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The Repertoire of Killer Cell Ig-Like Receptor and CD94:NKG2A Receptors in T Cells: Clones Sharing Identical αβ TCR Rearrangement Express Highly Diverse Killer Cell Ig-Like Receptor Patterns[1]

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Killer cell Ig-like receptor (KIR) and CD94:NKG2A molecules were first defined as human NK cell receptors (NKR), but now are known to be expressed and to function on subpopulations of T cells. Here the repertoires of KIR and CD94:NKG2A expression by T cells from two donors were examined and compared with their previously defined NK cell repertoires. T cell clones generated from peripheral blood of both donors expressed multiple NKR in different combinations and used the range of receptors expressed by NK cells. In both donors αβ T cells less frequently expressed the inhibitory receptors CD94:NKG2A and KIR2DL1 than either γδ T cells or NK cells. In contrast to NK cells, not all NKR$^+$ T cells expressed an inhibitory receptor for autologous HLA class I. This lack of specific inhibitory NKR was especially apparent on αβ T cells of one donor. Overall, αβ T cells exhibited a distinct pattern of NKR expression different from that of γδ T and NK cells, which expressed highly similar NKR repertoires. In one donor, analysis of TCR rearrangement revealed a dominant subset of NKR$^+$ T cells sharing identical TCR α- and β-chains. Remarkably, among 55 T cell clones sharing the same TCRαβ rearrangement 18 different KIR phenotypes were seen, suggesting that KIR expression was initiated subsequently to TCR rearrangement. *The Journal of Immunology,* **2001, 166: 3923–3932.**

The physiological functions of NK cells appear to be regulated by arrays of activating and inhibitory receptors on the NK cell surface, some of which interact with oligomorphic determinants of autologous MHC class I molecules (1–3). In humans, NK cell receptors (NKR)[7] consist of two broad classes of membrane glycoprotein: the lectin-like receptors encoded by genes in the NK cell complex on chromosome 12 (4–6), and Ig-like receptors encoded by genes in the leukocyte receptor complex on chromosome 19 (7–9). The predominant HLA class I specificity of the NK cell complex-encoded receptors is determined by CD94:NKG2A (inhibitory) and CD94:NKG2C (activating) receptors that recognize composite ligands consisting of a peptide derived from the leader sequence of an HLA-A, -B, -C, or -G heavy chain bound to HLA-E (10–12). Among the leukocyte receptor complex-encoded receptors, certain members of the killer cell Ig-like receptor (KIR) family have specificity for HLA-A (13, 14), -B (15, 16), -C (17), or -G (18) molecules and are of either the inhibitory or the activating type. In addition, the ILT2 molecule (19), also called LIR 1 (20), has specificity for HLA class I.

Although first characterized on NK cells, the KIR and CD94:NKG2 families are also found on subpopulations of peripheral blood αβ T cells (21–23), γδ T cells (24, 25), and T cells in the liver (26). Both CD4 and CD8 αβ T cells can express NKR, although the latter are much more common (27). The NKR expressed by T cells have been shown to be functional; stimulatory signals coming from the TCR can be overridden by inhibitory signals generated through a KIR or CD94:NKG2A receptor, thereby inhibiting target cell cytolysis and cytokine release (22–24, 27–33). A common feature of NKR$^+$ T cells is a cell surface phenotype that is characteristic of memory T cells; they lack CD28 and CD45RA, mostly express CD45RO, and have high levels of CD18, CD44, CD29, and CD57 (28, 34). This has suggested that induction of NKR expression on T cells occurs only after Ag stimulation.

The overall question to be addressed here is how the repertoire of expression of the different NKR in T cells compares with that which has been described for NK cells. Previous investigations have shown that individual NK cells express a variable number of different NKR (35–37), which can be as many as nine (38). Through expression of different combinations of NKR, a substantial diversity of NKR phenotype is present within an individual's NK cell population. This diversity may help NK cell populations respond to cells infected by diverse viruses and other pathogens. A

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[7] Abbreviations used in this paper: NKR, NK cell receptors; KIR, killer-cell Ig-like receptor; CDR, complementarity-determining region.

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critical factor determining an individual's repertoire of NKR expression is the cohort of NKR genes they inherit. Whereas the CD94 and NKG2 genes appear conserved within human populations, that is not the situation for the KIR gene family. KIR haplotypes differ in the number of genes they contain, with most variation being in the number (between 1 and 5) of activating receptors (9, 39, 40). Adding to the variation is polymorphism at some of the KIR genes (41). The consequence of such genetic differences is that the repertoire of NKR expression by NK cells varies between individual donors. A second factor that influences NKR expression by NK cells is the apparent requirement for each NK cell to be inhibitable by healthy cells expressing the autologous HLA class I type. This requirement for self tolerance is met by each NK cell expressing one or more inhibitory NKR with specificity for one or more autologous HLA class I allotypes (38).

Analysis of the cell surface phenotype using Abs specific for different types of NKR has shown that T cells, like NK cells, can express more than one type of NKR (21, 23). Another form of analysis has been to examine the heterogeneity of $\alpha\beta$ TCRs within cultured lines of T cells that were selected for expression of a particular KIR. Mingari et al. (34) found such lines to be restricted to particular Vβ families. A similar restriction was seen in the NKR^+ T cell populations in the peripheral blood. However, different Vβ gene families dominated the T cells that were derived from different donors but selected for expression of the same KIR. In contrast to the results of Mingari et al., Andrea and Lanier (27) did not find as consistent a picture when they compared Vβ usage in peripheral blood T cells that did or did not express KIR3DL1. Although in some donors they found a dominance of certain Vβ families in $KIR3DL1^+$ T cells, in others the differences were more subtle, and in no donor was there evidence for monoclonality or oligoclonality of the KIR3DL1-expressing $\alpha\beta$ T cells.

A factor limiting previous studies to assess NKR expression by T cells was the reliance on Abs for detection and discrimination of NKR. The available Abs detect only a subset of KIR; some are cross-reactive with different types of KIR, and others have polymorphic specificity, so that only the allotypes expressed by certain donors are detected. A second limiting factor was incomplete knowledge of the KIR genotypes of the donors studied. This hinders assessment of whether the observed differences between donors were a consequence of KIR genetics or of functional interactions, either with the products of other host genes or with environmental factors such as pathogens. To address these issues we have taken the following two approaches: first, to focus analysis on T cells from blood donors who have been well characterized for KIR type, HLA type, and NK cell repertoire (38); and second, to supplement Ab-based assessment of NKR expression by T cells with RT-PCR typing, which can be both more specific and more comprehensive (39). Using this overall strategy we have studied the repertoire of NKR expression by T cell clones and peripheral blood T cells from two donors with distinctive KIR genotypes.

