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Genetic Control of Human NK Cell Repertoire

Heather G. Shilling,* Neil Young,2* Lisbeth A. Guethlein, * Nathalie W. Cheng,* Clair M. Gardiner,3* Dolly Tyan,† and Peter Parham4*

Through differential killer cell Ig-like receptor (KIR) and CD94:NKG2 gene expression, human NK cells generate diverse repertoires, each cell having an inhibitory receptor for autologous HLA class I. Using a new method for measuring repertoire difference that integrates multiple flow cytometry parameters, we found individual repertoire stability, but population variability. Correlating repertoire differences with KIR and HLA genotype for 85 sibling pairs reveals the dominant influence of KIR genotype; HLA genotype having a subtle, modulating effect on relative KIR expression frequencies. HLA and/or KIR genotype also influences CD94:NKG2A expression. After HLA-matched stem cell transplantation, KIR repertoires either recapitulated that of the donor or were generally depressed for KIR expression. Human NK cell repertoires are defined by combinations of variable KIR and HLA class I genes and conserved CD94:NKG2 genes. The Journal of Immunology, 2002, 169: 239–247.

Natural killer cells are lymphocytes of innate immunity that kill cells and produce cytokines (1). NK cells contribute to early defense against viral infection and have been implicated in alloresponses following bone marrow transplantation (1–3). NK cell clonal diversity is due to the combinatorial expression of different cell surface molecules, including several receptors for polymorphic epitopes of MHC class I, each encoded by a conventional, nonrearranging gene. NK cells can express receptors for allogeneic as well as autologous MHC molecules, and class I specificity of individual clones is determined by the array of receptors present at the cell surface, rather than any individual element (4, 5). In humans, the family of killer cell Ig-like receptors (KIR)3 provides inhibitory, and some activating, receptors for HLA-A, -B, -C, and -G (5). KIR are not present in mice, where analogous functions are performed by the Ly49 family of lectin-like molecules (6). KIR are encoded by a compact, rapidly evolving analogues are performed by the Ly49 family of lectin-like receptors (6). KIR are encoded by a compact, rapidly evolving gene family having a subtle, modulating effect on relative KIR expression frequencies. HLA and/or KIR genotype also influences CD94:NKG2A expression. After HLA-matched stem cell transplantation, KIR repertoires either recapitulated that of the donor or were generally depressed for KIR expression. Human NK cell repertoires are defined by combinations of variable KIR and HLA class I genes and conserved CD94:NKG2 genes. The Journal of Immunology, 2002, 169: 239–247.

A general rule is that NK cells cannot kill cells expressing a full complement of autologous MHC class I alleles, but can kill cells expressing some combinations of allogeneic MHC class I (23). In humans, this tolerance to self has been correlated with the expression of at least one inhibitory KIR or CD94:NKG2A receptor having specificity for self-HLA class I (4). Such observations, derived from functional assays, provided evidence that HLA class I type influences the repertoire of inhibitory HLA class I receptors expressed by peripheral blood NK cells. In contrast, population analysis of HLA-B- or -C-specific KIR expression revealed no difference between individuals who did, or did not, express a cognate HLA class I ligand (24, 25).

Conversely, one study reporting no HLA effect implicated undefined, non-HLA genes as factors affecting NK cell expression of the HLA-B-specific KIR (KIR3DL1) (24). Subsequent discovery of KIR population diversity made the KIR locus on chromosome 19 a candidate for the non-HLA genes (7, 26–31). The investigation described here tested this hypothesis and defined the magnitude of the role of HLA class I in NK cell repertoire selection. In this analysis, the confounding effects of KIR and HLA genetic diversity were controlled by comparing NK cell receptor repertoires in sibling pairs of known HLA and KIR types. The role of the KIR genotype in determining KIR repertoire was further assessed by following KIR expression after HLA-matched allogeneic and autologous stem cell transplantation. The results demonstrate a role for both KIR and HLA class I genes in determining human NK cell repertoires and resolve the seemingly paradoxical results of previous functional and genetic analyses.

**Materials and Methods**

*Healthy sibling donors*

Peripheral blood samples were obtained from 104 healthy individuals: 53 donors representing 17 families, of which 5 were multigenerational, from...
Cedars-Sinai Medical Center (Los Angeles, CA); 4 individuals representing 2 families from the City of Hope Histocompatibility Laboratory (Duarte, CA); and from 47 individuals representing 17 families from Stanford Medical Center Histocompatibility Laboratory (Stanford, CA).

