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Human CD8$^+$ T lymphocyte subsets that express HLA class I-specific inhibitory receptors represent oligoclonally or monoclonally expanded cell populations

(natural killer cell receptors/class I major histocompatibility complex/cytolytic T lymphocytes/monoclonal T cell expansions)

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ABSTRACT **A small percentage of human T lymphocytes, predominantly CD8$^+$ T cells, express receptors for HLA class I molecules of natural killer type (NK-R) that are inhibitory for T-cell antigen receptor (TCR)-mediated functions. In the present study, it is demonstrated that the various NK-R molecules typically expressed by NK cells are also expressed on periheral blood T lymphocytes. These CD3$^+$ NK-R$^+$ cells have a cell surface phenotype typical of memory cells as indicated by the expression of CD45RO and CD29 and by the lack of CD28 and CD45RA. Furthermore, by the combined use of anti-TCR Vβ-specific antibodies and a semiquantitative polymerase chain reaction assay, the TCR repertoire in this CD3$^+$ NK-R$^+$ cell subset was found to be skewed; in fact, one or two Vβ families were largely represented, and most of the other Vβs were barely detected. In addition, analysis of recombinant clones of the largely represented Vβ families demonstrated that these Vβs were oligoclonally or monoclonally expanded.**

Recent studies in humans and mice have elucidated the molecular mechanisms that explain why natural killer (NK) cells selectively lyse certain virus-infected or tumor cells but spare normal autologous cells (1–3). NK cells express several specialized receptors (NK-Rs) that recognize class I major histocompatibility complex (MHC) molecules on the surface of normal cells. The lack of expression of one or more class I MHC alleles on target cells (as may occur in virus-infected or tumor cells) leads to NK-mediated target cell lysis (4, 5). Different NK-Rs, specific for groups of HLA-C (p58.1 and p58.2), HLA-B (p70 and CD94), or HLA-A (p140) alleles, have been identified (5–8). In most instances, they function as inhibitory receptors, thus preventing the NK-mediated lysis of cells that express the relevant HLA class I allele(s) (9, 10). All NK cells express at least one type of inhibitory NK-R for a given self HLA class I allele (5, 11). Recently, we and others (12–14) have shown that also a minor subset of human T cells (mostly CD8$^+$) express NK-R for HLA class I molecules. Furthermore, these NK-R were found to exert inhibitory activity on T-cell antigen receptor (TCR)-mediated T cell functions (13, 14). The finding that NK-R could affect the antigen-dependent pathway of T cell activation posed a number of questions as to the phenotype and functional properties of the NK-R$^+$ T cell subset.

In the present study, we demonstrate that the CD8$^+$ NK-R$^+$ T cell populations display a surface phenotype typical of memory cells. Analysis of the TCR Vβ repertoire revealed that only one or two Vβ families were largely expanded in the CD3$^+$ NK-R$^+$ cell populations isolated from a given individual. Furthermore, cloning and sequencing of the expanded Vβ families demonstrated that these expansions were oligoclonal or monoclonal in nature.

MATERIALS AND METHODS

Antibodies and Reagents. mAbs GL183 (IgG1 anti-P58.2), Y249 (IgM anti-P58.2), EB6 (IgG1 anti-P58.1), XA141 (IgM anti-P58.1), XA185 (IgG1 anti-CD94), KD1 (IgG2a anti-CD16), HP26 (IgG2a anti-CD4), B9.4 (IgG2b anti-CD8) (7), Q66 (IgM anti-P140) (8), JT3a (IgG2a anti-CD3), and JTi4 (IgG2b anti-Vβ8) (15) were produced in our laboratory. mAbs anti-Vβ2 (IgM), anti-Vβ16 (IgG1), and anti-Vβ17.1 (IgG1) were kindly provided by Dr. F. Romagnè (Immunotech, Marseille). UCHL-1-FITC (IgG2a anti-CD45RO) was purchased from Dako, and 2H4 CD45RA-FITC (IgG1) was purchased from Coulter. Anti-CD28 mAb (IgG1) was a gift by Dr. D. Olive (Institut National de la Santé et de la Recherche Médicale U119, Marseille). CK248 (IgM anti-CD28) (16) and JT90 (IgG2a anti-CD18) (17) mAbs were produced in our laboratory. 7E3 (IgG1 anti-CD29) was kindly provided by Dr. L. Zardi (Istituto Scientifico per lo studio e la cura dei Tumori, Genova). Leu-12 mAb (IgG1 anti-CD19) was purchased from Becton Dickinson. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-isotype goat anti-mouse (GAM) were purchased from Southern Biotechnology Associates. The culture medium was RPMI medium 1640 (Seromed, Berlin) supplemented with 10% fetal calf serum (Boehringer Mannheim), 1% glutamine (GIBCO), and 1% antibiotic mixture. Recombinant interleukin 2 was kindly provided by Cetus. Ficoll/Hypaque was purchased from Sigma; phytohemagglutinin (PHA) was purchased from GIBCO.

