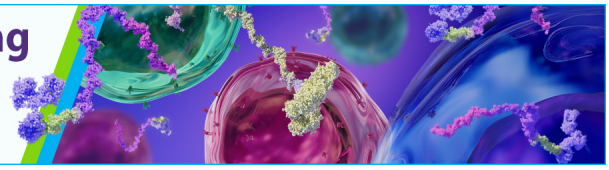

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Differential Expression of Leukocyte Receptor Complex-Encoded Ig-Like Receptors Correlates with the Transition from Effector to Memory CTL[1]

Neil T. Young,[2]* Markus Uhrberg,[3]* Joseph H. Phillips,† Lewis L. Lanier,[4]† and Peter Parham*

The human leukocyte receptor complex (LRC) on chromosome 19q13.4 encodes Ig superfamily receptors expressed on hemopoietic cells. Killer Ig-like receptors (KIR) are expressed in cytotoxic lymphocytes but other LRC molecules (Ig-like transcript(ILT)/leukocyte Ig-like receptor (LIR)) are more ubiquitous. We investigated expression of the ILT2/LIR1 inhibitory receptor compared with the related KIR. Both ILT2/LIR1 and KIR were expressed by peripheral $CD8^+$ T cells with a memory/effector phenotype. ILT2/$LIR1^+$ T cells demonstrated diverse TCRBV repertoires in contrast to KIR^+ T cells, while numbers of peripheral ILT2/$LIR1^+$ T cells were greater than KIR^+ T cells and the majority of ILT2/$LIR1^+$ T cells did not coexpress KIR. Analysis of $CD8^+$ T cells with specific HLA class I tetramers confirmed this pattern of expression, indicating differential regulation of LRC gene expression in T lymphocytes. Only a minor proportion of ILT2/$LIR1^+$ KIR^- clones survived in vitro cloning, were more susceptible to anti-CD3 or cognate peptide induced cell death than KIR^+ T cells and exhibited lower levels of the Bcl-2 survival molecule. Our results indicate a sequential program of LRC-encoded receptor expression with initial ILT2/LIR1 expression in effector T cells and KIR gene transcription in the minor proportion of expanded clones which survives activation-induced cell death to become long term memory T cells. *The Journal of Immunology,* **2001, 166: 3933–3941.**

Killer cell Ig-like receptor (KIR)[5] molecules are members of the Ig superfamily, containing either two (KIR2D) or three (KIR3D) extracellular Ig domains (1). The genes for these receptors are located in a polygenic cluster on chromosome 19q13.4 (2). Further molecules with homology to KIR have been described and shown to be encoded by genes located in the same genomic region, now designated the leukocyte receptor complex (LRC) (3). This additional family of receptors, known either as Ig-like transcripts (ILT) (4), leukocyte Ig-like receptors (LIR) (5), or monocyte inhibitory receptors (MIR) (6, 7) have structural and functional similarities to KIR including multiple isoforms. ILT/LIR possess either four or two extracellular Ig-like domains and can be classified as inhibitory forms with long cytoplasmic tails containing immunoreceptor tyrosine inhibitory motifs (ITIMs) which recruit SHP-1 phosphatase (8) or short-tailed, activating forms which associate with the FcRIγ signaling subunit (9). ILT/LIR proteins differ from KIR by being principally expressed on cells with a phagocytic and Ag-presenting function such as monocytes, macrophages, dendritic cells, and B lymphocytes, although a subset of these inhibitory receptors are expressed on peripheral NK and T lymphocytes (4, 5).

The functional activity of human NK cells and a subset of CTL is influenced by the interaction of KIR with epitopes of HLA class I molecules. Upon binding their specific ligands, KIR recruit SHP-1 phosphatase via ITIMs located in their cytoplasmic tail, inhibiting the signaling processes associated with cellular activation (1). In NK cells, KIR genotype and expression repertoire determine self tolerance (10) and allow these cells to detect "missing self," i.e., the loss of cell surface HLA class I expression resulting from infection with certain viruses or malignant transformation. Isoforms of the KIR molecules that have short cytoplasmic tails lacking ITIM motifs may trigger effector cell activation through association with the adaptor molecule DAP12 (11). The precise role of KIR expression in CTL is unclear, although these receptors can modulate signals transmitted through the TCR/CD3 complex (12–14). KIR expression in T lymphocytes appears to be restricted to cells with a differentiated "memory" phenotype (15).

As the KIR and ILT/LIR receptors are encoded within the same genomic cluster, it is relevant to assess the features of ILT/LIR expression in peripheral lymphocytes and the extent of any coincidence with KIR expression, particularly as inhibitory receptors such as ILT2/LIR1 appear to be expressed on both NK and T lymphocytes and recognize HLA class I ligands (16). Investigation of these features will provide information on cell-type specific expression of genes within the LRC and may have important implications for the functional repertoires of cells with cytotoxic capacity. We have thus analyzed characteristics of the ILT2/LIR1 inhibitory receptor on T lymphocytes in comparison with expression of the related KIR. Previous studies have demonstrated the

*Departments of Structural Biology and Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305; and †DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, CA 94306

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[2] Current address: Nuffield Department of Surgery, University of Oxford, John Radcliffe Hospital, Oxford, U.K.

[3] Address correspondence and reprint requests to Dr. Neil Young at his current address: Nuffield Department of Surgery, University of Oxford, John Radcliffe Hospital, Headington Oxford, OX3 9DU U.K. E-mail address: neil.young@nds.ox.ac.uk

[4] Current address: Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center, Düsseldorf, Germany.

[5] Current address: University of California San Francisco, Department of Microbiology and Immunology, San Francisco, CA 94143.

[6] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; ILT, Ig-like transcript(s); LRC, leukocyte receptor complex; LIR, leukocyte Ig-like receptor(s); CDR3, complementarity-determining region 3.

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expression of ILT2/LIR1 by a proportion of T cells but have not addressed the coordination of ILT2/LIR1 and KIR expression or defined the specific types of T cells that express ILT2/LIR1. Our results indicate a certain overlap in expression of the two types of receptors but also demonstrate a differential regulation of Ig-like receptor acquisition which correlates with the development of resistance to activation-induced cell death in memory CTL.

Materials and Methods

Normal donor leukocytes

Buffy coats obtained by leukapheresis of normal blood donors were purchased from Stanford Medical Center Blood Bank. Cord blood samples were obtained from normal term deliveries at the Maternity Unit, Packard Children's Hospital, Stanford. Mononuclear cells were obtained from these products by Ficoll-Hypaque gradient centrifugation. HLA class I typing was performed on genomic DNA derived from PBMC using PCR followed by direct sequencing and sequence-specific primer amplification.

Flow cytometry

Three-color flow cytometry was performed on mononuclear cells stained in a two-stage procedure with the ILT2/LIR1-specific mAb HPF1 (16) followed by FITC- or PE-labeled goat anti-mouse reagent. After blocking with normal mouse serum (Sigma, St. Louis, MO), cells were counterstained with directly conjugated (FITC or CyChrome) Abs specific for the T cell markers CD3, CD4, CD8, TCR$\gamma\delta$, TCR$\alpha\beta$, CD45RO, CD45RA, CD57, CD28, CD27, CD38, and the NK cell markers CD16 and CD56. A mixture of PE-coupled KIR-specific mAbs consisting of the anti-KIR reagents EB6 (anti-KIR2DL1, anti-KIR2DS1; Coulter, Hialeah, FL), DX27 (anti-KIR2DL2/L3, anti-KIR2DS2), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2) was also used. T cell clones as well as peripheral mononuclear cells were analyzed by flow cytometry using FITC-coupled mAbs specific for the TCRV region families AV2, AV12.1, BV3.1, BV5a, BV5b, BV5c, BV6.7, BV8a, BV12, BV13, BV14, BV16, BV21, BV22, and BV23 (Coulter and Serotec, Raleigh, NC). For analysis of intracellular Bcl-2 levels by four color flow cytometry, PBMC were first stained with appropriate PE, Cychrome or APC-labeled Abs specific for cell surface markers, permeabilized, and stained with FITC-labeled anti-Bcl-2 or IgG1 isotype control (Becton Dickinson, San Jose CA).