**Stem cell patients and donors**

Eighteen patients receiving allogeneic stem cell transplants at the Stanford University Medical Center for treatment of chronic myelogenous leukemia (CML; n = 12) or acute myelogenous leukemia (n = 6) were studied. Of these, 12 received bone marrow (n = 8) or G-CSF-mobilized peripheral blood stem cells (n = 4) from HLA-matched sibling donors. The remaining six patients received bone marrow from unrelated donors (MUD). This group included 6 males and 12 females, with a median age of 39 years (range, 21–54 years). Pretransplant myeloblastic regimens included a combination of busulfan, cyclophosphamide, and etoposide, with or without fractionated total-body irradiation. Posttransplant graft-vs-host disease (GVHD), antiviral, and antibacterial prophylaxis were comparable (32 out fractionated total-body irradiation. Posttransplant graft-vs-host disease (GVHD), antiviral, and antibacterial prophylaxis were comparable (32

For KIR genotyping, primers designed to discriminate allele-specific polymorphisms were paired with KIR2DL1, KIR2DL3, KIR3DL1, or KIR3DL2 locus-specific primers. KIR3DL1 and KIR3DL2 subtyping were described by Gardiner et al. (29) and Shilling et al. (39). KIR2DL1 and KIR3DL2 subtyping were as described by Shilling et al. (39). To supplement this, KIR2DL1, KIR2DL3, and KIR3DL1 locus-specific PCR products from some sibling pairs were purified using a QIAquick PCR Purification kit (Qiagen) and partially sequenced with the original amplification primers by a dye terminator automated sequencer (Applied Biosystems).

**Abs and flow cytometric analysis**

Eighteen patients receiving allogeneic stem cell transplants at the Stanford University Medical Center were studied. This group included 6 males and 12 females, with a median age of 39 years (range, 39–70 years). Patients underwent standard G-CSF stem cell mobilization and harvest and comparable myeloblastic conditioning (36). Samples were collected weekly before transplantation and on the first day that each patient showed clinical engraftment or on day 14 posttransplant. Samples were collected weekly for the first month following transplant and on days 60, 90, and 180 posttransplant.

PBMCs were isolated by Ficol-Hypaque gradient separation. All samples were collected with approval of the appropriate Institutional Review Board.

**KIR nomenclature**

KIR2DL1, KIR2DL3, KIR3DL1, and KIR3DL2 alleles were named according to guidelines used in naming HLA alleles. Briefly, an asterisk separates the accepted gene designations (37) from three digits which distinguish alleles that differ by nonsynonymous substitutions; fourth and fifth digits were assigned to alleles that differ only by synonymous substitutions. Numerical order was assigned based on date of submission to GenBank; partial or complete substitutions were excluded, as were sequences of single PCR-derived clones.

**Genomic DNA and cDNA preparation**

Genomic DNA was prepared from 2 × 10^6–1 × 10^7 PBMC using a QIAamp Blood kit (Qiagen, Valencia, CA). Total cellular DNA was prepared from 2 × 10^6–1 × 10^7 PBMC using RNeasy kit (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized from −5.0 μg RNA using oligo(dT) (PE Applied Biosystems, Foster City, CA) and avian myeloblastosis virus/reverse transcriptase (Promega, Madison, WI) at 42°C for 90 min.

**HLA class I determination and KIR epitope-typing**

HLA-A and HLA-B Ags, including Bw4 and Bw6, were determined serologically by the laboratories supplying the sample. HLA-C type was determined serologically or by PCR-sequence-specific primer analysis of genomic DNA using C locus Sequence-Specific Primer Unitray test kits (Pel-Freez Biologicals, Rogers, AR).