Isolation and Cloning of T Lymphocytes. Peripheral blood lymphocytes were isolated from blood of several normal donors by Ficoll/Hypaque density gradient centrifugation. Cells were plated under limiting numbers in round bottom microtiter plates in the presence of irradiated feeder cells, 0.5% PHA (vol/vol), and 200 units/ml of recombinant interleukin 2 as previously described (18). In some T-cell cloning experiments, peripheral blood lymphocytes were depleted of CD4$^+$ T cells by negative selection or were enriched in NK-R$^+$ cells by positive selection using appropriate mAbs and magnetic beads (Dynal, Oslo) coated with IgG anti-mouse antibody.

Immunofluorescence Analysis. The techniques used have been described in detail previously (16). Briefly, aliquots of 10^5 cells were stained with the corresponding mAb followed by

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Abbreviations: TCR, T-cell antigen receptor; NK, natural killer; NK-R, NK receptors; MHC, major histocompatibility complex; PHA, phytohemagglutinin A; FITC, fluorescein isothiocyanate.

either FITC- or PE-conjugated anti-isotype specific GAM antiserum (Southern Biotechnology Associates). Control aliquots were stained with the fluorescent reagent alone or with unrelated mAb. Samples were analyzed on a flow cytometer (FACsort, Becton Dickinson) equipped with an argon ion laser exciting FITC and PE at 488 nm. Results are expressed as log fluorescence intensity. In some experiments, we analyzed cell suspensions enriched in T lymphocytes by removal of $CD16^+$ and $CD19^+$ cells using magnetic beads.

Analysis of the TCR Vβ Repertoire. The TCR Vβ repertoire in the *in vitro* expanded $CD3^+$ $NK\text{-}R^+$ cell lines was analyzed by the use of anti-Vβ-specific mAbs and/or by a semiquantitative PCR assay as described previously (19–21). Briefly, 2 μg of total RNA, extracted by the RNAzol method (22), were reverse transcribed using random hexamers (20 μg ml; Promega), avian myeloblastosis virus reverse transcriptase (60 units; Life Science, St. Petersburg, FL) and each dNTP (Boehringer Mannheim). PCR analysis for the different Vβs used a 5′-specific Vβ primer and common 3′ constant-domain Cβ primer. 5′ and 3′ Cα primers were included in each PCR reaction as internal controls. To monitor the migration of Vβ and Cα bands, 3′ Cβ and 3′ Cα primers were radiolabeled with [γ-^{32}P]ATP (Amersham). Primer sequences of Vβ-specific oligonucleotides and of the control Cβ and Cα oligonucleotides have been reported (19). One aliquot of each PCR reaction was loaded and separated on 10% polyacrylamide gels containing 7 M urea. Gels were exposed overnight on Kodak storable phosphor screens, and the radioactive signal for each Vβ was quantified using a PhosphorImager (Molecular Dynamics).