Ag-specific T lymphocytes were identified using synthetic HLA-A*0201 tetrameric complexes labeled with PE fluorochrome. HLA-A2 tetramers were refolded with HCMV pp65 peptide NLVPMVATV or human EBV BZLF1 peptide GLCTLVAML. Tetramer staining was performed at room temperature to minimize low affinity binding.

Cells (2×10^5) of each T cell clone and 1×10^6 PBMC were incubated at 4°C for 45 min with the appropriate Abs, washed, and analyzed on a FACScan or FACSort flow cytometer using CellQuest (Becton Dickinson) and FlowJo (TreeStar, San Carlos, CA) analysis software.

T cell cloning

Single $CD3^+$ $HPF1^+$ T cells were sorted using a FACStar or FACSVantage cell sorter and Cyt-clone software (Becton Dickinson). Single-cell sorted T cell clones were established and maintained as described (17). Briefly, T cell clones were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 200 U/ml recombinant IL-2 (provided by C. Reynolds, National Cancer Institute/Biological Response Modifier Program, Frederick, MD) and 5% human T-STIM (Becton Dickinson). Mixed, allogeneic PBMC (1×10^6/ml; three donors) and 1×10^5/ml JY cells (EBV-transformed B lymphoblastoid cell line) 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 control for background proliferation.

RNA preparation and reverse transcription

Total cellular RNA was extracted from 3–5 $\times 10^6$ T cell clones and the "feeders-only" culture using RNAzol according to the manufacturer's instructions (Tel-Test, Friendswood, TX).

First strand cDNA was synthesized from 1 μg RNA by reverse transcription using oligo(dT) (Perkin-Elmer, Norwalk, CT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) in a volume of 50 μl at 42°C for 1.5 h.

PCR amplification

RT-PCR analysis of KIR expression on T cell clones was performed as described (18) using a panel of PCR primer pairs to enable the specific amplification of six groups of inhibitory KIR (KIR2DL1–4 and KIR3DL1–2) and six groups of noninhibitory KIR (KIR2DS1–5 and KIR3DS1). Amplifications were performed with specific primers at a concentration of 0.5 μM in 25-μl reactions for 30 cycles using 2 μl of cDNA per reaction. 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 RT-PCR controls to monitor the presence of residual transcripts from irradiated feeder cells. The use of this RT-PCR analysis allowed us to detect expression of all KIR genes, including those receptors that cannot be detected by specific Abs.

TCRBV family-specific PCR was performed as previously described (19). For each of 25 TCRBV families a specific sense primer and a common antisense primer matching the TCRB constant region were used. T cell clones were amplified for 25 cycles, PBMC for 35 cycles using 1 μl of cDNA per 25-μl reaction.

TCRB gene rearrangements of T cell clones were determined by direct sequencing of products 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) and cycle-sequenced using dye-labeled deoxynucleotide terminators and a 373A automated sequencer (Applied Biosystems, Foster City, CA). TCRB rearrangements were determined in both directions using the family-specific and constant region-specific primers used for PCR amplification.

Functional analysis

T cell clones (2×10^4) reactive with tetrameric HLA complexes were incubated at 37°C in 96-well plates with irradiated peptide-pulsed stimulator cells at a ratio of 5:1 for the indicated time periods in IL-2 medium. Stimulator cells comprised the 721.221 HLA class I-negative B lymphoblastoid cell line transfected with cDNA for HLA-A*0201 and incubated in the presence of relevant nonamer peptides at 37°C overnight before washing and irradiation. Cultures for each time point were established in duplicate. Peptides were synthesized by F-moc chemistry and purified to greater than 90% purity by reverse phase HPLC (Sigma Genosys, Austin TX). Cells were analyzed by flow cytometry with fluorochrome-labeled anti-CD8 and anti-CD3 Abs. Cell viability was assessed by forward and side scatter characteristics and staining with FITC-labeled annexin V (Becton Dickinson). For anti-CD3 stimulation, T cell clones (2×10^5) were incubated in the presence of 1 μg/ml anti-CD3 mAb (clone UCHT1; Coulter) and 2 μg/ml recombinant protein G (Sigma) in IL-2 medium at 37°C for 6 h, washed, and assayed for Annexin V binding and propidium iodide exclusion by flow cytometry. Control incubations consisted of T cell clones incubated in the presence of protein G or medium alone. For analysis of PBLs, $CD3^+$ $CD8^+$ T cells were sorted into $ILT2/LIR1^+$ KIR^- and $ILT2/LIR1^+$ KIR^+ populations by flow cytometry before anti-CD3 stimulation and flow cytometric analysis as described above.

Statistical analysis

Analysis of differences in Ig-like receptor expression between flow cytometry-defined subsets was accomplished using a two-tailed Student *t* test and ANOVA.

Results

Variation in ILT2/LIR1-inhibitory receptor expression in PBLs

Flow cytometric analysis of ILT2/LIR1 expression in PBMC of 20 normal donors using the specific mAb HPF1 demonstrated that the principal cell types expressing this inhibitory receptor were monocytes and B lymphocytes (Fig. 1*A*), as previously reported (16). In all individuals analyzed, ILT2/LIR1 was expressed on close to 100% of $CD3^-$ $CD19^+$ B lymphocytes and $CD14^+$ $CD16^+$ or $CD13^+$ $CD16^+$ monocytes. In contrast to the ubiquitous expression by B lymphocytes and monocytes, ILT2/LIR1 was expressed on a subset of peripheral adult $CD3^+$ T lymphocytes and $CD3^-$ $CD56^+$ NK lymphocytes (Fig. 1*A*) and a very small proportion of cord blood T lymphocytes (mean = 1.7% SD = 1.2, $n = 5$). ILT2/LIR1 was expressed on a mean of 48% (SD = 21.4) of $\gamma\delta$ T lymphocytes and a mean of 23.5% (SD = 14.9) of $\alpha\beta$ T-lymphocytes in the 20 normal adults. Expression of both ILT2/LIR1 and KIR was restricted to $CD8^+$ T lymphocytes, with $<1\%$ of $CD4^+$ T lymphocytes expressing Ig superfamily inhibitory receptors (Fig. 1*B*). Although the values for $CD3^+$ cells appear quantitatively