The presence of the class I HLA-C KIR epitopes was determined by RT-PCR amplification. In addition, the HLA-Bw4 and Bw6 serological typing results were confirmed by RT-PCR. The group 1 HLA-C epitope was detected using specific sense primer (5′-CGA GTG AGC CTG CCG AAC-3′) plus an HLA-C locus-specific antisense primer (5′-AGG ACA CTT AGT AGCA GCC-3′); the group 2 HLA-C epitope was detected with a specific sense primer (5′-CGG AGT GAA CCT GCG GAA A-3′) and the HLA-C generic primer. HLA-Bw4 epitopes were detected with a mixture of three sense primers (5′-CTT CCG CAG CAC GCT CCT CC-3′, 5′-CTT CCG GAT CGC CCT CC-3′, and 5′-CTT CCG GAG CCT CCT CC-3′) with an HLA-B locus-specific antisense primer (5′-TCC GAT GAC CAC AAC TGC TCC-3′). HLA-Bw6 epitopes were detected with a specific sense primer (5′-CTT CCG GAA CCT GCG CG-3′) paired with the same generic antisense primer. Primers were used at 0.5 μM in 25-μl reactions using 1–2 μl cDNA. Internal control primers specific for β-actin (sense, 5′-CTT CGA GCA AGA GAT GGC CAC-3′; antisense, 5′-TTG CTG ATC CAC ATC TGC TGG AAG-3′) were included at 0.05 μM. PCR conditions were: initial denaturation at 95°C for 5 min; 5 cycles of 97°C for 20 s, 62°C for 45 s, and 72°C for 90 s; followed by 26–30 cycles of 95°C for 20 s, 60°C for 45 s, and 72°C for 90 s; and a 7-min extension at 72°C.

**KIR genotyping**

Typing of genomic DNA for KIR was performed as described by Uhrberg et al. (7), with modification. KIR2DL1 (KIR2DL1*004) was detected with the KIR2DL1 forward primer and KIR2DS1 reverse primer. Detection of KIR2DS5 was as modified by Vilches et al. (38).

For KIR subtyping, primers designed to discriminate allele-specific polymorphisms were paired with KIR2DL1, KIR2DL3, KIR3DL1, or KIR3DL2 locus-specific primers. KIR3DL1 and KIR3DL2 subtyping were described by Gardiner et al. (29) and Shilling et al. (39), KIR2DL1 and KIR3DL2 subtyping were as described by Shilling et al. (39). To supplement this, KIR2DL1, KIR2DL3, and KIR3DL1 locus-specific PCR products from some sibling pairs were purified using a QIAquick PCR Purification kit (Qiagen) and partially sequenced with the original amplification primers by a dye terminator automated sequencer (Applied Biosystems).

**Calculation of differences in frequency and median fluorescence intensity of KIR expression from flow cytometry data**

The difference in frequency of KIR expression by NK cells for each KIR-specific Ab was calculated by gating on NK cells which stained brightly with a CD94-specific Ab (HP-3D9; BD Biosciences) instead of Z199. Anti-CD3 and anti-CD56 Abs were labeled with PerCP and FITC, respectively, anti-KIR, anti-NKG2A, and anti-CD94 Abs were PE conjugated. Flow cytometric analysis was performed on a FACScan flow cytometer using CellQuest software (BD Biosciences). The four KIR-specific Abs used here detect all KIR of known specificity, thereby providing a measure of functional KIR expression. As KIR-specific Abs bind subsets of KIR, rather than individual allotopes, fluorescence intensity levels likely encompass various Ab-binding affinities as well as cell surface expression levels, and therefore reflect physical properties of KIR repertoire.

**Statistical calculations**

Correlations coefficients (r) were calculated using the formula r = Cov(X,Y)/σ Xσ Y, where Cov(X,Y) = Σ(X − μX)(Y − μY); significance was evaluated by two-tailed t test with n − 2 df.

**Results**

NK cell KIR repertoire is stable within an individual but varies between individuals

Human NK cell clones differ in number and combination of KIR expressed (4, 40). This mode of gene expression generates a diverse repertoire of KIR expression in peripheral blood NK cells. To assess the stability of these expression patterns, we studied peripheral blood NK cells of five healthy donors over a period of about 1 year, analyzing cell surface KIR expression with four
mAbs of different KIR specificity. Flow cytometry analysis showed that the proportion of NK cells binding each Ab and their median level of binding were stable over time for all five donors (Fig. 1, A–E). These parameters appeared not to be perturbed by infection or other environmental stress, including running of a marathon by the donor in Fig. 1A. The results showed that donors have stable and characteristic patterns of KIR expression, or KIR repertoires, which can be described in terms of eight flow cytometry measurements: two parameters for each of four Abs.