cDNA Synthesis and Nucleotide Sequences of TCR Vβ. With regard to the cloning and sequencing of the different Vβ families, total RNA was extracted from the *in vitro* expanded cell line of donor K.K. cDNA synthesis was performed as described above. To enrich for the different Vβ families to be analyzed, total cDNA was amplified for 50 cycles (50 sec at 94°C; 30 sec at 55°C; 1 min at 72°C) in a GeneAmp PCR system 9600 (Perkin–Elmer). The sequences of the Vβ primers used and of a common 3′Cβ F have been previously reported (19). cDNA was purifed from low melting point agarose gel using a Gene Clean kit (Bio 101) and was ligated into TA cloning vector PCKII (Invitrogen) and transformed in DH5-α cells. Nucleotide sequences of the recombinant clones were performed using an Applied Biosystems model 377 sequencer following manufacturer's suggested protocols. For the Vβ genes, sequences were aligned to the published sequences Vβ17.1 (23) and Vβ16.1 (24).

RESULTS

Expression of Different HLA Class I-Specific NK-R in Human T Lymphocytes. More than 30 normal donors were studied for the presence of T cells that expressed at least one of the several NK-R that have been identified thus far. Double fluorescence and FACS analysis revealed that $CD3^+$ $NK\text{-}R^+$ T cells were present in peripheral blood of virtually all donors, ranging in proportion among different donors from <1% to 27% (mean 5.1 ± 0.74 SEM, as calculated on the most represented NK-R type in a given donor). Fig. 1*A–E* shows the expression of p58.1, p58.2, p70, p140, and CD94 in different donors. In most cases, $CD3^+$ $NK\text{-}R^+$ cells were $CD8^+$ (data not shown). Frequently, T lymphocytes from a single individual expressed more than one NK-R type. This could be readily