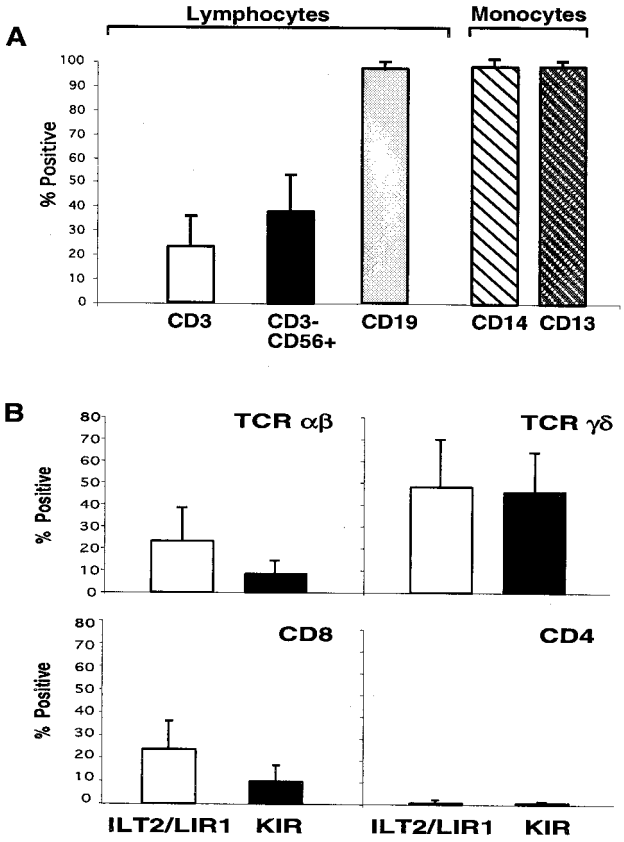

FIGURE 1. ILT2/LIR1 receptor is expressed on a subset of peripheral blood T and NK lymphocytes. *A*, Mean percentage of ILT2/LIR1$^+$ cells within each of the major mononuclear cell populations as determined by flow cytometric analysis of 20 normal adult individuals. Errors bars depict SD of the mean. Open columns, CD3$^+$ T lymphocytes; solid black columns, CD3$^-$ CD56$^+$ NK lymphocytes; solid gray columns, CD19$^+$ B lymphocytes; hatched white columns, CD14$^+$ monocytes; hatched black columns, CD13$^+$ monocytes. *B*, Mean percentage of ILT2/LIR1$^+$ cells within each of the major T cell subsets as determined by flow cytometric analysis of 20 normal adult individuals. Errors bars depict SD of the mean. Within the peripheral lymphocyte population, ILT2/LIR1 is principally expressed on cytotoxic cells. Open columns, ILT2/LIR1 (HPF1 Ab); filled columns, KIR (a mixture of EB6, DX27, DX9, and DX31 Abs).

similar to that for CD8$^+$ cells, this is an artifact of presenting the results as mean values. Analysis of individual donors demonstrates that these values are not correlated and are apparently influenced by variations in numbers of CD8$^+$ T cells between donors (data not shown).

ILT2/LIR1-inhibitory receptor is principally expressed by memory CTLs

In view of the low numbers of ILT2/LIR1$^+$ T lymphocytes observed in cord blood samples and the previously reported restriction of KIR expression to memory phenotype adult T lymphocytes, we performed a subset analysis of T cells in the panel of 20 normal individuals using the HPF1 Ab and a pool of Abs recognizing KIR2D and KIR3D molecules. This analysis demonstrated that both ILT2/LIR1$^+$ and KIR$^+$ T cells are predominantly contained within the CTL memory/effector population i.e., CD8$^+$ CD56$^+$ CD57$^+$ CD27$^-$ CD28$^-$ T cells (Fig. 2*A-D*).

However, analysis of subsets defined by markers previously recognized as distinguishing Ag-naive and Ag-experienced T cells showed no significant differences in ILT2/LIR1 or KIR expression between either CD45RA/RO or CD38$^+$/CD38$^-$ T cells (Fig. 2, *E* and *F*). ILT2/LIR1$^+$ T cells were of a higher proportion in the CD45RA$^+$ population, which may correlate with the reported effector function of this subset of cells (20). By comparison with KIR expression in the populations defined by CD8, CD56, CD57, CD27, or CD28, ILT2/LIR1 was expressed by a significantly higher number of T cells within each subset ($p < 0.0005$).

Coexpression of ILT2/LIR1 and KIR on CTLs

Our analysis of inhibitory receptor expression in T lymphocyte subsets revealed a consistently greater number of cells positive with the HPF1 Ab than the mixture of KIR-specific Abs within all subsets tested. This raised the possibility that ILT2/LIR1 and KIR expression may not be concomitant in these cells. To analyze this further, we tested coexpression of ILT2/LIR1 and KIR in the peripheral lymphocytes of twenty individuals. Although a consistent proportion of KIR$^+$ NK (gated on CD56$^+$ or CD16$^+$ lymphocytes) or T lymphocytes (gated on CD3$^+$ lymphocytes) coexpressed ILT2/LIR1 (Fig. 3*A*), this feature was not apparent when ILT2/LIR1$^+$ CD3$^+$ T-lymphocytes were analyzed for coexpression of KIR (Fig. 3*B*). Although equivalent levels of KIR expression were observed on ILT2/LIR1$^+$ NK lymphocytes, the majority of ILT2/LIR1$^+$ T cells expressed only this receptor and the minority coexpressed KIR. A comparison of the relative proportions of ILT2/LIR1 and KIR coexpression in the CD3$^+$ population is shown in Fig. 3, *C* and *D*.

This difference between ILT2/LIR1 and KIR expression in T lymphocytes and NK cells was strikingly apparent when the twenty individuals were analyzed individually (Fig. 4). In both CD3$^+$ CD8$^+$ (Fig. 4*A*) and TCR$\alpha\beta^+$ (Fig. 4*B*) populations, 19 of 20 individuals expressed ILT2/LIR1 on a higher percentage of T cells than KIR molecules. However, the majority of these individuals expressed ILT2/LIR1 on a lower percentage of their NK (CD3$^-$ CD56$^+$) cells (Fig. 4*C*) than KIR molecules with $\gamma\delta$ T cells demonstrating an intermediate phenotype (Fig. 4*D*).

Clonal analysis of ILT2/LIR1-positive T lymphocytes

To precisely investigate the characteristics of ILT2/LIR1 expression in T lymphocytes, we derived T cell clones by single cell sorting of CD3$^+$ ILT2/LIR1$^+$ lymphocytes from the peripheral blood of two donors who were previously analyzed in detail for the features of KIR expression in both NK (10) and T lymphocytes (21). The phenotype and TCR clonotype of the clones were analyzed in detail using flow cytometric and molecular techniques. Because the pool of KIR-specific Abs we used in the flow cytometric analysis detect all but four of the 12 known KIR molecules, a precise molecular analysis of T cell clones was required to unambiguously determine the full extent of overlap in Ig-like receptor expression.

ILT2/LIR1$^+$ T cell clones are representative of peripheral blood T lymphocytes

The panels of ILT2/LIR1$^+$ T cell clones derived from donors NV and PP have similarities to the populations of KIR$^+$ T cell clones we have previously analyzed (21) but also have significant differences (Table I). In short, although these cells typically displayed the phenotypes of activated memory cells, the panels comprised not only CD8$^+$ T cells but also included CD4$^-$ CD8$^-$ TCR$\alpha\beta$ cells and rare CD4$^+$ cells. Similar proportions of TCR$\alpha\beta$ and TCR$\gamma\delta$ cells were derived from cloning ILT2/LIR1$^+$ T cells as were found in KIR$^+$ cloning.

ILT2/LIR1$^+$ T cell clones exhibit a diverse TCR repertoire

The most striking finding of our previous study of KIR$^+$ T cell clones was the almost complete restriction of TCRBV region gene usage in TCR$\alpha\beta$ clones from donor NV. All such clones were

