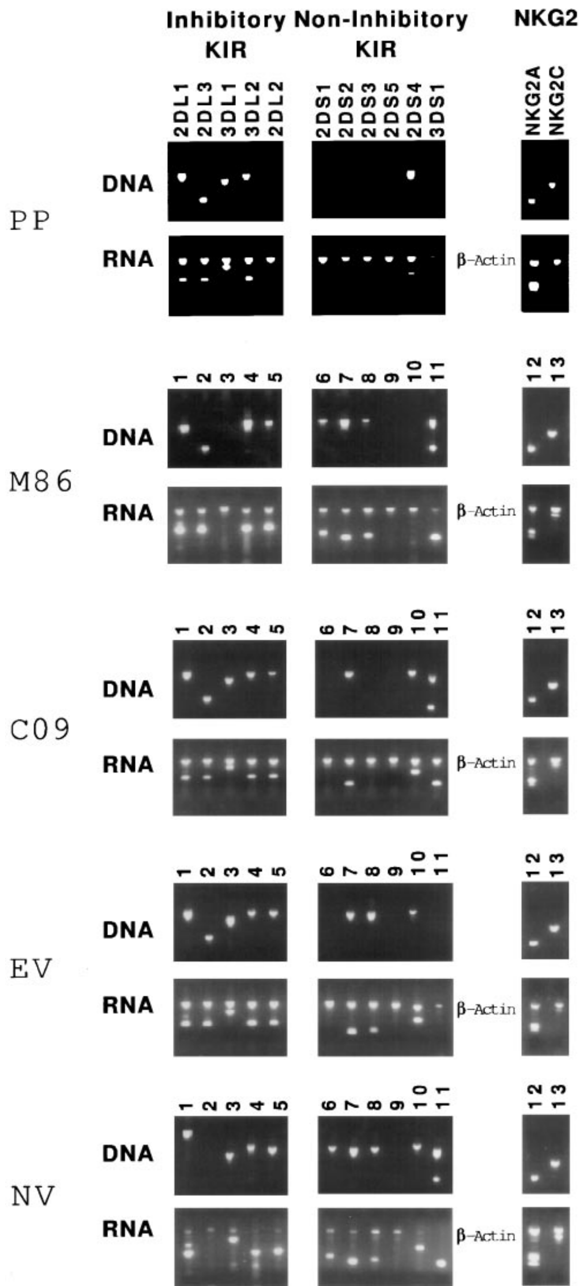

Figure 4. Variability of KIR Usage Is Determined at the Genomic Level

The presence of transcripts and genes corresponding to the indicated NK receptor groups was determined by RT-PCR and genomic PCR, respectively. A β-actin–specific primer pair was included in each RT-PCR amplification as an internal positive control and its product of 734 bp indicated. The results from 5 representative donors of a total of 12 analyzed are shown.

found in 11 of the 18 panel members, including the PP control. The remaining 7 members of the panel, including the NV control, had a variety of genotypes, generally characterized by an increased number of genes for non-inhibitory receptors. Among these were two additional genotypes that had not been found in the mostly caucasoid panel of 52 donors. The KIR genes of the ethnically more diverse panel of donors were also analyzed by Southern blotting. Those individuals with the most common KIR genotype showed the same banding pattern, as presented in Figure 5 for digestion with HindIII. Similar bands were also seen for individuals having less common KIR genotypes, but they were distinguished by an additional, large and prominent HindIII fragment of about 24 kb.

In contrast to the KIR gene family, all individuals analyzed possessed genes encoding NKG2-A, NKG2-C, NKG2-D, and NKG2-E. Southern blotting analysis showed no variation in the banding patterns for the NKG2 and CD94 genes (data not shown), although for some of the donors we were unable to detect NKG2-C transcripts.