Materials and Methods

Flow cytometry

Three-color flow cytometry was performed on PBMC, stained with FITC-coupled anti-CD16 mAb, Cy-Chrome-labeled anti-CD3 mAb (both from Becton Dickinson, Mountain View, CA), and a mixture of PE-coupled NKR-specific mAbs consisting of the anti-KIR reagents EB6 (anti-KIR2DL1, anti-KIR2DS1; Coulter, Hialeah, FL), DX27 (anti-KIR2DL2/3, anti-KIR2DS2), DX9 (anti-KIR3DL1), DX31 (anti-KIR3DL2), and the CD94-specific mAb DX22. No specific Abs were available for KIR2DL4, KIR2DS3–5, and KIR3DS1. Expression of the CD94:NKG2A heterodimer was determined by calculating the percentage of cells that stained brightly with the DX22 Ab. T cell clones and PBMC were analyzed by flow cytometry using FITC-coupled mAbs specific for the TCRVβ families 3, 5, 6, 8, 11, 12, 13, 14, 16, 17, 20, 21, and 22 (Coulter). mAbs specific for the T cell markers CD3, CD4, CD8, $\delta\gamma$ TCR, and $\alpha\beta$ TCR; the NK cell markers CD16 and CD56; the T cell memory markers CD45RO and CD57; as well as CD45RA Ag were used in different fluorochrome combinations (Becton Dickinson). Cells (2×10^5) of each T cell clone and PBMC (1×10^6) were incubated for 45 min with the appropriate Abs, washed, and analyzed with a FACScan flow cytometer using CellQuest software (Becton Dickinson).

T cell cloning

PBMC were isolated from whole blood by Ficoll-Hypaque gradient separation. $CD3^+CD16^-NKR^+$ T cells as well as $CD3^+CD16^-NKR^-$ control T cells were single-cell sorted using a FACStar cell sorter and cyt-clone software and hardware (Becton Dickinson). Sorted T cells were established in culture and maintained as previously described (42). Briefly, T cell clones were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 200 U/ml rIL-2 (provided by C. Reynolds, National Cancer Institute/Biological Response Modifier Program, Frederick, MD) and 0.1 μg/ml of PHA. Mixed allogeneic PBMC (1×10^6/ml; three donors) and cells of the JY B cell line (1×10^5/ml) were irradiated and used as feeder cells at the start of the culture and subsequently at weekly intervals. Irradiated feeder cells without T cell clones (feeders-only culture) were cultured in parallel to provide a control for background proliferation.

RNA preparation and RT

Total cellular RNA was extracted from 1×10^6 cultured T cells with feeders or with feeder cells alone using RNAzol according to manufacturer's instructions (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized from 1 μg of RNA by RT using oligo(dT) (Perkin-Elmer, Norwalk, CT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a volume of 25 μl at 42°C for 1 h.

NKR typing

PCR analysis of KIR and NKG2A expression was performed as described previously (39). In brief, a panel of PCR primer pairs was used to perform specific amplification of six groups of inhibitory KIR (KIR2DL1–4 and KIR3DL1–2) and five groups of noninhibitory KIR (KIR2DS1–4 and KIR3DS1) as well as the NKG2A gene. Amplifications were performed with NKR-specific primers at a concentration of 0.5 μM in 25-μl reactions for 30 cycles using 1 μl of cDNA. Internal control primers specific for β-actin were included in each PCR at a concentration of 0.05 μM. Feeders-only cultures were used as controls for RT-PCR that monitored the presence of residual NKR expression from irradiated feeder cells.

Typing for TCR Vα and Vβ families

TCRVβ family-specific PCR was performed as described previously (43). For each of 24 Vβ families a specific sense-primer and a common antisense primer matching the TCRβ constant region were used. Similarly, Vα family-specific PCR was performed as previously described (44) using sense primers for the specific amplification of 27 Vα families in combination with a common Cα-specific antisense primer. T cell clones were amplified for 30 cycles, and PBMC were amplified for 35 cycles using 1 μl of cDNA/25 μl reaction.

Nucleotide sequencing of TCR$\alpha\beta$ rearrangements

TCRα and TCRβ rearrangements in T cell clones were characterized by direct sequencing of template obtained by TCR family-specific PCR. Due to the monoclonality of the established T cell clones, no subcloning step was necessary to obtain unambiguous complementarity-determining region 3 (CDR3) sequences. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Subsequently, products were cycle-sequenced employing dye-labeled deoxynucleotide terminators and a 373A automated sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences of TCR α- and β-chain rearrangements were determined in both directions using as sequencing primers the family-specific and constant region-specific primers used for amplification.

Results

NKR^+ subsets of $\alpha\beta$ and $\gamma\delta$T cells express a diverse repertoire of NKR combinations

T cell clones from two healthy donors were cultured from PBMC that had been isolated by single-cell sorting using CD3-specific mAbs in combination with a cocktail of KIR-specific and CD94:

NKG2A-specific mAbs. Eighty-two NKR$^+$ T cell clones from donor NV and 71 clones from donor PP were obtained. As controls, 20 T cell clones expressing no NKR were also established from NKR$^-$ T cells of each donor using the same protocol. Flow cytometric analysis showed that 68 (83%) of the NKR$^+$ T cell clones from donor NV expressed $\alpha\beta$ T cell receptors, and 14 (17%) expressed $\gamma\delta$ TCRs. In contrast, 30 (42%) of the NKR$^+$ T cell clones from donor PP expressed $\alpha\beta$ TCRs, and 41 (58%) expressed $\gamma\delta$ TCRs. The numbers of $\alpha\beta$ vs $\gamma\delta$ T cell clones obtained from each donor reflected the relative abundance of $\alpha\beta$ and $\gamma\delta$ cells in the NKR$^+$ T cell population in their peripheral blood as determined by flow cytometry. For donor NV the NKR$^+$ population of peripheral blood T cells comprised 76% $\alpha\beta$ T cells and 24% $\gamma\delta$ T cells; for donor PP it was 45% $\alpha\beta$ cells and 55% $\gamma\delta$ T cells. For both donors, the $\alpha\beta$ T cell clones obtained were all CD4$^-$CD8$^+$, while the $\gamma\delta$ T cell clones were mostly CD4$^-$CD8$^-$, with a few being CD4$^-$CD8$^+$. All NKR$^+$ T cell clones from both donors had the T cell memory phenotype, CD28$^-$CD45RA$^-$CD45RO$^+$.

RNA was isolated from each T cell clone and typed for KIR and NKG2A by RT-PCR-based methods we described previously (39). Clones derived from NKR$^+$ T cells expressed NKR, whereas those derived from NKR$^-$ T cells did not, indicating that the culture conditions we used did not induce NKR expression in T cells. The expression of NKR by T cells (Fig. 1, *A* and *B*) was compared with that previously determined for NK cell clones obtained from the same two donors (38). For each donor all of his KIR genes are used by T cells as well as NK cells. None of the KIR was restricted to one or the other cell type. As for NK cells, the differential expression of KIR and CD94:NKG2A on T cells leads to a diverse repertoire of 31 and 24 different phenotypes in the 82 and 71 clones studied from donors NV and PP, respectively. The frequency of expression for most KIR genes was similar on NKR$^+$ T cells and NK cells. The KIR2DL4 gene was expressed on every NKR$^+$ T cell and NK cell. The HLA-Bw4-specific KIR3DL1 was infrequently expressed on the NK and NKR$^+$ T cells of donor NV, but was abundant on both kinds of lymphocytes from PP. The five noninhibitory KIR of donor NV (missing tyrosine-based inhibitory motifs in the cytoplasmic chain) were expressed at comparable frequencies on NK cells and NKR$^+$ T cells, as was the only noninhibitory KIR of donor PP, KIR2DS4.