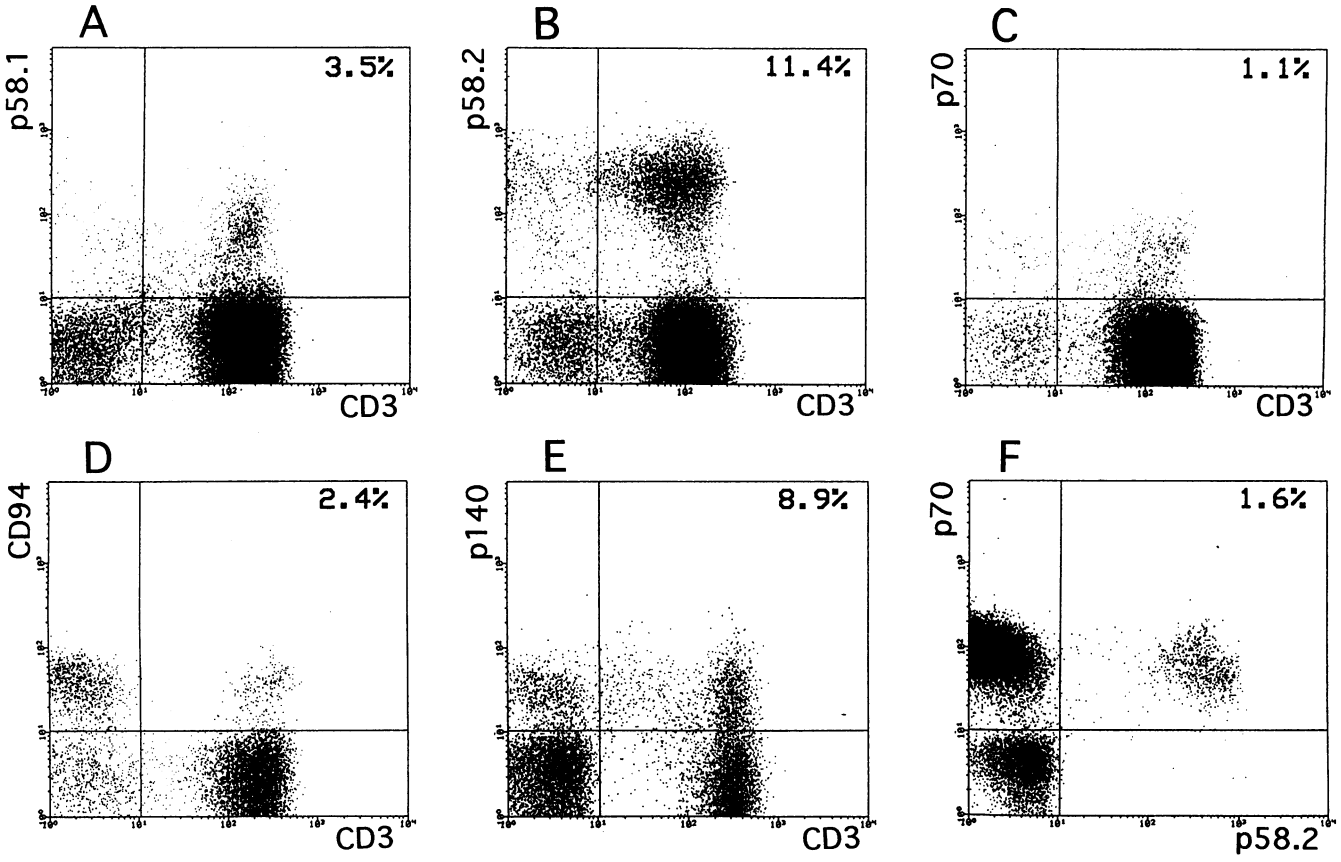

FIG. 1. Expression of different HLA class I-specific NK-R by human T lymphocytes. Lymphocytes were isolated from peripheral blood of different normal donors, depleted of adherent cells, and analyzed for the coexpression of CD3 antigen and of different NK-R including p58.1, p58.2, p70, p140, and CD94. (*A–E*) T cells were enriched by removal of $CD16^+$ (NK) and $CD19^+$ (B) cells. The coexpression of two different NK-R (p70 and p58.2) was analyzed in a $CD3^+$ $p70^+$ enriched cell population stimulated with PHA and cultured for 10 days in interleukin 2 (*F*). It is evident that a fraction of $p70^+$ coexpress p58.2 molecules.

documented in purified CD3$^+$ NK-R$^+$ populations cultured in interleukin 2. In Fig. 1*F*, a p70$^+$ enriched cell population cultured for 10 days is shown in which a fraction of p70$^+$ cells coexpress p58.2. The coexpression of different NK-R by single T cells could be further documented at the clonal level (data not shown).

The NK-R$^+$ Peripheral Blood T Lymphocyte Subsets Display a "Memory" Surface Phenotype. To define whether NK-R$^+$ T lymphocytes represent cells that had undergone *in vivo* stimulation, we analyzed a number of markers indicative of naive versus memory phenotype (25). In these experiments, we analyzed lymphocyte suspensions enriched in T cells by removal of adherent cells and cells expressing CD16 or CD19 antigens, thus allowing a direct analysis of the coexpression of p58 molecules with other relevant surface markers. In the representative donor A.L., 13% of CD3$^+$ cells expressed p58.2 molecules (Fig. 2*A*). In this donor, p58.2$^+$ cells were confined to CD8$^+$ cells (Fig. 2 *B* and *C*). Remarkably, essentially all p58.2$^+$ cells lacked CD28 (Fig. 2*D*), but expressed high levels of CD18 (Fig. 2*E*), CD29 (Fig. 2*F*), and CD45RO (Fig. 2*G*), while expressing little, if any, CD45RA (Fig. 2*H*). A similar pattern of naive/memory marker expression was detected in three other donors expressing different NK-R, including p58.1 and p70. In addition, CD3$^+$ NK-R$^+$ cells were found to express both CD57 and CD44 and to lack L-selectin (data not shown). Taken together, these data support the concept that the expression of NK-R is confined predominantly to T cells with a "memory" phenotype, thus suggesting that T cell activation is required for the induction of NK-R expression.