In combination, the results obtained from KIR genotyping and Southern blotting indicate that the KIR haplotypes segregating in the human population can be divided into two broad groups. We will refer to these as the group A and the group B haplotypes. Distinguishing the two groups of haplotypes is the 24 kb HindIII band seen on Southern blots, which is present in group B haplotypes and absent from group A haplotypes. Whereas the heterogeneity within the group B haplotypes is apparent from KIR genotyping, by that method of analysis the group A haplotypes appear homogeneous. Individuals who express two group A haplotypes are those having the common KIR genotype and phenotype. However, an underlying heterogeneity in the group A haplotypes is revealed by the sequence analysis of KIR from donor PP, who possesses two group A haplotypes. Determination of complete KIR sequences shows that donor PP expresses two different but closely related alleles for five of the six different types of KIR associated with the group A haplotypes.

Based on Southern blotting and KIR typing results, we can confidently characterize 27 individuals of the panel of 68 donors as homozygous for group A haplotypes and 17 individuals as homozygous for group B haplotypes. Each of the remaining 24 donors could be either heterozygous for group A and B haplotypes or homozygous for group B haplotypes. At one end of the range of possibilities, 40% of the haplotypes would be of group A and 60% of group B, while at the other end 57% of the haplotypes would be of group A and 43% of group B. Throughout this range, the frequencies of group A and B haplotypes both are between 0.4 and 0.6 and therefore of comparable magnitude.

Discussion

Human NK cells use both lectin-like and immunoglobulin-like molecules as their inhibitory receptors for HLA class I molecules (reviewed by Lanier et al., 1997; Valiante et al., 1997b). In addition to their distinctive structures, these two kinds of receptors have complementary specificities: the lectin-like receptor CD94:NKG2-A engages most HLA-A, -B, and -C allotypes, whereas the immunoglobulin-like KIR are specific for subsets of HLA-B or -C allotypes. Because their HLA class I ligands are polymorphic, we investigated whether the receptors also exhibit diversity within the human populations. From PCR-based molecular typing and Southern blotting, no evidence for variation in the number of CD94

Table 5. KIR Genotypes of Ethnically Diverse Panel

Donor	Ethnicity	Inhibitory KIR	Noninhibitory KIR	Haplotype Group[a]
C1 (PP)	Caucasoid	2DL1, 2DL3, 3DL1-2	2DS4	A
B1	Black American	2DL1, 2DL3, 3DL1-2	2DS4	A
E1	East Indian	2DL1, 2DL3, 3DL1-2	2DS4	A
F2	Filipino	2DL1, 2DL3, 3DL1-2	2DS4	A
C1	Chinese	2DL1, 2DL3, 3DL1-2	2DS4	A
K1	Korean	2DL1, 2DL3, 3DL1-2	2DS4	A
K2	Korean	2DL1, 2DL3, 3DL1-2	2DS4	A
J3	Japanese	2DL1, 2DL3, 3DL1-2	2DS4	A
J4	Japanese	2DL1, 2DL3, 3DL1-2	2DS4	A
H1	Hispanic	2DL1, 2DL3, 3DL1-2	2DS4	A
H2	Hispanic	2DL1, 2DL3, 3DL1-2	2DS4	A
C2 (NV)	Caucasoid	2DL1-2, 3DL1-2	2DS1-4, 3DS1	B
B2	Black American	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
E2	East Indian	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
F3	Filipino	2DL1-3, 3DL1-2	2DS2	B
C2	Chinese	2DL1, 2DL3, 3DL1-2	2DS1, 2DS3, 3DS1	B
A1	American Indian	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
B3	Black American	2DL1-3, 3DL1-2	2DS1-3, 3DS1	B

[a] Donors assigned haplotype group B may also have a group A haplotype.

and NKG2 genes was obtained, whereas striking differences in the KIR genes were apparent. All of the KIR genes possessed by an individual are expressed, though not necessarily on every NK cell. At the level of the NK cell population as a whole, the KIR phenotype correlated precisely with the KIR genotype.