KIR2DL1 was the only KIR expressed differentially by NKR$^+$ T cells and NK cells of both donors. Although NV expresses the HLA-C ligand for KIR2DL1, while PP does not, this receptor was expressed much more frequently on NK cells than on NKR$^+$ T cells of both donors. Differences were also seen for KIR2DL2 and KIR3DL1, which were found more frequently on NKR$^+$ T cells of NV and PP, respectively. Whereas the KIR repertoire of NKR$^+$ T and NK cells was mostly similar, CD94:NKG2A was expressed less frequently on NKR$^+$ T cells than on NK cells in both donors.

The expression of NKR by the $\alpha\beta$ and $\gamma\delta$ subsets of NKR$^+$ T cell clones obtained from the same individual showed specific differences (Fig. 1, *C* and *D*). Particularly impressive was the high frequency of expression of NKG2A by $\gamma\delta$ T cell clones compared with $\alpha\beta$ T cell clones; this difference was seen for both donors, but was more pronounced in NV. In expression of CD94:NKG2A, the $\gamma\delta$ T cells resemble NK cells, and it is the $\alpha\beta$ T cells that are different. Similarly, the higher frequency of KIR2DL2 expression by NV's T cell clones and that of KIR3DL1 by PP's T cell clones are due to $\alpha\beta$ T cells, while the expression of these receptors by $\gamma\delta^+$ cells resembles that of each individual's NK cell clones. By contrast, the low frequency of KIR2DL1 expression by T cell clones from both donors is a feature that distinguishes both $\alpha\beta$ and $\gamma\delta$ T cell clones from NK cell clones.

NKR$^+$ T cell clones from NV and PP expressed multiple NKR (two to eight receptors per cell for NV and two to seven receptors per cell for PP). For both donors a majority of the T cell clones expressed either three or four NKR (Fig. 2, *A* and *B*). In comparison with the autologous NK cell clones, the T cell clones expressed slightly fewer NKR, on the average. This can be seen in the reduction by one of the range in receptor number and in the reduction of the mean number of receptors expressed per cell (3.9 for NV's T cell clones compared with 4.2 for his NK cell clones, 3.7 for PP's T cell clones compared with 4.5 for his NK cell clones). These differences between NKR$^+$ T cells and NK cells are principally due to differences in the expression of inhibitory NKR (Fig. 2, *C* and *D*) rather than noninhibitory NKR (Fig. 2, *E* and *F*).

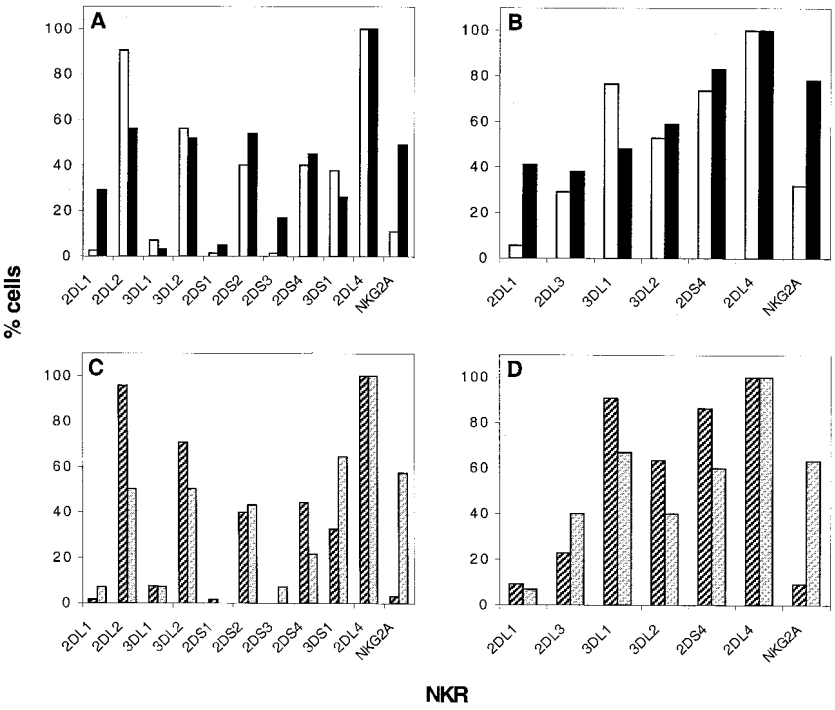

FIGURE 1. Comparisons of the frequencies with which individual KIR and NKG2A are expressed in the panels of NKR$^+$ T cell clones and NK cell clones from two healthy donors. In *A* (donor NV) and *B* (donor PP) the percentage of NKR$^+$ T cell clones (□) and NK cell clones (■) expressing each receptor are given. In *C* (donor NV) and *D* (donor PP) the percentage of NKR$^+$ $\alpha\beta$ T cells expressing each receptor (▨), and the percentage of NKR$^+$ $\gamma\delta$ T cells (▨) are shown. For donor NV, the data are from 82 T cell clones (68 $\alpha\beta$ and 14 $\gamma\delta$) and 111 NK cell clones; for donor PP, the data are from 71 T cell clones (30 $\alpha\beta$ clones and 41 $\gamma\delta$ clones) and 108 NK cell clones. Receptor expression was assessed by RT-PCR. The two donors have distinct sets of KIR genes, and the NK cell data are from the report by Valiante et al. (38).