Analysis of the TCR Vβ Repertoire in T Cells Expressing NK-R. We next analyzed purified CD3$^+$ NK-R$^+$ cell populations to determine their TCR Vβ repertoire. To this end, purified CD3$^+$ NK-R$^+$ cell populations were stimulated with PHA and cultured in the presence of exogenous interleukin 2. In addition, cells were cloned under limiting dilution conditions. Several CD3$^+$ NK-R$^+$ cell lines were derived from different individuals, and numerous clones were obtained that maintained their original surface phenotype. According to previous observations (13, 18), cell lines characterized by a CD8$^+$ phenotype efficiently lysed K562 target cells, whereas a CD3$^+$ NK-R$^+$ CD4$^+$ cell line was poorly cytolytic. Analysis of the TCR Vβ repertoire was performed by the combined use of mAbs specific for different human TCR Vβs and by a semiquantitative PCR assay. As shown in Fig. 3*A*, the CD3$^+$ p58.2$^+$ T cell line derived from donor K.K. expressed Vβ16.1 and Vβ17.1. Expression of all the other Vβ families was barely detected or absent. Unfractionated T cells cultured under the same conditions (used as control) expressed a wide Vβ repertoire (data not shown). Similar expansions of individual Vβs were documented in three other cell lines derived from different donors. In the cell line derived from donor L.M. (p70$^+$), Vβ4 and Vβ15 were detected; in the CD3$^+$ p70$^+$ cell line derived from donor S.D., Vβ8 was detected, while Vβ17.1 was detected in donor C.B. (p70$^+$) (data not shown). Thus, one or two TCR Vβ families were greatly expanded in the CD3$^+$ NK-R$^+$ cell lines analyzed. In addition, all the NK-R$^+$ T cell clones isolated from the above individuals expressed the same Vβ phenotypes detected in the corresponding cell line (data not shown). We further analyzed the TCR Vβ expressed in the original CD3$^+$ NK-R$^+$ populations present in fresh peripheral blood. This analysis was performed by directly assessing TCR Vβ$^+$ cells by the use of TCR Vβ-specific mAbs in conjunction with the relevant anti-NK-R mAb. In donor K.K., the majority of CD3$^+$ p58.2$^+$ peripheral blood cells expressed Vβ16.1 (Fig. 3*B*). In addition, a small proportion of p58.2$^+$ cells expressed Vβ17.1 (data not shown). mAbs to other Vβs, including Vβ8 and Vβ2, did not react with p58.2$^+$ cells. Therefore, similar to the results obtained by analysis of the Vβ repertoire in CD3$^+$ NK-R$^+$ cells that were cultured *in vitro*, the same Vβ families (Vβ16.1 and Vβ17.1) were found to be expanded *in vivo* in the CD3$^+$ NK-R$^+$ cell subset.

Oligoclonality or Monoclonality of CD3$^+$ NK-R$^+$ Cell Populations. The selective expansion of a few TCR Vβ$^+$ families *in vivo* suggested that this skewed TCR repertoire could result from chronic antigen (or superantigen) stimulation. To determine the oligoclonal, monoclonal, or polyclonal nature of these