The molecular typing we used was designed to distinguish the major kinds of KIR that have been defined by the cloning and sequencing of cDNA. Three kinds of KIR were expressed by all 68 blood donors we examined: the inhibitory KIR2DL1, which is specific for group 2 HLA-C allotypes; KIR3DL2, an inhibitory receptor of less clear specificity, which includes certain HLA-A allotypes (Döhring et al., 1996b; Pende et al., 1996); and the divergent KIR2DL4, of unknown specificity. Most donors have genes encoding inhibitory KIR specific for the three major ligands: group 1 HLA-C, group 2 HLA-C, and Bw4 HLA-B. However, a minority (∼8%) of individuals lack an inhibitory KIR (KIR3DL1) specific for Bw4 HLA-B, consistent with a previous population study of this receptor that used serological methods (Gumperz et al., 1996). In contrast to the relatively conserved phenotype of the inhibitory KIR, there is substantial variation in the number and type of noninhibitory KIR.

A minimum of 14 different KIR haplotypes can account for the 20 KIR phenotypes found for the 68 individuals analyzed in this study. Given the small size of the population analyzed and its limited coverage of the world's populations, these haplotypes probably represent a minority of the total number. The KIR haplotypes divide into two groups distinguished by the absence (group A haplotypes) or presence (group B haplotypes) of a 24 kb HindIII fragment on Southern blotting. The two kinds of haplotype have relatively even frequencies, and for the panel of individuals we analyzed there appears to be an excess of homozygotes over that expected by random association. Although this feature could well be due to the artificial nature of our sample population, it raises the possibility of a role for selection.

In PCR genotyping, haplotypes of group A type identically. However, heterogeneity was revealed by nucleotide sequencing of KIR from donor PP, who types only for group A haplotypes and expresses closely related pairs of alleles for the four inhibitory KIR genes

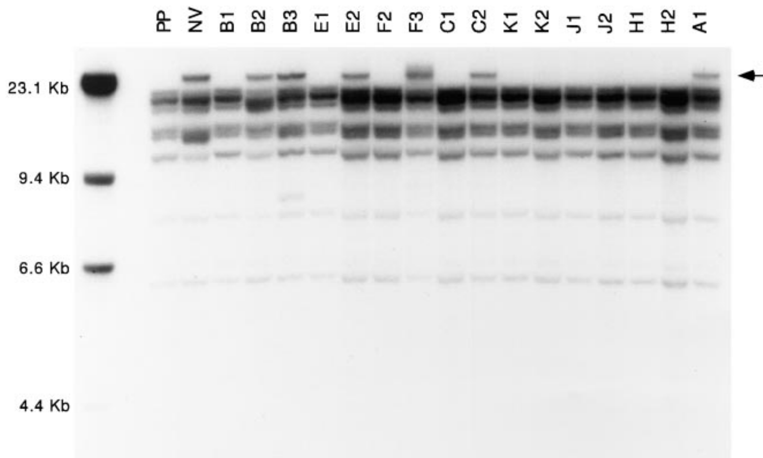

Figure 5. Genomic Polymorphism of KIR

Genomic DNA from 18 samples (see Table 5) of diverse ethnic origins were digested with HindIII and analyzed by Southern blotting using the full-length cDNA probe for KIR3DL1 (KIR-NKB1). An identical pattern was seen when a probe for KIR2DL4 (KIR-103AS), the most divergent member of the KIR gene family, was used (data not shown), indicating that all of the KIR genes hybridize with both KIR probes. The arrow indicates the 24 kb band that distinguishes the group B KIR haplotypes from the group A KIR haplotypes.

(KIR2DL1, KIR2DL3, and KIR3DL1–2). This result indicates that the four kinds of inhibitory KIR are encoded by separate genes. Of the other two kinds of KIR that characterize the group A haplotypes, two alleles for KIR2DL4 are expressed by PP and KIR2DS4 is represented by a single nucleotide sequence. Thus, these two KIR must also be encoded by separate genes. In total, the evidence supports a model in which the group A haplotypes consist of six KIR genes.