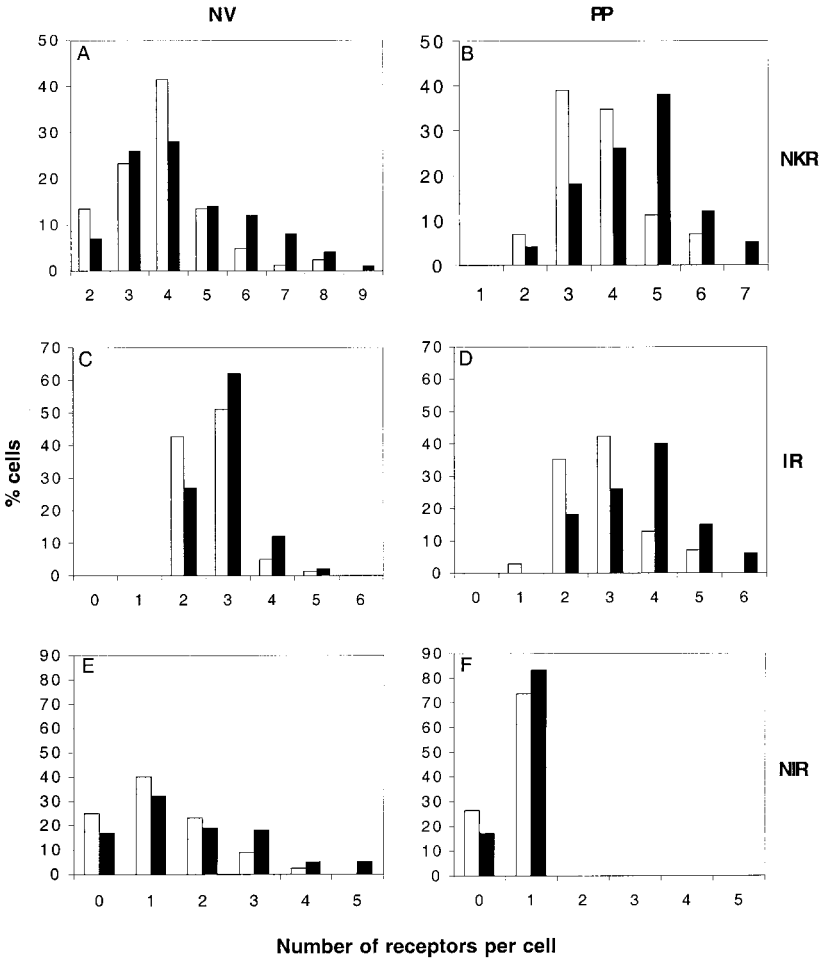

FIGURE 2. Clones of NKR$^+$ T cells express multiple NKR in different combinations. The percentage of NKR$^+$ T cell clones expressing the given numbers of receptors (□) are compared with the percentage of NK cell clones expressing those same numbers of receptors (■). In *A* (donor NV) and *B* (donor PP) all of the receptors analyzed are considered together. In *C* (donor NV) and *D* (donor PP) only inhibitory receptors (IR) are considered, while in *E* (donor NV) and *F* (donor PP) only the noninhibitory (NIR) or activating receptors are considered. A description of the clones analyzed is given in Fig. 1.

The two donors differ in their KIR types, with NV having genes for several noninhibitory KIR that are not possessed by PP. This disparity results in NV's NKR$^+$ T cells expressing up to five noninhibitory receptors, whereas PP's cells express only one (Fig. 2, *E* and *F*).

From knowledge of the NKR expressed by the NKR$^+$ T cell clones and of the HLA class I allotypes expressed by donors PP and NV, we assessed the extent to which each NKR$^+$ T cell clone expressed an inhibitory receptor that reacted with an autologous (self) HLA class I ligand (Fig. 3). For donor NV, every NKR$^+$ T cell clone expressed an inhibitory receptor specific for an autologous HLA class I allotype. By contrast, this criterion was met by only 41 of the 71 NKR$^+$ T cell clones from donor PP. Of the 30 clones that did not express a receptor for autologous HLA class I, 24 were $\alpha\beta$ T cells, and six were $\gamma\delta$ cells. Whereas 80% of PP's $\gamma\delta$ T cell clones had an inhibitory receptor, this was only true for 27% of the $\alpha\beta$ T cell clones. This difference between the two types of T cell clone can largely be attributed to the more frequent expression of CD94:NKG2A (Fig. 1, *C* and *D*). Similar differences in CD94:NKG2A expression between peripheral blood $\gamma\delta$ and $\alpha\beta$ cells were seen using flow cytometry; CD94:NKG2A was expressed by 25% (NV) and 17% (PP) of NKR$^+\alpha\beta$ T cells, and by 58% (NV) and 62% (PP) of NKR$^+\gamma\delta$ T cells (data not shown).

Cloned NKR$^+\alpha\beta$ T cells with identical $\alpha\beta$ TCR can be highly diversified for KIR expression

To examine the clonal diversity of NKR$^+$ T cells, their expression of TCR Vβ segments was analyzed by family-specific PCR. Each of the NKR$^+\alpha\beta^+$ T cell clones from donor NV expressed the Vβ16 gene segment. None of the control NKR$^-$ T cells expressed Vβ16; instead, they used various other Vβ segments (data not shown). NKR$^+\alpha\beta$ T cell clones from donor PP expressed one of three different Vβ families: Vβ11 (eight clones), Vβ14 (eight clones), and Vβ21 (six clones). Cell surface expression of the Vβ-chains assigned by PCR typing was confirmed by flow cytometric analysis of the T cell clones using Vβ family-specific mAbs (data not shown).

To determine the extent to which the restricted use of Vβ segments found in the NKR$^+$ $\alpha\beta$ T cell clones reflects the in vivo situation, PBMC from the two donors were analyzed by flow cytometry (Fig. 4*A*). For donor NV, 35% of NKR$^+$ T cells expressed Vβ16 (Fig. 4*D*), and 85% of the peripheral T cells expressing Vβ16 were NKR$^+$. Vβ16$^+$ T cells were also predominantly of CD8$^+$ type (Fig. 4*C*). The subset of peripheral T cells expressing both Vβ16 and NKR (Fig. 4*D*) was estimated to comprise approximately 4% of NVs PBMC. No other Vβ family was found on peripheral blood NKR$^+$ T cells of donor NV at such high frequency, although other Vβ families were clearly represented. Flow cytometry was also used to assess the expression of different KIR within the subset of peripheral blood NKR$^+$Vβ16$^+$ T cells, and the results were similar to those obtained by KIR typing of the cultured Vβ16$^+$ T cell clones by RT-PCR typing (Fig. 5). Thus, in pattern of NKR and TCR expression, the panel of NKR$^+$ T cell clones from donor NV represents the dominant Vβ family expressed by NKR$^+$ T cells in the peripheral blood.

For donor PP the results were less clear cut. Of the three Vβ families expressed by the NKR$^+$ T cell clones, only Vβ21 was expressed by substantial numbers (15%) of NKR$^+$ T cells in the

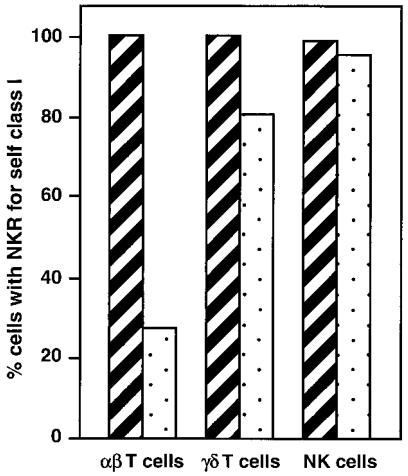

FIGURE 3. NKR$^+$ T cell clones need not express an inhibitory NKR with specificity for autologous class I. The percentages of NKR$^+$ $\alpha\beta$ T cell clones, NKR$^+$ $\gamma\delta$ T cell clones, and NK cell clones with one or more inhibitory NKR for autologous HLA class I are shown for donors NV (▨) and PP (⊡).