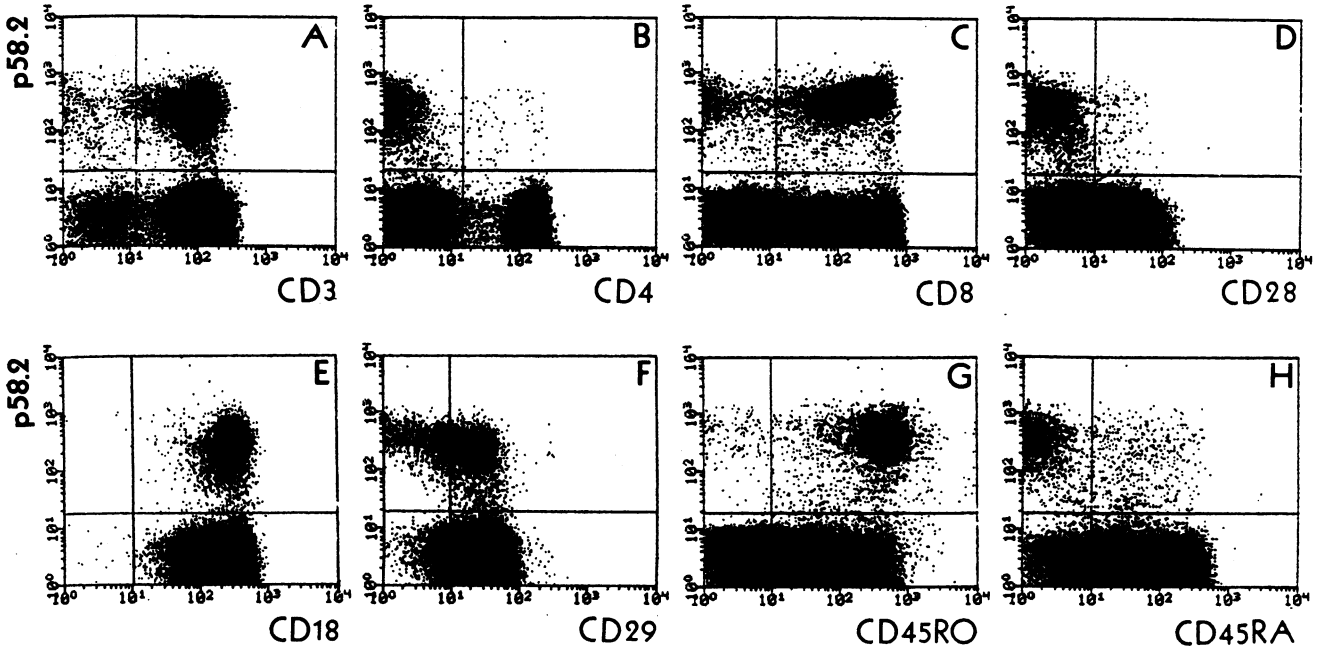

FIG. 2. NK-R$^+$ T lymphocytes express a "memory" surface phenotype. Lymphocyte suspensions were derived from a representative individual (A.L.) characterized by high proportions of CD3$^+$ p58.2$^+$ cells. After removal of cells adherent to plastic, cells expressing CD16 or CD19 were further removed. Lymphocytes enriched in T cells were analyzed for the expression of p58.2. Since >95% of p58.2$^+$ cells were CD3$^+$ (*A*), cells coexpressing p58.2 and other surface markers could be considered as T cells. p58.2$^+$ cells were CD4$^-$ (*B*) and CD8$^+$ (*C*). Remarkably, they did not express CD28 (*D*). In addition, cells expressed high levels of CD18 (*E*) and were CD29$^+$ (*F*). While most cells expressed CD45RO (*G*), they essentially lacked CD45RA (*H*).