All six KIR genes that characterize group A haplotypes are also found in group B haplotypes, but individual group B haplotypes can lack certain of these genes. For example, whereas KIR2DS4 is common to all group A haplotypes, it is present on only a subset of group B haplotypes. Several lines of evidence suggest that there are additional KIR genes present on the group B haplotypes that are not present on the group A haplotypes. Indirect evidence comes from Southern blots revealing the 24 kb HindIII fragment, which is characteristic of group B haplotypes and indicative of an expansion of the KIR gene family. More direct evidence comes from consideration of the closely related KIR2DL2, KIR2DL3, and KIR2DS2, which are expressed simultaneously by certain individuals. KIR2DL3 is characteristic of group A haplotypes, whereas KIR2DL2 and KIR2DS2 are characteristic of group B haplotypes. This indicates that KIR2DL2 and KIR2DS2 derive from two different genes that are present on some group B haplotypes and probably related by gene duplication or gene deletion to the single KIR2DL3 gene on the group A haplotypes. A third piece of evidence is that the noninhibitory KIR2DS3, which is found on some group B haplotypes, is relatively divergent (Döhring et al., 1996b; Valiante et al., 1997a) and may represent a distinct locus rather than an allele of one of the other genes. KIR2DS5, which was not detected in the panel we analyzed, could be a rarer allele of the same gene as KIR2DS3.

Although genomic analyses of two KIR genes and a preliminary map of the KIR complex have been reported (Selvakumar et al., 1997; Wagtmann et al., 1997; Wilson et al., 1997), the number and organization of the KIR genes on human chromosome 19 have yet to be defined. The number of KIR genes also cannot be predicted by comparison of KIR cDNA sequences, because the cDNA sequences cannot be confidently sorted into groups corresponding to the alleles of different loci. This property of KIR sequences contrasts with those of their HLA class I ligands, for which locus assignments can readily be made on the basis of coding region sequences (Parham et al., 1995). Uncertainty in assigning KIR to loci stems in part from the apparently independent evolution of sequences encoding the extracellular and intracellular domains. Thus certain pairs of inhibitory and noninhibitory KIR are very similar in the extracellular part of the molecule but divergent in their cytoplasmic tails. KIR2DL1/KIR2DS1 and KIR3DL1/KIR3DS1 are examples of such pairs, which could represent alleles either of the same locus or of two closely related loci. For the case already discussed above, the triplet of related KIR (KIR2DL2, KIR2DL3, and KIR2DS2) that is expressed by some individuals proves that there are at least two loci involved, but for the other pairs of KIR the evidence is not so clear. Evidence in support of allelism would be the expression by certain, putatively homozygous, individuals of one member of a pair but not the other. In the panels we analyzed this prediction is met for KIR3DL1 and KIR3DS1, suggesting they could be alleles of the same locus.

For the pair KIR2DL1/KIR2DS1, the segregation appears to be different: individuals either have both of the KIR or have just KIR2DL1. No individual in the panel has KIR2DS1 without also having KIR2DL1. This pattern is consistent with encoding of KIR2DS1 and KIR2DL1 by different genes. It may also be consistent with encoding of the two KIR by alleles, providing that a role for selection is allowed. The rule governing receptor expression by NK cells is that every cell carries an inhibitory receptor that binds an autologous HLA class I allotype; in contrast, expression of noninhibitory receptors appears not to be under similar constraint (Valiante et al., 1997b). A possible effect of this requirement could be selection for individuals who have the allele encoding the inhibitory receptor KIR2DL1 and against individuals who are homozygous for the noninhibitory receptor KIR2DS1, who would then be much rarer than predicted by the square of the allele frequency. Resolution of these alternative explanations should come from pedigree studies and genomic analysis of individuals who are homozygous by consanguinity for KIR haplotypes.

Our results support a model in which the group A KIR haplotypes consist of six genes: four encoding inhibitory receptors, one encoding a noninhibitory receptor, and one encoding a divergent receptor of uncertain category. Within the population sampled, 40% of the individuals carry two group A haplotypes, showing that these six genes are sufficient to provide an adequate set of KIR for regulation of NK cells in the human immune system. The group B KIR haplotypes are more variable in their organization, containing one additional gene, and possibly more, that encode noninhibitory receptors. The functions of these additional genes have yet to be established, but the relatively even frequency of group A and B haplotypes suggests that both kinds of haplotype are actively maintained in the population.