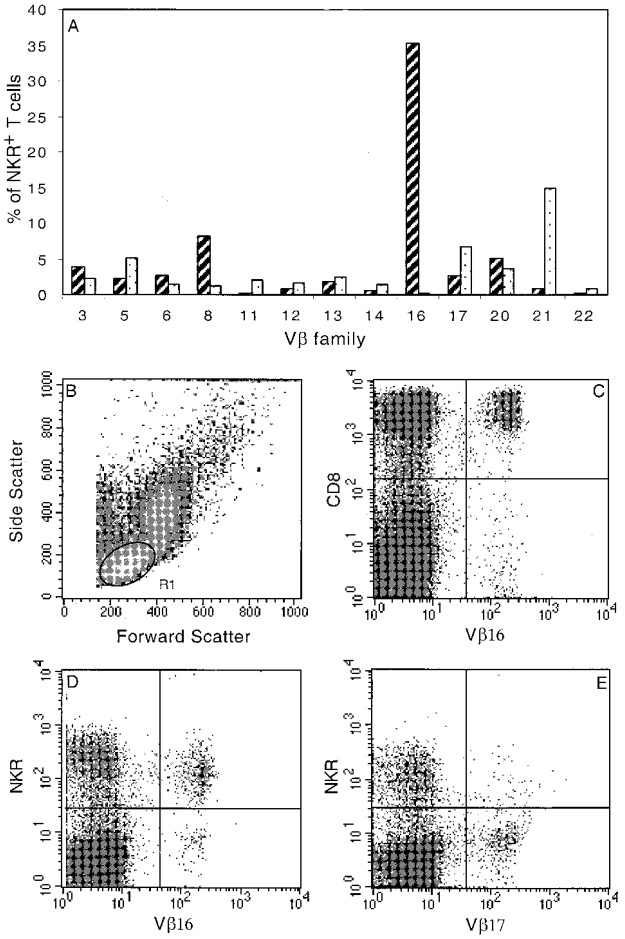

FIGURE 4. The Vβ16 family is highly expressed in donor NV's peripheral blood NKR$^+$ T cells. *A*, The relative expression of different Vβ families within peripheral blood NKR$^+$ T cells of donors NV (▨) and PP (⊡). *B–E*, Flow cytometric data from analysis of PBMC from donor NV. *B*, The population of lymphocytes selected for analysis of cell surface phenotypes. *C*, Staining with anti-Vβ16 and anti-CD8. *D*, Staining with anti-Vβ16 and anti-NKR. *E*, Staining with anti-Vβ17 and anti-NKR. Anti-NKR was a mixture of five Abs specific for different KIR or CD94:NKG2A.

peripheral blood. By contrast, the Vβ11 and Vβ14 families, which were the two Vβ families most commonly expressed by PP's NKR$^+$ T cell clones, were expressed by relatively few NKR$^+$ T cells in the peripheral blood (Fig. 4*A*).

Since NKR$^+\alpha\beta^+$ T cell clones from donor NV were strongly biased toward usage of the Vβ16 gene segment, we were interested to determine the heterogeneity of their TCR β-chain gene rearrangements. Five different Vβ rearrangements were represented in the panel of 68 $\alpha\beta$ T cell clones (Fig. 6*A*). A predominant rearrangement (II) was common to 55 clones, while four less frequent rearrangements (IV, V, I, and III) were represented by one, two, three, and seven clones, respectively. All five gene rearrangements led to shared characteristic features in the CDR3 region of the TCR Vβ-chain. Aspartic acid followed by arginine was found in all five rearrangements at positions 97–98 encoded by the D gene-segment and its flanking N nucleotides. This motif is determined either by the D1 germline segment (probably rearrangements II, III, and IV) or by N nucleotide addition (rearrangements I and V). Moreover, three of the five rearrangements share the same J element, and they differ from each other by no more than two amino acids in the CDR3 region. In the clones expressing rearrangement II, the Vβ19 gene segment was also rearranged and expressed at the mRNA level (data not shown). However, its nucleotide sequence showed this to be an unproductive rearrangement, resulting in a stop codon in the CDR3 region, consistent with the assignment of Vβ19 as a pseudogene (45). Thus, in clones with rearrangement II, Vβ16 is the only functionally rearranged TCR-Vβ gene segment.

To define the complete TCR $\alpha\beta$ clonotype for each NKR$^+$ T cell clone in the NV panel, their Vα gene rearrangements were determined. Among the 66 NKR$^+$ T cell clones having Vβ rearrangement I, II, III, or IV, different Vα gene rearrangements were found in combination with each Vβ rearrangement, and all clones with identical Vβ rearrangements had identical Vα rearrangements. Clones with the type I, II, or III Vβ16 rearrangement expressed two different α-chains. Thus, a total of seven different α rearrangements were expressed by the 66 T cell clones, and these involved five different Vα segments and five different Jα segments (Fig. 6*B*). The seven Vα rearrangements had no shared characteristics, contrasting with their Vβ counterparts. Even in cases where one Vα segment was held in common among cells having the same Vβ rearrangement, they differed substantially in their Vα CDR3 region and were also joined to different Jα segments. All seven rearrangements are in-frame and could give rise to full-length α-chains. Not known is whether any of the T cell clones of clonotype I, II, or III expresses both the α-chains at their cell surfaces, since their simultaneous cell surface expression could not be analyzed with the Abs available.

Finding that only four different $\alpha\beta$ TCR clonotypes were represented in 66 NKR$^+$ $\alpha\beta$ T cell clones provided an unprecedented opportunity to assess the extent to which NKR expression distinguished clones of identical clonotype. Typing was performed by both RT-PCR and flow cytometry, and both techniques gave concordant results (Fig. 7). Whereas only the single $\alpha\beta$ T cell of clonotype IV was distinguished by expression of NKG2A:CD94, there were many differences in the expression of KIR (Fig. 8). Most informative was the analysis of clonotype II that accounted for 55 NKR$^+\alpha\beta$ T cell clones from donor NV. Among these cells with identical TCR were 18 different KIR phenotypes that varied in frequency and number of KIR expressed. All the clones expressed KIR2DL2 and KIR2DL4, and diversity was mediated by the differential expression and combination of seven other KIR. The frequency of expression of these KIR was in the order

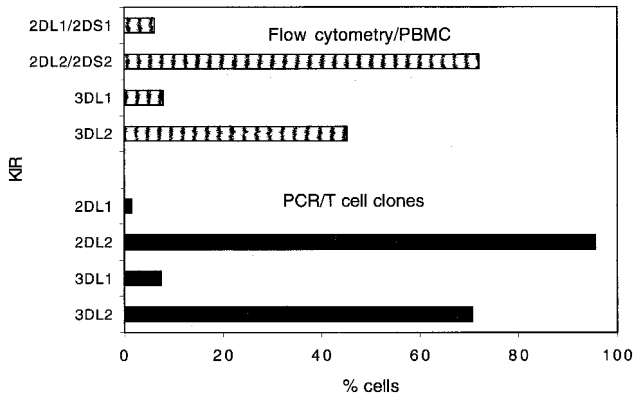

FIGURE 5. Peripheral blood Vβ16$^+$ T cells and cloned NKR$^+$ T cells from donor NV have similar patterns of KIR expression. The frequencies of KIR expression determined by three-color flow cytometry of PBMC are compared with the results obtained from RT-PCR typing of the panel of NKR$^+$ T cell clones. For flow cytometric analyses the percentages of cells expressing KIR were calculated relative to the total Vβ16$^+$ T cell subpopulation. The KIR-specific mAbs used were EB6 (anti-KIR2DL1), DX27 (anti-KIR2DL2/3 and anti-KIR2DS2), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2).

KIR3DL2>2DS4>2DS2>3DS1>3DL1>2DL1~2DS1. KIR-mediated diversification of T cell clones with clonotypes I, II, and V was also seen (Fig. 8), although to a lesser extent because of the fewer numbers of clones analyzed.