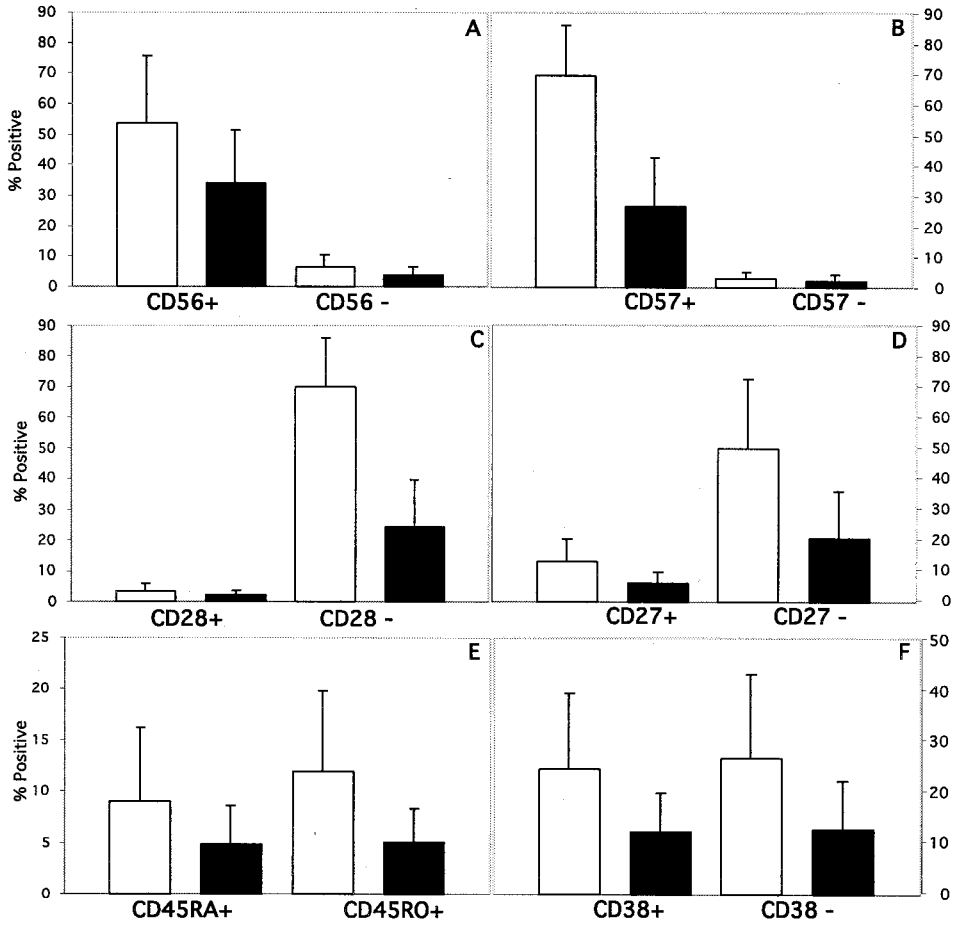

FIGURE 2. ILT2/LIR1 receptor expression is principally restricted to memory/effector T lymphocytes. ILT2/LIR1 and KIR expression was determined by flow cytometry in $CD3^+$ T lymphocytes positive or negative for the following markers of T cell memory and activation: *A*, CD56; *B*, CD57; *C*, CD28; *D*, CD27; *E*, CD45; *F*, CD38. Mean percentage of positive cells within each subset is shown ($n = 20$). Error bars depict SD of the mean. Open columns, ILT2/LIR1 (HPF1 Ab); filled columns, KIR (a mixture of EB6, DX27, DX9, and DX31 Abs).

TCRBV16 and expressed a highly restricted CDR3 sequence (21). We used PCR amplification and nucleotide sequencing of TCRBV gene transcripts from ILT2/LIR1$^+$ T cell clones from donors NV and PP to assess the extent of TCR diversity in these cells. A proportion of the ILT2/LIR1$^+$ TCR$\alpha\beta$ clones from donor NV represented the population cloned previously as they expressed the same TCRBV16 clonotype as the KIR$^+$ T cell clones. However, a further population of ILT2/LIR1$^+$ T cell clones expressed TCRB chain genes of diverse families and clonotypes including TCRBV2, BV6, BV17, BV21, and BV23 (Table II). No motifs common to all represented TCRBV families were found when the CDR3-encoding regions of these transcripts were sequenced, suggesting that these clones originally proliferated in response to unique peptide Ags. Similar results were found in clones derived from donor PP, where clonal TCRBV transcripts included TCRBV1, BV2, BV3, BV6, BV10, BV13, BV14, BV16, BV17, and BV21 (Table II).

In addition, flow cytometric analysis of peripheral blood CD8$^+$ lymphocytes with TCRBV-specific mAbs in six normal donors indicated that ILT2/LIR1 was expressed on a proportion of all families tested (mean = 25.4% 1SD = 10.3) whereas the mixture of KIR-specific Abs only detected low percentages in each family (mean = 6.1% 1SD = 4.1) or expansions within a small number of families.

Clonal analysis of ILT2/LIR1 and KIR coexpression

Typing of KIR gene expression in each of the T cell clones by RT-PCR and flow cytometry revealed a surprising finding. Although all clones were ILT2/LIR1$^+$, only a very small number displayed the Ig-like receptor phenotype of the cells that predominated in peripheral blood, i.e., ILT2/LIR1$^+$ KIR$^-$. From a total of 104 clones (including 20 from a third donor), only 19 did not react with KIR-specific mAbs and the majority of these expressed KIR2DL4, KIR2DS4, or KIR3DS1, which are not detectable by currently available Abs but can be detected at the cDNA level (Fig. 5). Thus, only 3 of the 104 clones displayed a truly KIR$^-$ phenotype. The remaining 101 clones displayed KIR phenotypes, as determined by RT-PCR typing, similar to those described in our previous study (21). These results indicate that our use of the pooled KIR-specific Abs in the analysis of PBLs detected the majority (~80%) of the KIR-expressing T cells.

To examine this apparent differential expression of LRC-encoded receptors more precisely, we analyzed Ag-specific peripheral blood CD8$^+$ cells of normal donors with tetrameric HLA class I molecules (Fig. 6). Using an HLA-A*0201-HCMVpp65 tetramer, it is evident that the majority of these peptide-specific CD8$^+$ T cells express ILT2/LIR1 (Fig. 6*B*) but only a very small proportion express KIR or CD94 receptors (Fig. 6*C*). Similar results were detected in another five donors

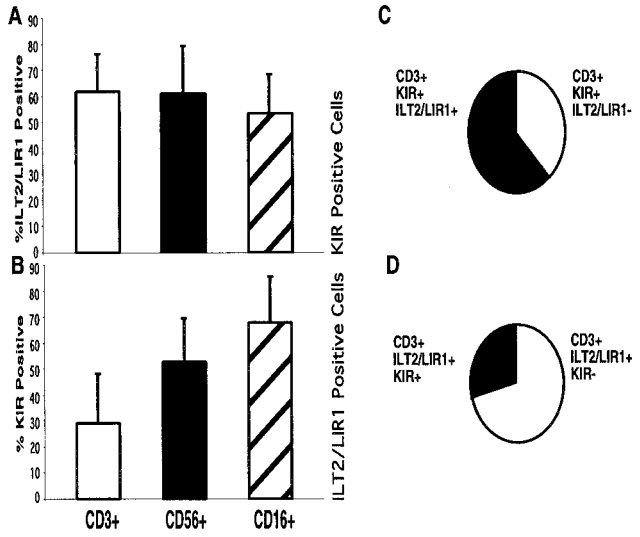

FIGURE 3. The majority of CD3$^+$ ILT2/LIR1-expressing T lymphocytes do not coexpress KIR. Flow cytometric analysis of receptor coexpression in peripheral lymphocyte subsets reveals differences in ILT2/LIR1 and KIR coexpression in the CD3$^+$ T cell subset. Mean percentage of positive cells within each subset is shown ($n = 20$). Error bars depict SD. Open columns, CD3$^+$ cells; filled columns, CD56$^+$ cells; hatched columns, CD16$^+$ cells. *A*, Percentage of ILT2/LIR1$^+$ cells of each subset within the KIR$^+$ population. *B*, Percentage of KIR$^+$ cells of each subset within the ILT2/LIR1$^+$ population. *C* and *D*, Proportions of single and double positive CD3$^+$ cells within either KIR$^+$ or ILT2/LIR1$^+$ populations, respectively.