To compare KIR repertoires, paired sets of flow cytometry data of the type shown in Fig. 1, A–E, were used to calculate summed differences for the proportion of NK cells binding each Ab and for the median level of Ab binding. In this way, a comparison between two data sets was reduced to a single point on a two-dimensional plot (Fig. 1F; see Materials and Methods for further details). Control comparisons confirmed the validity of this method; when frozen aliquots of the same sample of PBMC were thawed and analyzed in duplicate, and, on different days, the total differences were small (data not shown). As expected from Fig. 1, A–E, comparison of the repertoires measured from PBMC obtained from the same donor on different occasions produced small differences. In contrast, pairwise comparison of unrelated individuals revealed a wide range of differences (Fig. 1F). In sum, these results show that an individual’s NK cell KIR repertoire is defined and stable over time, but that repertoires are highly diversified within human populations.

**Differences in KIR repertoire are principally determined by KIR genotype**

Functional and genetic studies have given contradictory results regarding the role of HLA class I polymorphism in determining NK cell KIR repertoires, while the effects of KIR gene variation on NK cell KIR repertoire remain unexplored. To define the contributions of these two gene families, we compared the KIR repertoires of 85 healthy sibling pairs and correlated differences in KIR expression with identity or disparity at the HLA class I and KIR loci.

A panel of 104 individuals from 36 families was studied (Fig. 2). Of 85 sibling pairs, 21 (25%) were HLA class I identical and 64 (75%) were disparate at one or both HLA class I haplotypes, as determined by serology (HLA-A and -B) and/or PCR typing (HLA-C). KIR identity of sibling pairs was determined through a combination of low- and high-resolution PCR typing and selected DNA sequencing; results were confirmed by segregation for 43 of the 85 sibling pairs. Twenty-seven sibling pairs (32%) were KIR identical and 58 (68%) were disparate at one or both KIR haplotypes. These numbers demonstrate random segregation of parental HLA and KIR haplotypes. That the number of KIR-identical sibling pairs exceeds 25% is because some families segregate more than one copy of a common KIR haplotype. HLA class I and KIR polymorphisms independently segregated in the 36 families, as expected from location of HLA and KIR genes to different chromosomes (chromosomes 6 and 19, respectively). Seven pairs (8%) were KIR and HLA identical; 20 (24%) were KIR identical, HLA disparate; 14 (16%) were KIR disparate, HLA identical; and 44 (52%) were KIR and HLA disparate (Fig. 2).

Peripheral blood NK cells were analyzed for KIR expression by flow cytometry (as in Fig. 1, A–E). For each sibling pair, the difference in NK cell KIR repertoire was calculated (as in Fig. 1F), the results being displayed in four two-dimensional dot plots in Fig. 3 according to whether the sibling pairs were KIR identical, HLA identical (Fig. 3A); KIR identical, HLA disparate (Fig. 3B); KIR disparate, HLA identical (Fig. 3C); or KIR disparate, HLA disparate (Fig. 3D). Each dot plot has a distinct distribution, demonstrating the influence of KIR and HLA genes on the NK cell KIR repertoire. However, KIR type is by far the dominant factor. In the context of incompatibility at the other genetic complex, KIR identity gave greater similarity in repertoire than did HLA class I identity. Thus, siblings who are KIR identical, HLA disparate have a much tighter distribution with lower summed difference (Fig. 3B) than do siblings who are KIR disparate, HLA identical (Fig. 3C).

The dominance of KIR over HLA is well illustrated when the data points for all sibling pairs are placed on the same dot plot and differentiated according to either KIR (Fig. 3E) or HLA identity (Fig. 3F). The data points for all KIR-identical pairs cluster close to the origin, irrespective of their HLA status (Fig. 3E), whereas the data points for HLA-identical pairs distribute throughout all but the outer fringe of the distribution (Fig. 3F). The distributions for KIR-disparate sibling pairs split into two subpopulations (Fig. 3, C–F), where the subpopulation with higher difference consists of pairs in which one sibling (but not the other) lacks reactivity with a KIR Ab (DX9 or EB6), due either to the absence of a KIR gene (KIR3DL1 or KIR2DL1/2DS1) or presence of an allele giving a