Discussion

Previous investigation has shown that CD94:NKG2A and KIR receptors (NKR) are expressed on subpopulations of human peripheral blood T cells with cell surface phenotypes that are characteristic of memory T cells. Signals generated through HLA class I ligands binding to NKR can influence the effects of Ag engagement through the TCR, a modulatory activity that has been likened to T cell costimulation. The results described here demonstrate that T cells can express diverse combinations of NKR involving as many as eight different receptors. More specifically, we have characterized T cell clones from one donor that have identical an $\alpha\beta$ TCR clonotype, but different KIR phenotypes. Particularly striking are the 55 clones of clonotype II that divide into 18 distinct groups on the basis of differential expression of five KIR genes. These data reveal the considerable potential for NKR expression to modulate the functional activities of T cells.

We previously showed that mature NK cells express at least one inhibitory KIR or CD94:NKG2A receptor with specificity for self class I (38). This restriction in the NKR repertoire is in accord with the missing self model and ensures self tolerance of the peripheral NK cell compartment (46). A similar restriction was not apparent in our panel of NKR$^+$ T cell clones. Whereas NV's T cell clones all express an inhibitory receptor for autologous HLA class I determinants, this is only true for some 60% of PP's clones. This suggests either that there is no requirement for T cells to express an inhibitory NKR for autologous class I or that additional receptors that we have not considered here are fulfilling that role.

ILT2, also called LIR1, is another type of inhibitory receptor with Ig-like domains that is distinct from KIR (19, 20). ILT2/LIR1 is specific for the viral homologue UL18, but exhibits also weak binding to relatively nonpolymorphic sites on HLA class I molecules (47). In peripheral blood of both donors NV and PP, expression of ILT2/LIR1 and KIR was found on distinct but overlapping T cell subsets. Similarly, ILT2/LIR1 expression was found on a substantial fraction of NKR$^+$ T cell clones by flow cytometry. However, in studies with a soluble LIR1 fusion protein, LIR1 was found to bind to one of NV's HLA-B allotypes, but none of PP's class I allotypes (data not shown). It therefore seems unlikely that those NKR$^+$ T cells in PP that lack KIR and CD94:NKG2A receptors for autologous class I allotypes are functionally inhibited by ILT2/LIR1. On the other hand, the existence of mature NKR$^+$ T cells without self-specific inhibitory NKR should not pose a principal problem to the host. In contrast to NK cells that rely on engagement of their inhibitory receptors by self class I epitopes to be self tolerant, T cells are already selected for self tolerance by TCR-driven processes in the thymus.

Although the differences in NKR expression by the two donors are many, within each individual the expression of NKR by NK cell clones and that by T cell clones from each individual are largely similar, but with a few potentially important differences. The biggest difference is the reduced frequency with which NKR$^+$ T cells express CD94:NKG2A compared with NK cells, which approaches zero for NKR$^+$ $\alpha\beta$ T cells. It is this difference that largely accounts for the absence of an inhibitory receptor for autologous HLA class I on 40% of PP's NKR$^+$ T cells. Also common to both donors was a reduced frequency of KIR2DL1, the inhibitory receptor for HLA-C allotypes with the N77, K80 amino acid sequence motif. Although for NV the frequency of KIR2DL2 expression by $\alpha\beta$ T cells was higher than that for either NK cells or $\gamma\delta$ T cells, this may not be a general property because of the limited number of T cell clonotypes represented in the clones and their ubiquitous expression of KIR2DL2. Overall, the similarities in frequency with which KIR are expressed by NK cells and NKR$^+$ T cells indicate that the mechanisms that diversify the expression of KIR in populations of these cells are very similar.

Much of the analysis we report was made upon NKR$^+$ T cell clones that were stimulated and cultured in vitro, and in this regard T cells expressing NKRs are more difficult to isolate than NK cells because of their lack of a generic marker. The extent to which the clones reflect the in vivo populations is therefore of importance; to address this question we have, wherever possible, compared the clones with the NKR$^+$ T cells in peripheral blood. Such comparison shows that clones we have isolated and analyzed do not provide complete representation of the peripheral blood NKR$^+$ T cells of either donor. One possible source of bias is that cells expressing certain combinations of NKR were not captured by the Ab cocktail used in isolating NKR$^+$ T cells. This cocktail did not include Abs that bind to either KIR2DL4 or the recently described KIR2DL5 (9, 48), and the weak affinity of the CD94-specific Ab for the CD94:NKG2C heterodimer may have meant that this Ab was inefficient in capturing cells via interaction with CD94:NKG2C. A second potential source of bias is that the in vitro cloning procedure did not equally promote the growth of all cells isolated with the Ab cocktail.

For donor NV, one-third of the peripheral blood NKR$^+$ T cells and all 68 of the $\alpha\beta$ T cell clones express Vβ16. Clearly, the in vitro culture was strongly biased toward furthering the growth and survival of Vβ16-expressing cells; however, this selection involved the dominant Vβ family of the peripheral blood NKR$^+$ T cells, and the data collected from these cells are therefore likely to have physiological relevance to this particular donor's history of immune response. The dominance of Vβ16 NKR$^+$ T cells of the type seen in donor NV is a phenomenon that may not have generality, being seen in just a fraction of the human population, or may even be unique to this donor. Indeed, the high diversity and polymorphism of KIR genotype and the low frequency of unrelated individuals with identical KIR genes provide a genetic context in which individuality in the patterns of NKR expression by human T cells could actually be the common theme (39, 40).

A

No	Rearrangement	n	BV16	N1-DB-N2	BJ	BC
I	BV16S1J1S1BC1	3	A S S H GCC AGC AGC CA	D R L C GAC AGA CTA	E A F F G Q G G T R L T V V GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA G	E D L N AG GAC CTG AAC
II	BV16S1J2S7BC2	52	A S S Q GCC AGC AGC CAA	D R F GAC AGG TT	Y E Q Y F G P G T R L T V T C TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	E D L K AG GAC CTG AAA
III	BV16S1J2S7BC2	7	A S S GCC AGC AGC C	P D R CG GAC AGG A	T Y E Q Y F G P G T R L T V T CC TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	E D L K AG GAC CTG AAA
IV	BV16S1J2S7BC2	1	A S S Q GCC AGC AGC CAA	D R A GAC AGG GCG	Y E Q Y F G P G T R L T V T TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	E D L K AG GAC CTG AAA
V	BV16S1J2S1BC2	2	A S S Q GCC AGC AGC CAA	D R V L GAT CGG GTA CT	Q F F G P G T R L T V L G CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA G	E D L K AG GAC CTG AAA