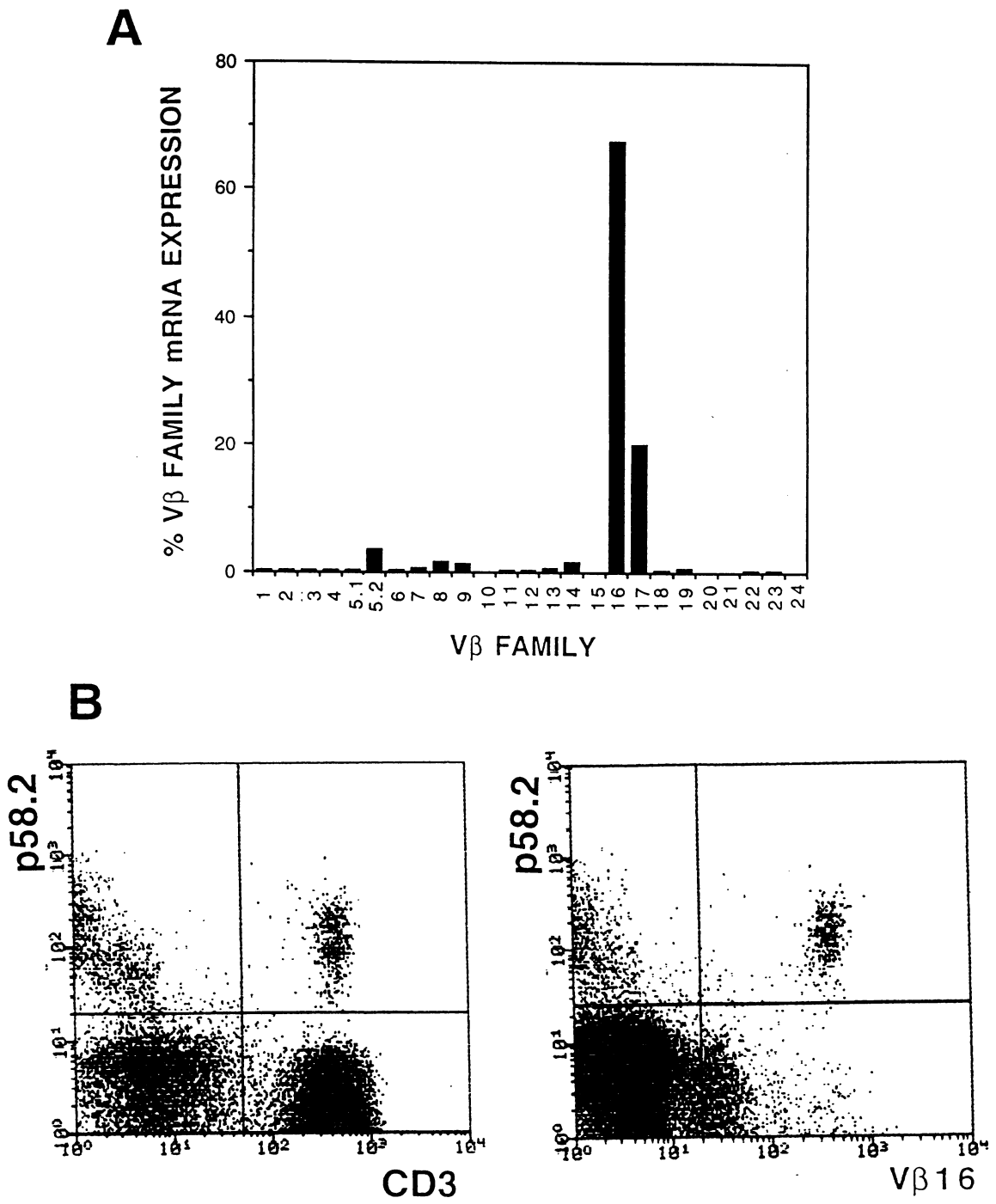

FIG. 3. NK-R$^+$ T lymphocytes express a limited TCR Vβ repertoire. (*A*) *In vitro* expanded CD3$^+$ p58.2$^+$ cells from donor K.K. were analyzed with regard to TCR Vβ repertoire by a semiquantitative PCR assay as described. (*B*) The expression of TCR Vβ16.1, i.e., the predominant Vβ expressed in cultured CD3$^+$ p58.2$^+$ cells from donor K.K., was directly evaluated in fresh peripheral blood from the same donor using two color cytofluorometric analysis. It is evident that TCR Vβ16.1$^+$ cells account for the majority of CD3$^+$ p58.2$^+$ cells.

Vβ expansions, several recombinant clones of the expanded Vβ families were derived from a cell line that had been expanded *in vitro* and sequenced. As mentioned above, CD3$^+$ NK-R$^+$ cells from donor K.K. expressed predominantly Vβ16.1 and Vβ17.1 (Fig. 3). As shown in Fig. 4, 9 of 12 Vβ16.1 clones rearranged to Jβ1.2, and these clones exhibited identical rearrangements. Similarly, 12 of 12 Vβ17.1 clones rearranged to Jβ2.5, and 11 of 12 clones had an identical rearrangement.