In a previous population study we used a monoclonal antibody (MAb) to examine the cell surface expression of the HLA-Bw4–specific KIR-NKB1 (now designated KIR3DL1) by NK cells of individuals with different HLA class I types (Gumperz et al., 1996). Although variations in KIR3DL1 expression were detected, they were not correlated with expression of the Bw4 HLA-B ligand. In the present study these results are confirmed and extended to the other KIR of known HLA specificity. Expression of a KIR does not depend on expression of the HLA class I ligand, and individuals who lack expression of a particular KIR do so because they lack the gene. A considerable majority of individuals have genes for inhibitory KIR that recognize the three well-defined HLA-B and -C ligands, and they express these receptors irrespective of their HLA type. By this mechanism the KIR genes on human chromosome 19 can segregate independently from the HLA genes on chromosome 6 while still maintaining compatibility between receptors and the KIR ligands presented by a particular HLA class I type.

The number of KIR ligands an individual possesses

varies from one to three and affects the extent to which KIR are used as inhibitory receptors by their NK cells (Valiante et al., 1997b). The CD94:NKG2-A inhibitory receptor is able to compensate for such differences because the HLA class I type of most, if not all, individuals includes a ligand for CD94:NKG2-A. The broad specificity of the CD94:NKG2-A receptor for HLA-A, -B, and -C allotypes suggests that the origin of this receptor:ligand combination predates the diversification of HLA-A, -B, and -C from a common ancestral class I gene. In contrast, the specificity of KIR for subsets of HLA-B or -C allotypes suggests that these receptor:ligand combinations arose subsequent to the divergence of the HLA-B and -C genes from HLA-A (Parham, 1994; Valiante et al., 1997a). Correlating with this difference in age of the two class I receptor systems, we find that the CD94 and NKG2 genes are relatively conserved from one individual to another, whereas the KIR genes exhibit considerable polymorphism.

Our results show that humans express the products of six or more KIR genes. In addition to this diversity of KIR within an individual's immune system, there is considerable diversity in the KIR type that distinguishes individuals within the population. This polymorphism of KIR appears analogous to that seen for MHC class I and II genes but contrasts with the variability of B and T cell antigen receptors, where diversity is played out largely within the individual. At present, the functions of only a subset of the KIR are known, but if they all contribute to the regulation of the NK cell response, then the diversity of KIR and HLA class I types within the human population has the potential to modify the NK cell response in ways that are highly individualized.

Experimental Procedures

Cells

Peripheral Blood Cells

Samples were obtained from a total of 68 donors: 52 blood donors (Stanford Blood Center, Stanford, CA) and 16 unrelated donors of different ethnic origins (Cedars-Sinai Medical Center, Los Angeles, CA). PBMC were isolated from whole blood by Ficoll-Hypaque gradient separation.

NK Cells

$CD3^-CD56^+$ NK clones were generated and maintained in culture as described previously (Yssel et al., 1984; Litwin et al., 1993).

Transfected Cells

KIR-NKAT1–6 (KIR2DL1–3, KIR3DL1–2, and KIR2DS2) cDNA were transfected into the murine BaF/3 pre-B cell line (Lanier et al., 1997). The NKG2-A cDNA was transfected into the murine P815 mastocytoma (Lazetic et al., 1996).

HLA Class I Typing

Two donors, NV and PP, were HLA class I typed by cDNA cloning and sequencing as described previously (Domena et al., 1993). Donor PP typed as A*0101/0301, B*1501/0702, and Cw*0304/0702; donor NV typed as A*0201/0301, B*2702/0702, and Cw*0202/0702.

Flow Cytometry Analysis

The MAb HP-3E4 (anti-KIR2DL1) was generously provided by M. López-Botet (Melero et al., 1994). The DX27 (anti-KIR2DL2–3), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2) MAbs have been described previously (Litwin et al., 1994; Lanier et al., 1997). All anti-KIR MAbs were detected using a fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG antiserum (Caltag, Burlingame, CA). The CD94:NKG2-A–specific polyclonal rabbit antiserum was used in combination with an FITC-conjugated goat anti-rabbit IgG antiserum as the secondary reagent (Caltag, Burlingame, CA).