B

No	Rearrangement	AV	N	AJ	AC
I	AV8S6J37AC1	C A V S TGT GCT GTG AGT G	V S T TG TCT AC	N T G K L I F G Q G T T L Q V K P C AAC ACA GGC AAA CTA ATC TTT GGG CAA GGG ACA ACT TTA CAA GTA AAA CCA G	D I Q N AT ATC CAG AAC
	ADV23S1J34AC1	C A A TGT GCA GCA A	K AA G	D S G Y A L N F G K G T S L L V T P AT TCC GGG TAT GCA CTC AAC TTC GGC AAA GGC ACC TCG CTG TTG GTC ACA CCC C	H I Q N AT ATC CAG AAC
II	AV16S1J23AC1	A L GCT CT	I M T ATC AT	I Y N Q G G K L I F G Q G T E L S V K P G ATT TAT AAC CAG GGA AAG CTT ATC TTC GGA CAG GGA ACG GAG TTA TCT GTG AAA CCC A	N I Q N AT ATC CAG AAC
	ADV23S1J34AC1	C A A TGT GCA GCA A	T R L R CC CGT CTA AGG G	D N T D K L I F G T G T R L Q V F P AT AAC ACC GAC AAG CTC ATC TTT GGG ACT GGG ACC AGA TTA CAA GTC TTT CCA A	N I Q N AT ATC CAG AAC
III	ADV14S1J37AC1	C A M R E G TGT GCA ATG AGA GAG GG	S C TCT A	S N T G K L I F G Q G T T L Q V K P GC AAC ACA GGC AAA CTA ATC TTT GGG CAA GGG ACA ACT TTA CAA GTA AAA CCA G	D I Q N AT ATC CAG AAC
	AV19S1J20AC1	C A TGT GC	Q A T R C CAG GCC ACA CG	L S F G A G T T V T V R A G CTC AGC TTT GGA GCC GGA ACC ACA GTA ACT GTA AGA GCA A	N I Q N AT ATC CAG AAC
IV	AV19S1J34AC1	C A L S E A TGT GCT CTG AGT GAG GC	G G	D N T D K L I F G T G T R L Q V F P AT AAC ACC GAC AAG CTC ATC TTT GGG ACT GGG ACC AGA TTA CAA GTC TTT CCA A	N I Q N AT ATC CAG AAC
V	n.d.				

FIGURE 6. Five combinations of Vβ and Vα rearrangements (clonotypes) account for the TCRs expressed by the 68 NKR$^+$ $\alpha\beta$ T cell clones from donor NV. Nucleotide and amino acid sequences encompassing the recombinatorial regions of the TCR Vβ-chain (*A*) and Vα-chains (*B*) of NKR$^+$ $\alpha\beta$ T cell clones from donor NV are shown. The five clonotypes are designated I, II, III, IV, and V. The α- and β-chain rearrangements given the same Roman numeral are expressed together. Conserved nucleotides at positions 97–98 of the CDR3 region of TCRVβ are indicated in bold. The borders of the V and J segments were based on published germline sequences (56, 57). TCR rearrangements were named using World Health Organization-International Union of Immunological Societies nomenclature (58). The Vα rearrangement of clonotype V could not be determined unequivocally because of background amplification of Vα rearrangements from feeder cells.

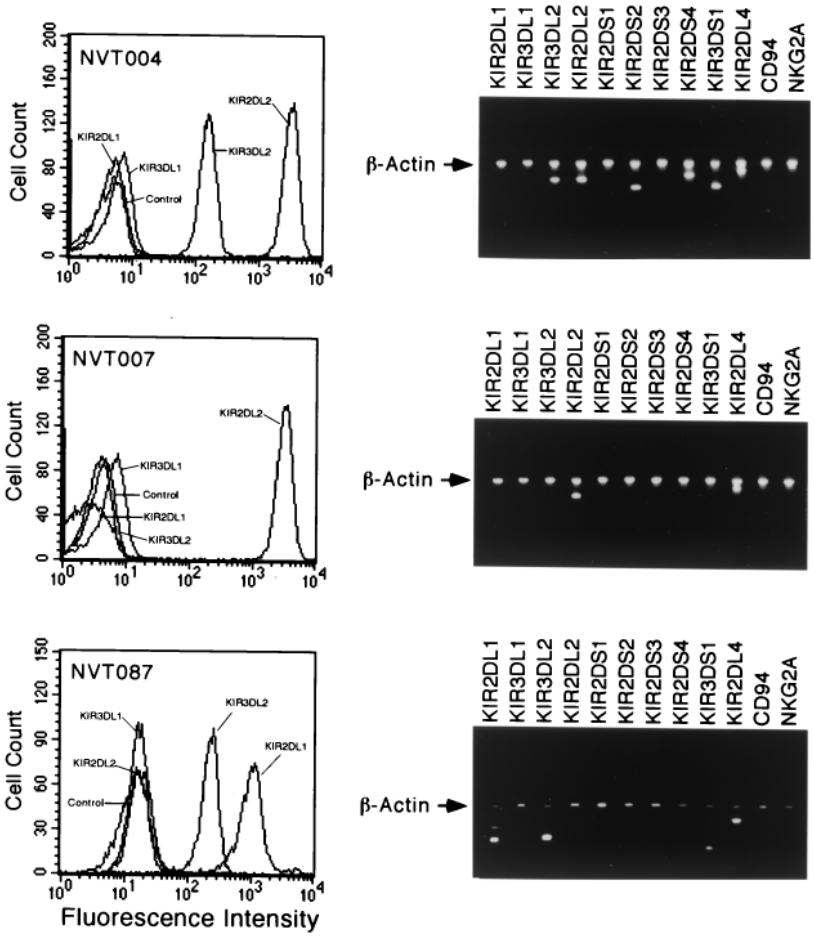

FIGURE 7. NKR gene expression in NKR$^+$ T cell clones with identical $\alpha\beta$ TCR correlates with NKR cell surface phenotype. Three representative NKR$^+$ T cell clones (NVT004, NVT007, and NVT087) with $\alpha\beta$ TCR of clonotype II were typed for expression of NKR by RT-PCR (*right panels*) and for cell surface expression of NKR as determined by flow cytometric analysis (*left panels*) using mAbs specific for KIR2DL1 and KIR2DS1 (EB6), KIR2DL2/3 and KIR2DS2 (DX27), KIR3DL1 (DX9), and KIR3DL2 (DX31).

In donor NV, five $\alpha\beta$ TCR clonotypes account for the 68 clones, and for all four clonotypes that were represented by more than one clone, there is diversification due to differential KIR expression. That different combinations of KIR are expressed by T cells with the same TCR strongly indicates that KIR expression was turned on in mature T cells that had already undergone TCR gene rearrangement and thymic selection. This model is consistent with the memory phenotype of Vβ16$^+$NKR$^+$ T cells in peripheral blood. The alternative model, that KIR expression preceded TCR gene rearrangement, is not consistent with the memory phenotype and is inherently unlikely because it requires the occurrence of 18 independent, but identical, sets of TCR α and β gene rearrangements in immature T cells with different KIR phenotypes.

It is possible that KIR-mediated diversification of T cells with identical clonotype occurred during in vitro culture. It was recently reported that murine CD8 T cells acquire Ly-49 receptors upon in vitro culture with IL-2, IL-4, or IL-15 (49). For several reasons we consider an analogous induction of KIR genes during the cloning procedure unlikely. First none of the control clones cultured from NKR$^-$ T cells expressed KIR at any stage during culture. Second stimulation of CMV-specific NKR$^-$ T cells with stimulatory cytokines and cognate peptide did not result in acquisition of KIR. Many other deliberate attempts to induce KIR expression in T cells in vitro have failed, although Mingari et al. have reported the induction of CD94:NKG2A (50). Recently, down-regulation of KIR was reported on NKR$^+$ T cell clones that were deprived of specific Ag (51). Since we do not know the Ag specificity of our NKR$^+$ T cell clones, we were unable to perform a similar experiment with our T cell clones. However, analysis of KIR expression levels over time did not reveal any signs of KIR gene down-regulation during culture. Therefore, it is likely that the diverse KIR phenotypes seen on single-cell sorted T cells with identical TCR are not a consequence of in vitro changes, but represent changes in the NKR expression status that occurred in vivo.