DISCUSSION

The present study demonstrates that CD3$^+$ NK-R$^+$ cells represent activated T lymphocytes comprising a limited number of TCR Vβs. Perhaps more importantly, these cells represent oligoclonal or monoclonal T cell expansions. In our experience, mitogen-driven T cell proliferation *in vitro* is not sufficient to induce the expression of NK-R; in addition, preliminary data indicate that *in vivo* activated peripheral blood T cells in patients with acute Epstein–Barr virus or hepatitis B virus infections do not express NK-R. These observations suggest that unique microenvironmental conditions and/or a particular set of cytokines are required to induce NK-R expression; these cytokines may be produced by T cells at various stages of cellular activation. In this context, it is of interest that preliminary experiments demonstrated that

Clone	Vβ	N1-Dβ-N2	Jβ	ID	n
A.					
3	TGT GCC AGC AGC C A S S	CCG GAC AGG GGA AGG P D R G R	GGC TAC ACC TTC G Y T F	Vβ16.1Jβ1.2	9/12
1	TGT GCC AGC AGC C A S S	CAA GAT CGA Q D R	GAT ACG CAG TAT TTT D T Q Y F	Vβ16.1Jβ2.3	3/12
B.					
2	TGT GCC AGT AGT C A S S	ATA GGC GGA CTG ATA CAA I G G L I Q	GAG ACC CAG TAC TTC E T Q Y F	Vβ17.1Jβ2.5	11/12
17	TGT GCC AGT AGT C A S S	ATA GGC GGA CTG ATT TCA I G G L I S	GAG ACC CAG TAC TTC E T Q Y F	Vβ17.1Jβ2.5	1/12

FIG. 4. Nucleotide sequences of Vβ16.1 and Vβ17.1 recombinant clones derived from an *in vitro* expanded CD8$^+$ NK-R$^+$ cell line from donor K.K. (*A*) Nucleotide sequence analysis of 12 recombinant clones of Vβ16.1. Nine of 12 cloned used Jβ1.2 and had identical clonotype (PDRGR). (*B*) Nucleotide sequence analysis of 12 recombinant clones of Vβ17.1. Eleven of 12 recombinant clones used Jβ2.5 and had identical clonotype (IGGLIQ). Cloning of Vβ16.1 and Vβ17.1 and nucleotide sequences were performed as described.

a fraction of CD3$^+$ NK-R$^+$ clones produced interleukin 5 and, less frequently, interleukin 4; these two cytokines are not released by normal CD8$^+$ cytolytic clones (26). Although interleukin 5 could not induce expression of NK-R in T cells, we are further investigating the possible role of different cytokines used alone or in combination in the induction of NK-R expression.

The finding that CD3$^+$ NK-R$^+$ cells expressed a memory phenotype and an oligoclonal or monoclonal TCR Vβ repertoire is reminiscent of previous observations that oligoclonal cell expansions are frequent and express a CD8$^+$ CD45RO$^+$ phenotype in normal individuals (27). In addition, clonal CD8$^+$ T cell expansions have been detected both in autoimmune diseases and in chronic viral infections, including cytomegalovirus and HIV infections (21, 28–30).

The finding that, in different individuals, CD3$^+$ cells expressing a given NK-R are characterized by expression of different Vβ phenotypes that are oligoclonally or monoclonally expanded is not consistent with the hypothesis that proliferation of these cells is driven by a superantigen(s). It is possible that oligoclonally or monoclonally expanded CD8$^+$ T cells expressing NK-R may mediate cytotoxic function in response to a variety of antigens. The expression of these inhibitory receptors (NK-R) on these CD8$^+$ cytotoxic T cells may be detrimental to the host in certain viral infections. For example, in the case of chronic viral infections, this inhibitory phenomenon may lead to a less efficient control of virus replication and spread mediated by virus-specific cytotoxic T lymphocytes. We are presently analyzing whether the expression of NK-R actually occurs in virus-specific cytotoxic T lymphocytes and if so, whether a correlation exists with particular stages of the disease. Along this line, increased percentages of CD3$^+$ p58$^+$ cells have been reported in HIV-infected individuals (31).

In conclusion, the finding that chronically activated T cells may express TCR and NK-R, both of which are specific for class I MHC molecules but each of which perform opposing functions (13, 14), may offer new perspectives in our understanding of the complexity of the regulation of T cell responses.

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