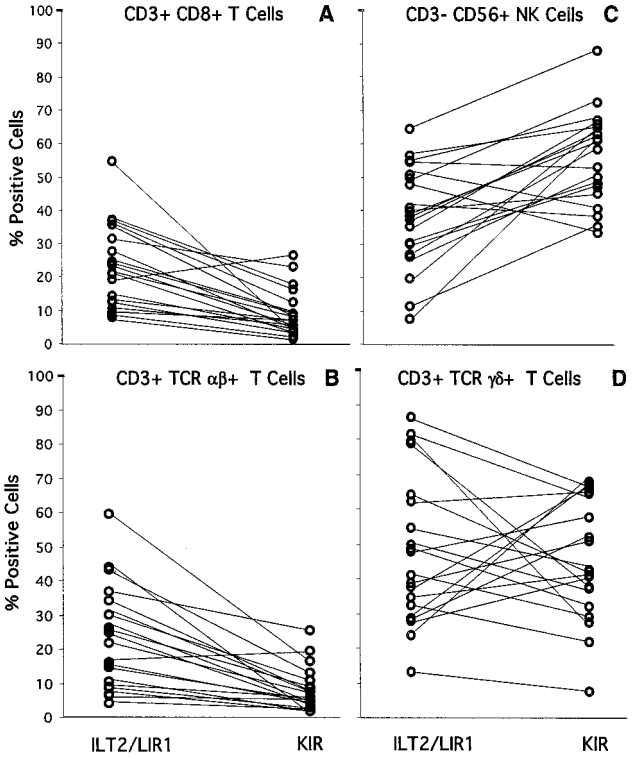

FIGURE 4. Discordant expression of ILT2/LIR1 and KIR in peripheral blood cytotoxic lymphocytes. Each panel depicts the percentage of PBL subsets (*A*, CD8$^+$ T cells; *B*, TCR$\alpha\beta$ T cells; *C*, CD3$^-$ CD56$^+$ NK cells; *D*, TCR$\gamma\delta$ T cells) expressing either ILT2/LIR1 (*left*, HPF1 Ab) or KIR (*right*, a mixture of EB6, DX27, DX9, and DX31 Abs) in 20 normal individuals. Values for each receptor are linked for individual donors and demonstrate a consistently higher proportion of ILT2/LIR1 expression in CD8$^+$ TCR$\alpha\beta$ T cells that is not observed in NK or TCR$\gamma\delta$ cells.

using tetramers containing either HCMV- or EBV-specific peptides (data not shown) confirming the validity of the LRC receptor phenotype found in our analysis of peripheral blood CD8$^+$ lymphocytes (Fig. 4).

ILT2/LIR1$^+$ KIR$^-$ clones are prone to activation-induced cell death

In view of the discrepancy observed between the phenotype of peripheral blood ILT2/LIR1$^+$ T cells and that of the in vitro expanded panel of T cell clones, we reasoned that the majority of ILT2/LIR1$^+$ T cells did not survive the cloning procedure and only those clones, which were also KIR$^+$, exhibited long-term in vitro growth characteristics. To investigate whether possible differences in the survival ability of T cell clones correlated with their expression of particular Ig-like receptors we performed another two experiments. After isolation and limited expansion of HCMVpp65 peptide-specific T cell clones using tetramers and single cell sorting, we tested the response of these ILT2/LIR1$^+$ KIR$^-$ clones to in vitro stimulation with their cognate peptide/HLA class I restriction element in the presence of IL-2.

As shown in Fig. 7*A*, incubation of T cell clones with their cognate peptide resulted in the death of ~80–90% of these cells by 48 h of culture, with a slightly more rapid rate of cell death induced by stimulator cells pulsed with higher peptide concentrations. In contrast, incubation with stimulators pulsed with an equivalent concentration of irrelevant peptide resulted in a decrease of viable CD8$^+$ cells of only 5–10%. To compensate for any potential differences in the affinity of clonally distributed TCRs for peptide-HLA complexes, we also stimulated ILT2/LIR1$^+$ KIR$^-$ and ILT2/LIR1$^+$ KIR$^+$ T cell clones with soluble anti-CD3 Ab in the presence of protein G and IL-2. After 6 h of incubation under these conditions, ~50% of cells of the KIR$^-$ clone bound annexin V, indicating the translocation of plasma membrane phospholipids characteristic of the early stages of apoptosis (22). In contrast, KIR2DL4$^+$ T cell clones displayed only a background level of such annexin V binding (Fig. 7*B*).

Flow cytometric sorting of peripheral CD8$^+$ T cells expressing ILT2/LIR1 alone or in combination with KIR demonstrated a similar finding, with the KIR$^-$ cells being more susceptible to anti-CD3-induced cell death (Fig. 8*A*). This population also contained significantly ($p < 0.005$) lower intracellular levels of the survival molecule Bcl-2 (Fig. 8*B*). The results of these two experiments

Table I. *Phenotypic characteristics of ILT2/LIR1$^+$ T cell clones derived from two normal donors*[a]

Phenotype	NV Clones ($n = 45$)	PP Clones ($n = 39$)
TCR$\alpha\beta$	80[b]	59[b]
TCR$\gamma\delta$	20	41
CD8$^+$	67	33
CD4$^+$	0	8
CD4$^-$8$^-$	13[c]	21[c]
CD56$^+$	71	28
CD57$^+$	67	77
CD28$^+$	0	5
CD16$^+$	20	5
CD45RO$^+$	100	100

[a] Clones were derived by single cell sorting of CD3$^+$ ILT2/LIR1$^+$ peripheral blood lymphocytes and expanded in vitro. Clones were phenotyped by flow cytometry and T cell receptor type was confirmed by RT-PCR.
[b] Percentage of total clones expressing the indicated cell surface marker.
[c] Percentage calculated from the number of TCR$\alpha\beta$ CD4$^-$8$^-$ clones.

Table II. *Comparison of the range of TCRBV gene usage in T cell clones selected on the basis of KIR expression or ILT2/LIR1 expression*[a]

Donor NV		Donor PP	
KIR[b]	ILT2/LIR1[c]	KIR[b]	ILT2/LIR1[c]
BV16	BV16	BV11	
	BV2	BV14	BV14
	BV6	BV21	BV21
	BV12		BV1
	BV17		BV2
	BV21		BV3
	BV23		BV6
			BV10
			BV13
			BV16
			BV17

[a] TCRBV gene usage was determined by RT-PCR and nucleotide sequencing. The overlap between the two populations is apparent but ILT2/LIR1$^+$ clones display a greater diversity of TCR usage.
[b] KIR$^+$ T cell clones. Data from Ref. 21.
[c] ILT2/LIR1$^+$ T cell clones derived in the present study.

indicate that expression of KIR by T cells correlates with the acquisition of resistance to activation-induced cell death by apoptosis.

Discussion

The LRC contains a large number of genes encoding Ig-superfamily proteins that have both structural and functional homologies.

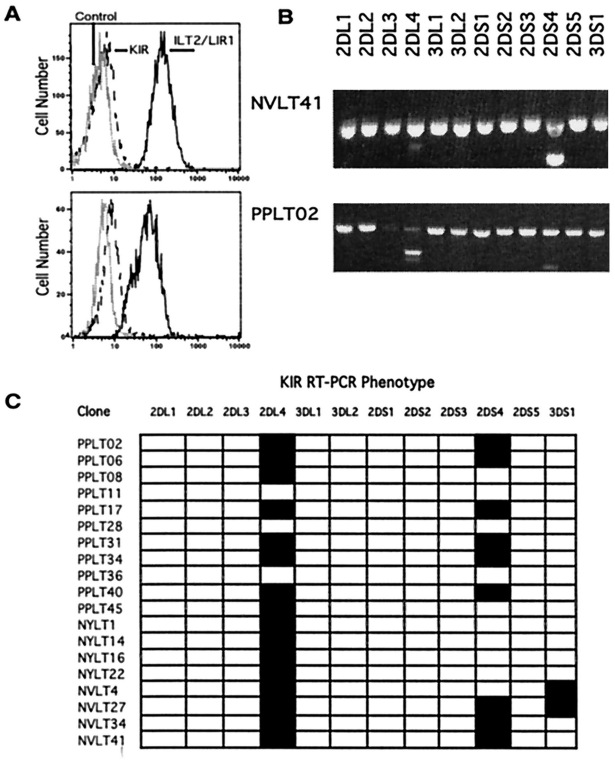

FIGURE 5. Phenotypic analysis of CD3$^+$ ILT2/LIR1$^+$ T cell clones detects KIR2DL4 expression. Flow cytometric analysis of a panel of T cell clones demonstrates only a minor proportion display the commonest phenotype observed in peripheral blood. *A* and *B*, Flow cytometric and RT-PCR analysis of KIR expression in two representative clones. *C*, KIR phenotype defined by RT-PCR in 19 clones (from a total of 104) that did not react with KIR-specific Abs, demonstrating that the majority of these cells express KIR2DL4. Open boxes indicate a negative typing result, and filled boxes represent a positive result.