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**FIGURE 1.** Patterns of KIR expression by peripheral blood NK cells can be used to define and compare NK cell KIR repertoires. A–E, Flow cytometry data for five donors whose peripheral blood NK cells were assayed at intervals over a period of 13 mo for binding for KIR-specific Abs. Each panel shows the data from one donor. The Abs encompass the HLA-A, -B, and -C-specific KIR and were EB6 (☐: KIR2DL1/S1), DX27 (☒: KIR2DL2–3/S2), DX9 (▲: KIR3DL1), and DX31 (△: KIR3DL2). Each identical symbol in a panel represents a different time point. F, The data from A–E is used to compare KIR repertoires. Each symbol on the two-dimensional plot derives from two sets of flow cytometry data and provides a measure of the overall differences in frequency of cells binding each Ab and the overall differences in the level of binding. Comparisons are for pairs of unrelated individuals (●) and, as controls, for data sets obtained from the same person, but from blood samples drawn on different occasions (△). The range of possible value for each sum is 0–8.
null phenotype ($KIR3DL1*004$) with DX9 (29). Thus, $KIR$ genes dominate over $HLA$ class I genes in determining the NK cell repertoire of $KIR$ expression in peripheral blood NK cells.

Apparent from the distribution of repertoire differences between sibling pairs is that the two summed differences are not completely independent variables. Thus, siblings who have large differences in Ab-binding frequencies tend to have large differences in Ab-binding levels (Fig. 3E). This correlation between the sum of differences in frequency and median levels of Ab binding is statistically significant ($r = 0.72, p < 0.01$; Fig. 3E). It demonstrates that polymorphisms in the $KIR$ gene family influence the frequency of NK cells that express a particular KIR as well as other characteristics assessed by Ab binding, including the level of cell surface expression and Ab-binding affinity.

HLA genotype influences the frequencies of KIR-expressing NK cells

To determine the contribution of HLA class I to the NK cell KIR repertoire, we considered just the subset of $KIR$-identical sibling pairs. For these 27 pairs the differences in repertoire between siblings in HLA-identical pairs were compared with the differences between the siblings in HLA-disparate pairs. As seen in Fig. 4, the 7 HLA-identical pairs have similarly low values for the sum of differences for the frequency of Ab-binding cells, whereas the 20 HLA-disparate HLA-identical siblings exhibited a much wider range of values. This difference has statistical significance in a $t$ test ($p < 0.0001$). In contrast, the HLA-identical and HLA-disparate pairs exhibited a similar range of values for the summed
differences in the median level of Ab binding. Thus, the effect of the HLA class I genotype on the NK cell KIR repertoire is to modify the relative frequencies of cells expressing particular KIR, but not the surface levels of KIR expression.

Best characterized of the HLA class I-specific inhibitory KIR are those specific for epitopes of HLA-B or -C, which correlate with polymorphisms in the carboxyl terminal part of the α1 helix. The HLA-Bw4 epitope, formed by some sequence motifs at positions 77–83 in HLA-B, is recognized by KIR3DL1. HLA-C epitopes, defined by alternative motifs at positions 77 and 80, are bound by KIR2DL2/2DL3/2DS2 and 2DL1/2DS1, respectively (41–44). Because different HLA types can have the same KIR epitopes, we distinguished KIR-identical, HLA-disparate sibling pairs according to whether they were identical or different for KIR epitopes (Fig. 4). Repertoire differences were similar, there being no statistically significant difference between the two HLA-disparate groups. When the expression of individual KIR was analyzed for these sibling pairs, neither KIR frequency nor fluorescence intensity differences between siblings were consistently correlated with the presence or absence of a relevant KIR epitope. However, KIR- and HLA-identical sibling pairs and KIR-identical, HLA-disparate, KIR epitope-matched pairs were significantly different \((p < 0.01)\). Likewise, KIR- and HLA-identical pairs were different from KIR-identical, HLA-disparate, KIR epitope-disparate pairs \((p < 0.001)\). Together, these results imply that HLA modification of the NK cell KIR repertoire cannot be explained simply in terms of the HLA-B and -C sequence motifs.

CD94:NKG2A expression is affected by HLA and KIR genotypes

The stability of CD94:NKG2A expression was studied using the same five donors and samples used for KIR (Fig. 1). For each individual, the frequency of NK cells expressing CD94:NKG2A and their median level of surface expression were stable over time. However, these parameters varied among the five donors (data not shown). The stability of the individual CD94:NKG2A phenotype parallels that seen for KIR.

CD94:NKG2 expression by NK cells from the 85 sibling pairs was measured. A range of difference between siblings, in both frequency and level of CD94:NKG2A expression, was observed (Fig. 5). The differences were least for KIR- and HLA-identical siblings (Fig. 5A), showing that the combination of KIR and HLA influences the repertoire of CD94:NKG2A expression. The differences were lower for KIR-disparate, HLA-identical siblings than for either KIR-identical, HLA-disparate siblings or siblings disparate at both KIR and HLA. Thus, HLA type appears to override KIR type in affecting CD94:NKG2 expression. However, the effect is weaker than the influences of HLA and KIR on KIR expression and for this sample size did not reach statistical significance.