The memory phenotype of all NKR$^+$ T cells in peripheral blood implies that induction of NKR expression occurs only after T cells have been stimulated by Ag, a model supported by the absence of KIR on T cells in fetal thymus and cord blood (27). That all $\alpha\beta$ T cell clones from donor NV comprise five clonotypes using Vβ16 chains with common residues in their CDR3 loops is also evidence for clonal expansion driven by some form of Ag. Vβ16 was shown to dominate the clonal expansions of CD8$^+$CD57$^+$ T cells that occurred in four patients following transplantation of allogeneic bone marrow from an HLA-identical sibling (52). Steinle et al. showed that an HLA-B35-specific alloreactive T cell expressed a similar type of Vβ16 chain (53). Such associations raise the possibility that T cells bearing such Vβ16-containing receptors could have specificity for alloantigens. In healthy donors expansions of such cells might arise as a result of blood transfusion or pregnancy. Alternatively, some types of superantigen or specific Ag could be responsible for the expansion of Vβ16-expressing cells, and these might be associated with infections that either occur or are reactivated following transplantation. In this context it is important to note that the culture system we used to generate T cell clones could have favored the growth of NKR$^+$ T cells with allospecificity, because the feeder cells were pooled PBMC from allogeneic donors. Selection by alloantigens could also explain why a majority of the $\alpha\beta$ T cell clones from donor PP expressed Vβ families that were represented at relatively low frequency in the NKR$^+$T cell population of the peripheral blood.

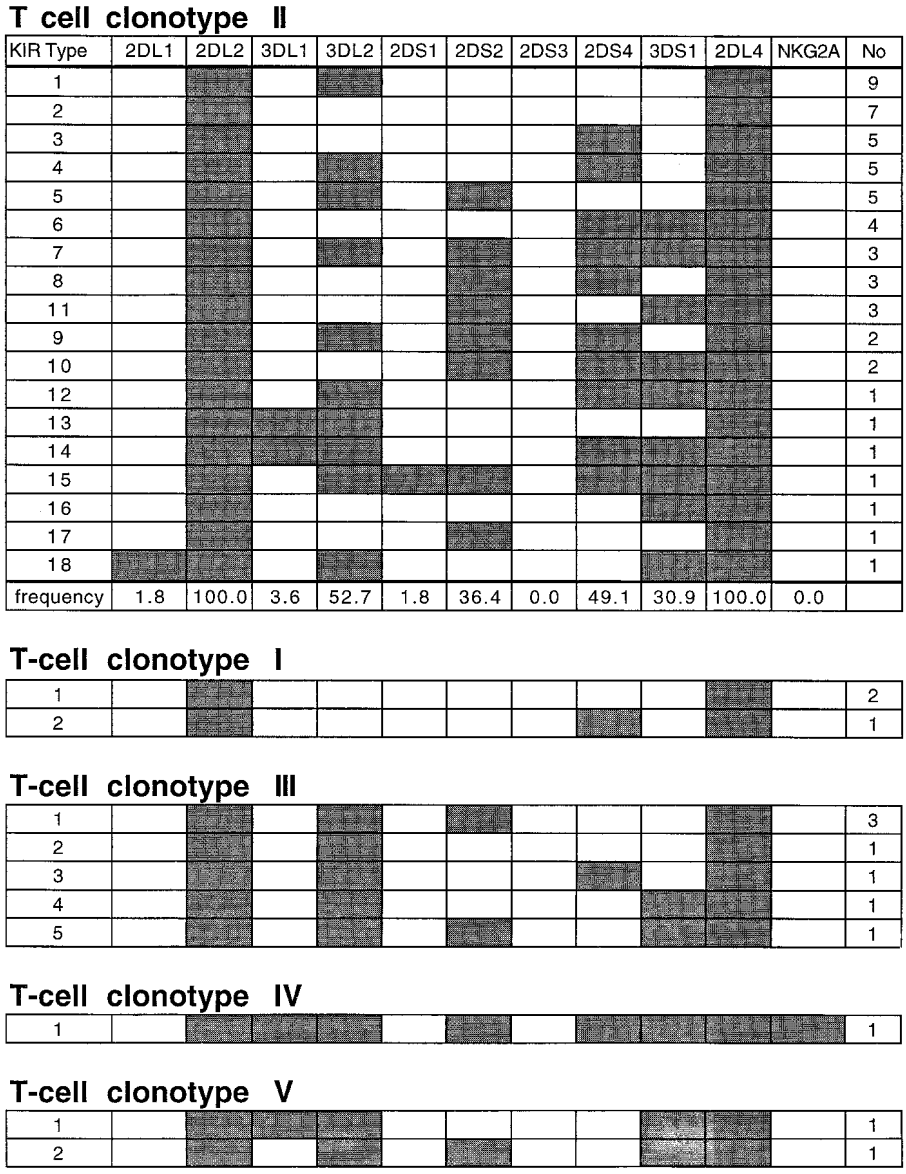

FIGURE 8. NKR$^+$ T cell clones with an identical TCR $\alpha\beta$ clonotype can be highly diversified for KIR expression. The results of typing the 68 NKR$^+$ $\alpha\beta$ T cell clones from donor NV for TCR α- and β-chains and for NKR expression are summarized. Expression of a particular NKR is indicated by stippled shading. The number of T cell clones sharing a particular NKR expression pattern is indicated in the *right column*.

The results of this study demonstrate that individual NKR$^+$ $\alpha\beta$ and $\gamma\delta$ T cells express variable combinations of receptors that collectively have a complexity approaching that of NK cells. This complexity can also introduce considerable heterogeneity within the populations of memory-type T cells that constitute a clone as defined by the TCR. The functional consequences of differential KIR expression on clones of NKR$^+$ T cells in the course of an immune response are largely unknown. Redirected lysis experiments show that both types of inhibitory NKR, KIR and CD94: NKG2A, are functional in the analyzed T cell clones (data not shown). Other studies have shown that inhibitory signaling through NKR can affect TCR-mediated functions, and in the case of a melanoma-specific response this property has the deleterious effect of preventing T cell-mediated killing of tumor cells (31). Very little is known about the role of noninhibitory NKR on T cells. It was recently shown that the adapter molecule DAP12, also known as KARAP, is involved in the activation of NK cells through noninhibitory KIR (54, 55). RT-PCR analyses show that a fraction of approximately 20% of donor NVs NKR$^+$ T cells expresses DAP12. Differential expression of DAP12 was also seen within T cells with identical KIR phenotype and TCR (data not shown). If DAP12 is essential for signal transduction through noninhibitory KIR, its differential expression would introduce another level of heterogeneity into clones of NKR$^+$ T cells. In summary, the present study shows that expression of inhibitory and noninhibitory KIR in a clone of T cells produces 18 different KIR phenotypes, each of which involves two to seven different receptors that individually or in combination have the potential to modify T cell responses to Ag.

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