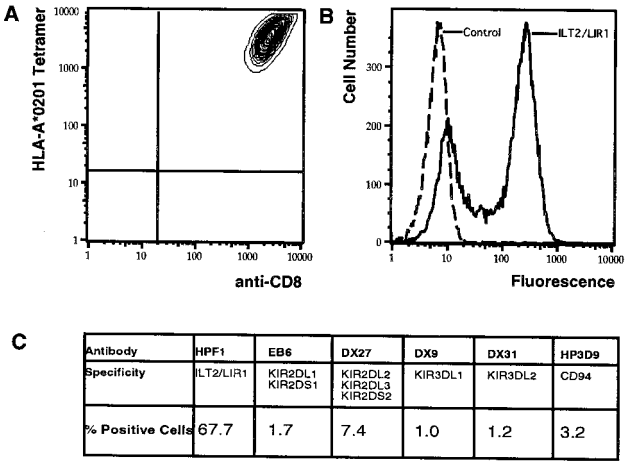

FIGURE 6. Analysis of peripheral Ag-specific CD8$^+$ T cells confirms the ILT2/LIR1$^+$ KIR$^-$ phenotype. Ex vivo analysis of bulk-sorted CD8$^+$ cells reactive with an HLA-A*0201-HCMVpp65 tetramer (*A*) demonstrates a phenotype consistent with the investigation of peripheral blood T cells. These cells display a proportion of ILT2/LIR1 expression (*B*) at least 10-fold greater than KIR expression (*C*).

Although certain of these receptors (e.g., ILT2/LIR1) are expressed on several hemopoietic cell types, particular genes such as KIR are only expressed in cells with a specific function. The mechanisms that control this cell type-specific gene expression are currently unknown. Our initial results demonstrated the expression of the ILT2/LIR1 inhibitory receptor on virtually all B lymphocytes and monocytes but emphasized the differences between individuals in terms of T lymphocyte expression. This feature suggested clonal variation in ILT2/LIR1 expression and prompted us to investigate the detailed characteristics of ILT2/LIR1 expression in T lymphocytes in regard to that of the related KIR genes. Unlike certain KIR genes (18), haplotypic diversity in the presence of the ILT2/LIR1 gene did not contribute to the apparent clonal variation because all donors typed positively for ILT2/LIR1 at the level of genomic DNA (data not shown).

The phenotypic assessment of peripheral blood T cell subsets definitively shows that ILT2/LIR1 and KIR genes are expressed in the same types of T cells, i.e., differentiated, Ag-experienced cytotoxic cells which may have lost the requirement for costimulation. However, this overlap in expression of the related genes is not necessarily coordinate as the proportions of ILT2/LIR1$^+$ cells always exceeded that of KIR$^+$ cells within each subset. The more widespread expression of ILT2/LIR1 is also supported by phenotypic analysis of T cell clones and Ag-specific T cells. ILT2/LIR1$^+$ clones and peripheral T cells displayed a diverse assortment of TCRV region gene usage in contrast to KIR$^+$ cells. Also, ILT2/LIR1$^+$ clones included CD4$^-$CD8$^-$ and rare CD4$^+$ T cells in addition to the CD8$^+$ cells characteristically expressing KIR.

Analysis of coexpression of the two receptor types demonstrated the predominance of ILT2/LIR1$^+$ KIR$^-$ T cells in peripheral blood and the use of tetrameric HLA class I constructs confirmed this pattern of receptor expression within a population of CD8$^+$ T cells specific for distinct viral peptides.

The derivation of ILT2/LIR1$^+$ T cell clones allowed us to define a major functional difference between cells expressing ILT2/LIR1 alone or in concert with KIR. The use of established in vitro cloning procedures failed to produce clones representative of the major phenotype observed ex vivo, as the only cells that displayed significant in vitro proliferation were KIR$^+$ and not ILT2/LIR1$^+$ KIR$^-$. Further functional analysis of these clones demonstrated

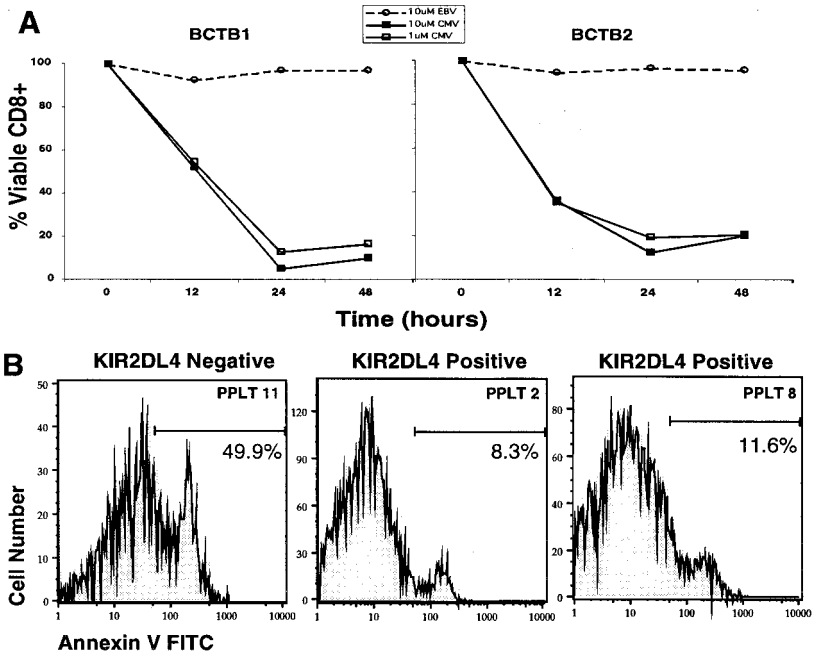

FIGURE 7. Stimulation of TCR of T cell clones defines resistance to activation-induced cell death associated with expression of KIR. *A*, HLA-A*0201-HCMVpp65 tetramer-reactive ILT2/LIR1$^+$ KIR$^-$ T cell clones (BCTB1 and BCTB2) were incubated with 721.221-HLA-A*0201 cells pulsed with 10 μM HCMVpp65 peptide (■), 1 μM HCMVpp65 peptide (□), or 10 μM EBV BZLF1 peptide (○), and the viability of CD8$^+$ cells was determined by flow cytometry at indicated time points. *B*, ILT2/LIR1$^+$ KIR2DL4$^-$ and ILT2/LIR1$^+$ KIR2DL4$^+$ T cell clones were incubated with 1 μg/ml soluble anti-CD3 in the presence of 2 μg/ml protein G for 6 h and analyzed for binding of annexin V by flow cytometry.

that ILT2/LIR1$^+$ KIR$^-$ clones were susceptible to apoptosis when activated through the TCR/CD3 complex, whereas KIR$^+$ clones were not. This result was confirmed by analysis of these T cell populations derived from peripheral blood, which additionally demonstrated a lower level of the cell survival molecule Bcl-2 in ILT2/LIR1$^+$ KIR$^-$ CD8$^+$ T cells.

It could be argued that without an Ab to specifically identify KIR2DL4, we do not currently know the true extent of KIR2DL4 expression in the serologically KIR$^-$ population. However, if all of the ILT2/LIR1$^+$ KIR$^-$ cells expressed KIR2DL4 we would expect a far higher proportion of the in vitro expanded clones to display this phenotype than was actually detected (~18% of ILT2/LIR1$^+$ clones compared with ~60% of peripheral blood ILT2/LIR1$^+$ T cells). This suggests that the data on KIR expression we obtained by flow cytometry are not a gross underestimate of peripheral KIR$^+$ T cells.