We also compared expression of CD94:NKG2A and KIR in the donor panel, independently of familial relationships. This revealed a statistically significant inverse correlation between the total frequency of cells expressing KIR and the frequency of cells expressing CD94:NKG2. This was seen either when the total frequency of KIR-expressing cells was roughly estimated by summation of the frequency of cells expressing KIR and the frequency of cells expressing CD94:NKG2 (Fig. 5E, \(r = -0.39 \) \((p < 0.01)\)) or when it was calculated from individual KIR frequencies, taking account of knowledge that KIR are expressed in random combinations (4) (Fig. 5F, \(r = -0.40 \) \((p < 0.01)\), respectively). This result demonstrates some coupling between expression of the CD94:NKG2A receptor and KIR receptors.

![FIGURE 3. KIR genes dominate HLA class I genes in determining human peripheral blood NK cell KIR repertoires. Sibling NK cell KIR repertoires were compared for 85 HLA- and KIR-typed sibling pairs. Sibling pairs are organized into four groups: KIR identical, HLA identical (A), KIR identical, HLA disparate (B), KIR disparate, HLA identical (C) and KIR disparate, HLA disparate (D). Each symbol () represents the repertoire difference between two siblings. E and F, Both contain all of the data points from A–D. E, Sibling pairs are distinguished according to KIR identity (■) or difference (○); F, they are distinguished according to HLA identity (●) or difference (○). Included in E is a linear regression trend line, calculated using all data, which shows the correlation between summed differences in frequency and surface level of KIR expression.](http://www.jimmunol.org/)

![FIGURE 4. HLA class I influences the relative frequencies of cells expressing KIR but not their levels of surface expression. Shown are KIR repertoire comparisons for the 27 KIR-identical sibling pairs. They are distinguished as HLA identical (■), HLA different but having identical HLA-B and -C KIR epitopes (□) or HLA and KIR epitope different (○).](http://www.jimmunol.org/)
Reconstitution of NK cell receptor repertoire following stem cell transplantation

Reconstitution of NK cell receptor expression was followed in 18 patients undergoing stem cell transplantation for treatment of CML or acute myelogenous leukemia. Twelve of these transplants involved HLA-matched sibling donors, five involved HLA-matched unrelated donors (MUD), and one an unrelated donor that was HLA-C disparate, but otherwise HLA matched. Consistent with random segregation of parental KIR and HLA haplotypes, 3 (25%) of the 12 (25%) sibling donor/recipient pairs were KIR identical. All of the HLA-matched unrelated donor/recipient pairs were KIR disparate.

The three donor/patient pairs with identical KIR genotypes had similar KIR repertoires (Fig. 6A), exhibiting summed differences comparable to those of healthy KIR- and HLA-identical sibling pairs (Fig. 3A). Thus, the hematologic malignancy suffered by the patients in these pairs had little effect on their KIR expression, indicating that, in general, the KIR repertoires measured in patients before transplant will reflect their healthy repertoires before the onset of malignant disease. The KIR-disparate donor/recipient pairs gave a wide range of summed differences, as did the MUD pairs (Fig. 6A), similar to those observed for healthy HLA-identical, KIR-disparate sibling pairs (Fig. 3C).

Following transplantation, patients’ peripheral blood was sampled at intervals over the course of 1 year. Posttransplant KIR repertoires were compared with those of the donor and patient before transplant. The patients could be divided into three groups based on reconstitution of KIR and CD94:NKG2A expression. For the eight patients of group 1 (five MUD, three sibling donors), the reconstituted repertoire of KIR expression was like that of the donor and distinct from that of the patient before transplant (Fig. 6, B and C). Upon initial engraftment, few peripheral blood NK cells expressed KIR, while the majority were CD94:NKG2A positive. The frequency of KIR expression gradually increased during the subsequent 6–9 mo, reaching levels comparable to those of the donor. Concomitant with increasing KIR expression, the proportion of NK cells expressing CD94:NKG2A gradually lessened. Patients in this group (group 1) had good recovery from transplantation with no major clinical complications.