DNA Extraction and Southern Blot Analysis

DNA for PCR typing was prepared from 1×10^7 B lymphoblastoid cell lines (BLCL) using the QIAamp Blood Kit according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). High-molecular-weight DNA for Southern blot analysis was prepared from 3×10^8 BLCL, and 10 μg of genomic DNA was digested to completion with restriction enzymes. The resulting DNA fragments were separated by electrophoresis through 0.6% agarose gels in 0.5× TBE buffer. Hybridizations were performed as described previously (Shum et al., 1996), using full-length cDNA probes for KIR-NKB1 (KIR3DL1), KIR-103AS (KIR2DL4), CD94, and NKG2-A.

RNA Extraction and Reverse Transcription

Total cellular RNA was prepared from 1×10^6 NK cell clones or 1×10^7 PBMC using RNAzol according to the manufacturer's instructions (Tel-test, Friendswood, TX). First-strand cDNA was synthesized from NK clones and PBMC-derived RNA (0.5 μg and 5 μg RNA, respectively) by RT using oligo(dT) and Moloney murine leukemia virus reverse transcriptase (GIBCO–BRL, Gaithersburg, MD) at 42°C for 1 hr.

NK Receptor–Specific PCR Typing

PCR amplification was performed with a pair of sense and antisense primers, each possessing a 3′ residue matching a polymorphic position on a given NK receptor gene (Table 1). The primer length was adjusted to result in an annealing temperature of the primers between 56°C and 62°C. This enabled amplification of all NK receptors under the same PCR conditions. Internal control sense (5′-CGC GAG AAG ATG ACC CAG ATC -3′) and antisense (5′-TTG CTG ATC CAC ATC TGC TGG-3′) primers, specific for a 734 bp β-actin fragment, were included in each RT-PCR at a concentration of 0.1 μM for the analysis of NK clones and 0.05 μM for PBMC. Genomic PCR was performed without internal positive controls to increase amplification efficiency of the larger fragments. Each experiment included negative control reactions containing distilled water as the surrogate template. KIR typing of NK clones included as a negative control a feeder cell culture without NK clones to monitor possible amplification of residual KIR transcripts from the irradiated feeder cells.

The NK receptor–specific primers were used at a concentration of 0.5 μM and stored in ready-to-use PCR plates at −20°C. Amplification of cDNA was performed in 25 μl reactions in a model 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) using 0.625 U AmpliTaq polymerase, 2.5 μl 10× Buffer (Perkin-Elmer), and 0.2 mM dNTPs (Promega, Madison, WI) under the following conditions: initial denaturation at 95°C for 2 min; then 60 s at 62°C, 45 s at 72°C, and 60 s at 94°C for the first five cycles; and then 45 s at 60°C, 45 s at 72°C, and 30 s at 94°C for 25–30 cycles. Genomic PCR analysis of KIR and NKG2 genes was performed with 100–300 ng DNA in a model 9700 thermal cycler (Perkin-Elmer) under the following conditions: initial denaturation for 5 min at 95°C; then 20 s at 97°C, 45 s at 62°C, and 90 s at 72°C for the first five cycles; and then 20 s at 95°C, 45 s at 60°C, and 90 s at 72°C for 25 cycles. Amplification products were analyzed on ethidium bromide–prestained 1.5% (RT-PCR typing) or 0.9% (genomic typing) agarose gels.

Nucleotide Sequencing of KIR

NK receptor-specific PCR products were purified using a QIAquick PCR Purification Kit according to manufacturer's instructions (QIAGEN) and directly sequenced in both orientations using dye-labeled deoxy-terminators and a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Full-length KIR coding sequences were obtained from donor PP and NV by amplification of KIR cDNAs with primers matching sequences in the 5′ and 3′ untranslated regions (Valiante et al., 1997b). PCR products were cloned into a pBLUESCRIPT SK^+ vector and partially sequenced with a standard T7 oligonucleotide primer. Based on the partial sequences, three to four representatives of each KIR were selected and were sequenced completely on both strands to obtain a consensus sequence.

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