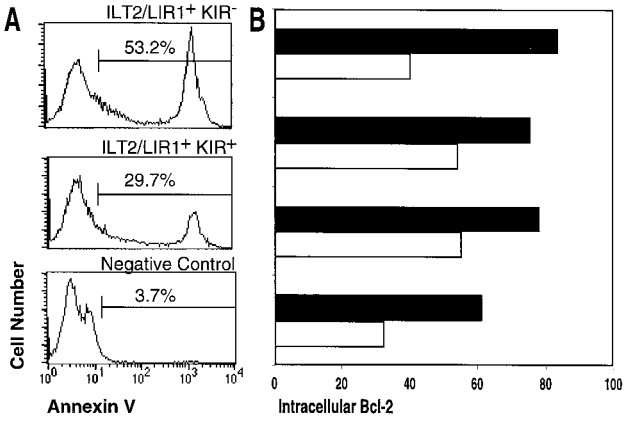

FIGURE 8. Peripheral CD8$^+$ lymphocytes expressing ILT2/LIR1 are more susceptible to activation-induced cell death and have lower intracellular levels of Bcl-2. *A*, Bulk-sorted ILT2/LIR1$^+$ KIR$^-$ (*top*) and ILT2/LIR1$^+$ KIR$^+$ (*middle*) peripheral CD3$^+$ CD8$^+$ lymphocytes were incubated with 1 μg/ml soluble anti-CD3 in the presence of 2 μg/ml protein G for 6 h and analyzed for binding of annexin V by flow cytometry. *Bottom*, Profile of cells incubated with protein G alone. Results shown are representative of four donors. *B*, PBLs were stained to identify CD3$^+$ CD8$^+$ ILT2/LIR1$^+$ (open columns) and CD3$^+$ CD8$^+$ KIR$^+$ (filled columns) T cells, permeabilized, and stained with a labeled anti-Bcl-2 Ab. The graph shows intracellular Bcl-2 levels (measured in arbitrary fluorescence units) in each subset of CD8$^+$ T cells of four donors. Significantly ($p < 0.005$) lower levels of Bcl-2 are found in the ILT2/LIR1$^+$ population.

These phenotypic and functional results allow us to propose a scheme for the sequential acquisition of LRC-encoded receptors by activated CTLs. In our previous study of KIR$^+$ T cell clones (21), we suggested that the diverse KIR phenotypes displayed by a dramatically expanded clone of T lymphocytes represented the end result of a program of KIR gene activation which began with the expression of KIR2DL4, a receptor which has been demonstrated to be expressed in all KIR$^+$ NK or T cells (23).

Our present study supports such a serial mechanism of LRC receptor expression and allows this to be extended to a stage before the expression of KIR2DL4. The dramatically increased proportion of activated T cells that are ILT2/LIR1$^+$ KIR$^-$, as exemplified by the tetramer binding population, can be regarded as effector CTL because they comprise the greater number of Ag-specific cells, are CD56$^+$ (24) and are prone to apoptosis when stimulated (25). This association of ILT2/LIR1 gene expression with T cell activation may be related to our finding of an NCAM (CD56)-associated transcription element in the 5′-untranslated region of ILT2/LIR1 cDNA clones (N.Y., unpublished data). However, the precise signals determining Ig-like receptor gene expression are currently unknown. Although certain cytokines, such as IL-15 (26), TGF-β (27), and IL-10 (28), have been reported to induce or modulate expression of the lectin-like receptors CD94 and NKG2A on T cells, no effects on LRC-encoded Ig-like receptor expression have been observed. The relevance of KIR2DL4 expression at the initiation of KIR gene expression in T cells is unclear. Although this receptor has been detected at the mRNA level in all KIR$^+$ cells, its expression on the cell surface is currently controversial. One study reports KIR2DL4 expression on all peripheral NK cells (23), whereas another suggests that KIR2DL4 is only expressed on the surface of decidual NK cells in pregnancy (29). The significance of the apparent specificity of KIR2DL4 for the nonclassical HLA-G class I molecule (23, 29) is also unclear because HLA-G expression is highly tissue restricted (30). Specific

reagents to reliably detect KIR2DL4 are required before these matters can be fully resolved.

The relatively small population of KIR^+ cells represent clones that have made the transition to a state of long-lived memory T cells in a scheme that is consistent with the recently demonstrated linear differentiation of specific memory CTL in transgenic mice (31). The proportions of KIR^+ and KIR^- cells within the ILT2/$LIR1^+$ T cell population are consistent with the numbers of effector and memory cells reported in the anti-HY response of these mice. This situation is likely to be observed in chronic viral infections because the pool of responsive peptide-specific T cells will continually be exposed to Ag (32), becoming activated and expanding in number before maintaining dynamic homeostasis through apoptosis, with a small percentage of cells remaining as KIR^+ memory cells. Such a predominance of effector cells may not be observed where viral infections are cleared and only the relatively scarce Ag-specific memory cells will be detectable (33).

Recently, it has been suggested that memory T cells can be distinguished into two types (effector and central) based on the expression of CCR7 chemokine receptors, correlating with their in vivo trafficking (34). Due to the unavailability of the Abs defining the CCR7 chemokine receptor we have been unable to directly assess how this correlates with the expression of specific Ig-like receptors. We would suggest that the major population of ILT2/$LIR1^+$ KIR^- cells represent the effector cells characteristic of an initial immune expansion, while future studies should allow us to define whether the ILT2/$LIR1^+$ KIR^+ cells we have identified are comparable with the effector memory subset defined by Sallusto et al. (34).

Our results also indicate a possible role for KIR in the maintenance of memory T cell survival and resistance to activation-induced cell death. Effector $CD8^+$ cells have lower levels of the cell protective molecule Bcl-2 (35), which results in sensitivity to apoptosis induced by fratricide (36) or TNF-α (37). Our findings support these reports and suggest that KIR expression in T cells is associated with resistance to activation-induced cell death mediated through stimulation of the TCR. At present, we cannot assign this anti-apoptotic role directly, as it is possible that KIR expression in memory cells is simply coordinate with that of another molecule(s) that confers this survival feature (38). However, KIR have been suggested to bind the p85α subunit of PI3-kinase leading to activation of the anti-apoptotic AKT kinase (39), and the functionally equivalent Ly49 receptors in mice may be involved in the selection and survival of individual NK clones (40).

The differential expression of ILT2/LIR1 and KIR in activated T lymphocytes is consistent with differences in the structure and ligand binding specificities of these molecules. Whereas KIR bind defined epitopes in the α1 domain of HLA-C or HLA-B molecules, the larger size of the four Ig-domain ILT2/LIR1 molecule is compatible with its binding to the common α3 domain of class I in an extended conformation, similar to that of CD8 (41). If such a model is correct, the molecular dimensions of ILT2/LIR1, in addition to the nature of its ligand binding site, would allow concomitant binding of both TCR and ILT2/LIR1 to the same HLA class I ligand molecule. This would enable the recruitment of phosphatases directly to the site of TCR signaling and the subsequent diminution of cellular activation.

In conclusion, our study indicates a program of sequential expression of Ig-superfamily receptors encoded within the LRC, with ILT2/LIR1 expression as an initial event in early stage, activated cytotoxic effector T cells followed by the acquisition of KIR at the stage of transition to differentiated memory cells. The expression of KIR appears to correlate with a resistance to activation-induced cell death in these cell populations, although the requirement for KIR interaction with self-HLA class I in this process is currently unknown.

In view of the close developmental and functional relationships between NK and $CD8^+$ T lymphocytes (42), further investigation of the events occurring in activated $CD8^+$ T cells should enable the use of such cells as an appropriate working model for analysis of LRC gene transcription and expression. Future studies will allow us to determine the potential role of these Ig-like receptors in the long-term survival of memory T cells.