Reconstitution in the five patients of group 2 (all sibling donors) was characterized by reduced frequencies of KIR-positive NK cells that persisted throughout the first year posttransplant, while CD94:NKG2A expression frequencies remained high (Fig. 6D). Although fewer NK cells expressed KIR, the hierarchy of KIR expression within the KIR-positive population resembled that of the donor. All but one of the group 2 patients experienced no major clinical complications during the first year posttransplant. A similar pattern of reconstitution was observed in patients receiving autologous stem cell transplants (Fig. 6E). Five patients (group 3) exhibited idiosyncratic patterns of NK cell receptor reconstitution (data not shown). They all suffered serious complications within the first year after transplant, including chronic GVHD, grade IV acute GVHD, and CML relapse.

Discussion

We developed a simple, robust, and quantitative method for comparing NK cell repertoires of KIR and CD94:NKG2A expression. Study of five healthy donors over a 1-year period showed that each person’s repertoire was stable and unaffected by infection or other environmental stress. This raises our previous observation of KIR3DL1 expression stability (24) to a general principal. The validity of this conclusion of individual repertoire stability is underscored by the fact that the same method of comparison showed that each donor has a different repertoire. Thus, each donor’s repertoire is genetically predetermined, a characteristic of innate defense mechanisms. Moreover, the variation from one donor to another shows that no one repertoire is optimal.

From a practical standpoint, repertoire stability meant that reliable assessment of a person’s repertoire could be based on a single blood donation. This made feasible the analysis of >100 individuals from whom correlations of NK cell repertoire difference with KIR and HLA genetics were made. By studying 85 sibling pairs from 36 different families, we were able to combine a fair sampling of the human population with valuable simplification of a complex genetics. Comparison of sibling pairs demonstrated that the KIR genotype is the dominant factor determining the repertoire
of KIR expression on NK cells, as is vividly seen from comparison of KIR-identical pairs with KIR-disparate pairs in the absence of any knowledge of HLA class I type (Fig. 3, E and F). Now evident is that the KIR genes themselves are the undefined, non-HLA genes previously implicated in controlling KIR expression (24).

In healthy individuals, the influence of the HLA genotype on NK cell KIR expression is subordinate to that of KIR, only being detectable when analysis was restricted to the subset of KIR-identical sibling pairs. Then it became clear that the KIR repertoires of KIR-identical, HLA-identical siblings were more similar than those of KIR-identical, HLA-disparate siblings. Importantly, the impact of HLA is to change the frequencies of KIR-expressing cells, not the surface levels of KIR expression. This provides good evidence that the HLA class I genotype imposes selection during development of the NK cell receptor repertoire and is consistent with functional observations showing that human NK cells express an inhibitory receptor for autologous HLA class I, though not necessarily for allogeneic HLA class I (4). Formally, it is possible that the HLA effect we observed is not due to HLA class I but to other linked genes of the HLA region. However, we consider this an unlikely possibility, given the well-established role of HLA class I polymorphisms in human NK cell receptor biology (23).

The subtlety of HLA class I selection on NK cell repertoires arises because NK cells express multiple KIR, so that selection for cells expressing inhibitory receptors specific for autologous HLA class I causes only small reductions in the relative frequencies of cells expressing other KIR. This subtlety may have contributed to the failure of previous studies to see any influence of HLA class I on the expression of HLA-B- or HLA-C-specific KIR (24, 25). In those studies, comparison of the effect of the HLA class I difference was not made in the context of KIR identity and any effect due to HLA would have been obscured by the greater effects of the KIR genotype difference. Also critical was that the earlier studies used KIR epitope motifs of HLA-B and -C molecules as measures of HLA identity; these are simplified measures of KIR ligands which, in this study, did not reveal the HLA effect in selection of the NK cell KIR repertoire. Thus, the results obtained here are compatible with and resolve the seemingly paradoxical results obtained in previous investigations. Moreover, our data suggest that the HLA effect on the KIR repertoire may be governed by complex interactions between KIR and HLA molecules; these could include allelic fine specificities for the human KIR and as yet undiscovered KIR specificities.

Clinical stem cell transplantation provides a system for examining the reconstitution of NK cell repertoires under conditions of HLA and/or KIR genetic difference. The patients we studied formed three groups, with those experiencing no major clinical complications following transplantation making up the first two. In the majority of allogeneic HLA-matched transplants, the patterns of KIR expression became like that of the donor, confirming the importance of the KIR genotype revealed in the comparison of healthy sibling pairs. In some allogeneic transplants and autologous transplants, the relative expression of the different KIR genes was like the donor, but the overall percentage of NK cells expressing KIR was reduced compared with the donor. One possibility was that CD56dim KIR+ cells were replaced by CD56bright KIR− cells (45, 46). This did not seem to be the case, because lack of KIR expression was seen in both the CD56bright and CD56dim populations. In contrast, allogeneic transplants between HLA-matched unrelated individuals tended to reconstitute NK cell populations with frequencies of KIR expression comparable to those of the donor. Thus, in the transplant situation, some degree of genetic incompatibility may facilitate induction of KIR expression. A potentially related phenomenon is that in vitro culture of human NK
cell precursors with xenogeneic mouse feeder cells facilitated induction of KIR expression (47). An alternative explanation for the distinct reconstitution patterns was the more common use of fractionated total-body irradiation in the conditioning regimens of group 1 patients (seven of eight) than group 2 patients (one of five), which may have more thoroughly ablated recipient hemopoietic cells and/or had a more damaging effect on the stromal environment.

The third group of patients was those suffering major clinical complications following transplantation. Their idiosyncratic patterns of NK cell receptor expression suggest that GVHD, early relapse, and other complications or clinical interventions can influence NK cell receptor expression. This is consistent with a previous study showing lower frequencies of KIR expression in patients with chronic GVHD compared with those without, although, in that study, the patient and donor KIR types were not taken into account (48).

Whereas HLA class I modifies the frequency of KIR-expressing cells in NK cell repertoires, shown here in healthy donors, it does not affect expression levels. This property of KIR contrasts with observations made of the Ly49 molecules of mice. Surface levels of Ly49 are lower in the presence of cognate MHC class I ligands than in their absence (49–51). The proportions of NK cells expressing various Ly49 molecules are also affected by the class I environment; the expression of a self-specific Ly49 decreases the subsequent expression of additional Ly49 receptors capable of binding autologous MHC. The results support a scheme whereby each NK cell expresses receptors stochastically until a threshold level of functional interaction with a self-MHC molecule is reached (51–55). Whether the observed differences in humans and mice reflect their biology or are due to the very different, and often complementary, approaches taken to their investigation remains to be seen.

KIR are expressed in stochastic fashion, which does not always end with expression of an inhibitory KIR with specificity for autologous HLA class I. For a proportion of NK cell clones, the CD94:NKG2A receptor fills that role. Comparison here of 104 healthy donors revealed a statistically significant inverse correlation between the proportions of NK cells expressing KIR and CD94:NKG2A. This relationship was also apparent during post-stem-cell transplant recovery of NK cell populations. Together, these data provide direct evidence for the two types of receptors sharing common purpose and for some coordination in their differential expression during NK cell development. Long-term follow-up of patients having NK cell receptor repertoires with diverse KIR specificities (group 1) vs repertoires biased toward CD94:NKG2A usage (group 2) may provide an indication of the relative importance of KIR and CD94:NKG2A to NK cell function in vivo.

That the repertoire of NK cell CD94:NKG2A expression is stable in healthy individuals but varies between them is consistent with genetic control, although data from both healthy sibling pairs and stem cell transplant patients suggests that regulation of CD94:NKG2A expression may be more complex than that of KIR. Variability in the CD94 and NKG2A genes could contribute to this effect. The evidence available shows these genes to be highly conserved in the human population, although the effect of potential allelic differences outside the coding region on CD94:NKG2A expression cannot be completely dismissed (14, 56, 57). Our study suggests that patterns of CD94:NKG2A expression are influenced by the combination of HLA and KIR genotypes, with the former having greater effect. Again, this is consistent with CD94:NKG2A and KIR sharing the function of providing inhibitory receptors for NK cells and for there being coordination in expression of the two types of genes. One explanation for the greater influence of HLA than KIR genotype on CD94:NKG2A expression is that HLA genotypes vary in the number of KIR ligands they provide, whereas the great majority of KIR genotypes provide all of the different types of inhibitory KIR with specificity for HLA class I. Consequently, the HLA class I genotype will usually dictate the number of KIR that can serve as inhibitory receptors for autologous HLA class I and thus the proportion of NK cells needing CD94:NKG2A expression to be tolerant of self. A second, and not necessarily mutually exclusive, possibility is that the HLA effect is due to polymorphism affecting either the expression or function of HLA-E, the ligand for CD94:NKG2A.

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