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References

1. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359.*
2. Baker, E., A. D'Andrea, J. H. Phillips, G. R. Sutherland, and L. L. Lanier. 1995. Natural killer cell receptor for HLA-B allotypes, NKB1: map position 19q13.4. *Chromosome Res. 3:511.*
3. Wende, H., M. Colonna, A. Ziegler, and A. Volz. 1999. Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. *Mamm. Genome 10:154.*
4. Colonna, M., H. Nakajima, F. Navarro, and M. Lopez-Botet. 1999. A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *J. Leukocyte Biol. 66:375.*
5. Borges, L., M.-L. Hsu, N. Fanger, M. Kubin, and D. Cosman. 1997. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J. Immunol. 159:5192.*
6. Wagtmann, N., S. Rojo, E. Eichler, H. Mohrenweiser, and E. O. Long. 1997. A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors. *Curr. Biol. 7:615.*
7. Arm, J. P., C. Nwankwo, and K. F. Austen. 1997. Molecular identification of a novel family of human Ig superfamily members that possess immunoreceptor tyrosine-based inhibition motifs and homology to the mouse gp49B1 inhibitory receptor. *J. Immunol. 159:2342.*
8. Fanger, N., D. Cosman, L. Peterson, S. C. Braddy, C. R. Maliszewski, and L. Borges. 1998. The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fc receptor-mediated signalling in monocytes. *Eur. J. Immunol. 28:3423.*
9. Nakajima, H., J. Samaridis, L. Angman, and M. Colonna. 1999. Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor γ-chain 1. *J. Immunol. 162:5.*
10. Valiante, N. M., M. Uhrberg, H. G. Shilling, K. Lienert-Weidenbach, K. L. Arnett, A. D'Andrea, J. H. Phillips, L. L. Lanier, and P. Parham. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity 7:739.*
11. Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature 391:703.*
12. Mingari, M. C., C. Vitale, A. Cambiaggi, F. Schiavetti, G. Melioli, S. Ferrini, and A. Poggi. 1995. Cytolytic T-lymphocytes displaying natural killer (NK)-like activity: expression of NK-related functional receptors for HLA class I molecules (p58 and CD94) and inhibitory effect on the TCR-mediated target cell lysis or lymphokine production. *Int. Immunol. 7: 697.*
13. D'Andrea, A., C. Chang, J. H. Phillips, and L. L. Lanier. 1996. Regulation of T cell lymphokine production by killer cell inhibitory receptor recognition of self HLA class I alleles. *J. Exp. Med. 184:789.*
14. Phillips, J. H., J. E. Gumperz, P. Parham, and L. L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science 268:403.*
15. Mingari, M. C., F. Schiavetti, M. Ponte, C. Vitale, E. Maggi, S. Romagnani, J. Demarest, G. Pantaleo, A. S. Fauci, and L. Moretta. 1996. Human $CD8^+$ T lymphocyte subsets that express HLA class I-specific inhibitory receptors represent oligoclonally or monoclonally expanded cell populations. *Proc. Natl. Acad. Sci. USA 93:12433.*
16. Colonna, M., F. Navarro, T. Bellón, M. Llano, P. García, J. Samaridis, L. Angman, M. Cella, and M. López-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med. 186:1809.*
17. Yssel, H., J. E. D. Vries, M. Koken, W. V. Blitterswijk, and H. Spits. 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods 72:219.*
18. Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity 7:753.*
19. Uhrberg, M., and P. Wernet. 1996. Quantitative assessment of the human TCRBV repertoire by competitive PCR. *J. Immunol. Methods 194:155.*
20. Hamann, D., S. Kostense, K. C. Wolthers, S. A. Otto, P. A. Baars, F. Miedima, and R. A. van Lier. 1999. Evidence that $CD8^+$ $CD45RA^+$ $CD27^-$ cells are induced by antigen and evolve through extensive rounds of division. *Int. Immunol. 11:1027.*

21. Uhrberg, M., N. M. Valiante, N. T. Young, L. L. Lanier, J. H. Phillips, and P. Parham. 2001. The repertoire of killer cell Ig-like receptor and CD94:NKG2A receptors in T cells: clones sharing identical $\alpha\beta$ TCR rearrangement express highly diverse killer cell Ig-like receptor patterns. *J. Immunol. 166:3923.*
22. Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med. 182:1545.*
23. Rajagopalan, S., and E. O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med. 189:1093.*
24. Pittet, M. J., D. E. Speiser, D. Valmori, J.-C. Cerottini, and P. Romero. 2000. Cytolytic effector function in human circulating $CD8^+$ T cells closely correlates with CD56 surface expression. *J. Immunol. 1148.*
25. Hamann, D., P. A. Baars, M. H. G. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. W. van Lier. 1997. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. *J. Exp. Med. 186:1407.*
26. Mingari, M. C., M. Ponte, S. Bertone, F. Schiavetti, C. Vitale, R. Bellomo, A. Moretta, and L. Moretta. 1998. HLA class I-specific inhibitory receptors in human T lymphocytes: interleukin 15-induced expression of CD94/NKG2A in superantigen- or alloantigen-activated $CD8^+$ T cells. *Proc. Natl. Acad. Sci. USA 95:1172.*
27. Bertone, S., F. Schiavetti, R. Bellomo, C. Vitale, M. Ponte, L. Moretta, and M. C. Mingari. 1999. Transforming growth factor-β-induced expression of CD94/NKG2A inhibitory receptors in human T lymphocytes. *Eur. J. Immunol. 29:23.*
28. Galiani, M. D., E. Aguado, R. Tarazona, P. Romero, I. Molina, M. Santamaria, R. Solana, and J. Pena. 1999. Expression of killer inhibitory receptors on cytotoxic cells from HIV-1-infected individuals. *Clin. Exp. Immunol. 115:472.*
29. Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta, and M. C. Mingari. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl. Acad. Sci. USA 96:5674.*
30. Crisa, L., M. T. McMaster, J. K. Ishii, S. J. Fisher, and D. R. Salomon. 1997. Identification of a thymic epithelial cell subset sharing expression of the class Ib HLA-G molecule with fetal trophoblasts. *J. Exp. Med. 186:289.*
31. Opferman, J. T., B. T. Ober, and P. G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science 283:1745.*
32. Emery, V. C., A. V. Cope, E. F. Bowen, D. Gor, and P. D. Griffiths. 1999. The dynamics of human cytomegalovirus replication in vivo. *J. Exp. Med. 190:177.*
33. Coles, M. C., C. W. McMahon, H. Takizawa, and D. H. Raulet. 2000. Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors. *Eur. J. Immunol. 30:236.*
34. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
35. Grayson, J. M., A. J. Zajac, J. D. Altman, and R. Ahmed. 2000. Increased expression of Bcl-2 in antigen-specific memory $CD8^+$ T cells. *J. Immunol. 164: 3950.*
36. Su, M. W., P. R. Walden, H. N. Eisen, and D. E. Golan. 1993. Cognate peptide-induced destruction of $CD8^+$ cytotoxic T lymphocytes is due to fratricide. *J. Immunol. 151:658.*
37. Alexander-Miller, M. A., M. A. Derby, A. Sarin, P. A. Henkart, and J. A. Berzofsky. 1998. Supraoptimal peptide-major histocompatibility complex causes a decrease in Bcl-2 levels and allows tumor necrosis factor α receptor II-mediated apoptosis of cytotoxic T lymphocytes. *J. Exp. Med. 188:1391.*
38. Screaton, G., and X.-N. Xu. 2000. T cell life and death signalling via TNF-receptor family members. *Curr. Opin. Immunol. 12:316.*
39. Marti, F., C. W. Xu, A. Selvakumar, R. Brent, B. Dupont, and P. D. King. 1998. LCK-phosphorylated human killer cell-inhibitory receptors recruit and activate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA 95:11810.*
40. Lowin-Kropf, B., and W. Held. 2000. Positive impact of inhibitory Ly49 receptor-MHC class I interaction on NK cell development. *J. Immunol. 165:91.*
41. Chapman, T. L., A. P. Heikema, and P. J. Bjorkman. 1999. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity 11:603.*
42. Spits, H., L. L. Lanier, and J. H. Phillips. 1995. Development of human T and natural killer cells. *Blood 85:2654.*