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Multiplex and Genome-Wide Analyses Reveal Distinctive Properties of KIR+ and CD56+ T Cells in Human Blood

Wing Keung Chan,* Piya Rujkijyanont,* Geoffrey Neale,† Jie Yang,* Rafijul Bari,* Neha Das Gupta,* Martha Holladay,* Barbara Rooney,* and Wing Leung*§

Killer cell Ig-like receptors (KIRs) on NK cells have been linked to a wide spectrum of health conditions such as chronic infections, autoimmune diseases, pregnancy complications, cancers, and transplant failures. A small subset of effector memory T cells also expresses KIRs. In this study, we use modern analytic tools including genome-wide and multiplex molecular, phenotypic, and functional assays to characterize the KIR+ T cells in human blood. We find that KIR+ T cells primarily reside in the CD56+ T population that is distinctively DNAM-1 high with a genome-wide quiescent transcriptome, short telomere, and limited TCR excision circles. During CMV reactivation in bone marrow transplant recipients, KIR+CD56+ T cells rapidly expanded in real-time but not KIR+CD56+ T cells or KIR+ NK cells. In CMV+ asymptomatic donors, as much as 50% of CD56+ T cells are KIR+, and most are distinguishably KIR2DL2/3+NKG2C+CD57+. Functionally, the KIR+CD56+ T cell subset lyases cancer cells and CMVpp65-pulsed target cells in a dual KIR-dependent and TCR-dependent manner. Analysis of metabolic transcriptome confirms the immunological memory status of KIR+CD56+ T cells in contrast to KIR+CD56− T cells that are more active in energy metabolism and effector differentiation. KIR+CD56+ T cells have >25-fold higher level of expression of RORC than the KIR− counterpart and are a previously unknown producer of IL-13 rather than IL-17 in multiplex cytokine arrays. Our data provide fundamental insights into KIR+ T cells biologically and clinically. The Journal of Immunology, 2013, 191: 1625–1636.

Human NK cells are part of the innate immune system and recognize microbe-infected cells and tumor cells through a combination of activating and inhibitory receptors that do not require somatic gene rearrangement, such as the killer-cell Ig-like receptor (KIR) family (1). T cells, in contrast, mediate adaptive immune response to MHC-bound Ags through recognition by rearranged TCRs (2). KIR generates diversity through variable haplotype gene content, allele polymorphism, and stochastic expression (3, 4), whereas TCR recombines αβ or γδ chains during development (5). Both KIR and TCR generate specificity and are useful developmental markers. KIR+ NK cells are usually CD56dim, cytotoxic, and developmentally more mature than KIR−CD56bright cytokine-secreting NK cells (6). γδ T cells bear more innate-like attributes and appear earlier in the thymus than αβ T cells (5). TCR is never found in NK cells, but a subset of terminally differentiated effector memory T cells expresses KIRs (7–9).

KIR+ T cells were first identified two decades ago (10) and were found in the CD8+, CD4+, TCR-γδ+, and αβ+ T cell fractions (8, 11–15). Most KIR+ T cells are αβ+CD8+, possess a memory phenotype, and are generated upon TCR recognition of HLA-E-associated viral peptides after monoclonal or oligoclonal expansion (16–18). Continuous TCR engagement sustains their KIR expression with resultant resistance to apoptosis (19–23). These cells are important in the control of infections such as CMV and hepatitis C virus infections (14, 15, 18, 24, 25). KIR expression and function are fundamentally different in T cells and NK cells (26). For instance, the KIR repertoire in NK cells is different from that in T cells from the same individual (27, 28). Although KIR acquisition during NK cell development is stochastic, essential for licensing and tuning of responsiveness to self-MHC, KIR is acquired in T cells after TCR rearrangement and Ag encounter, and its repertoire is independent of self-MHC (9, 28, 29). The KIR promoters on NK cells have a minimal size of 120–250 bp, are regulated by all-or-none methylation, and involve transcriptional factors such as YY1, CRE/ATF, RUNX3, and Sp1 (30–34). In contrast, the KIR promoter in T cells has a minimal size of 60 bp, patchy methylation, and involvement of different sets of transcriptional factors (35, 36). Although inhibitory KIRs have similar suppressive function in NK cells and T cells (37–39), activating KIRs appear unable to trigger T cells directly and serve rather in a costimulatory role without consistent DAP12 expression (40–42).

Similar to KIR, CD56 is another NK-related receptor that is expressed on a small subset of T cells characterized by reduced proliferative potential because of upregulation of P16 and P53 (43, 44). Most KIR+ T cells are CD56−, and the CD56 expression level correlates well with both NK and CD8+ CTL functions (45). In this study, we used modern genome-wide and high-throughput multiplex assays to characterize the KIR+ T cells in human blood. We found that KIR+ T cells primarily resided in the CD56− T cell population that was distinctively DNAM-1 high with a unique genome-wide quiescent transcriptome substantially different from that of the CD56+ T cells, NK cells, and Vc24+Vb11+ invariant (i) NKT cells. In addition, the KIR+ subset of CD56+ T cells was cytotoxic to cancer cells and CMV-infected cells in a dual KIR- and TCR-dependent manner, whereas the KIR+ subset was a unique metabolically active RORC+ cytokine producer of IL-13.

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Abbreviations used in this article: FDR, false-discovery rate; αGC, α-galactosylceramide; i, invariant; KIR, killer cell Ig-like receptor; TREC, TCR excision circle.

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In a bone marrow transplant cohort, KIR "CD56+" T cells expanded rapidly during CMV reactivation, and these KIR+ cells remained prevalent in asymptomatic CMV+ donors with a characteristic phenotype of KIR2DL2/3"NKG2C/CD57". Our findings provide a fundamental understanding of the biology of these unique subsets of T lymphocytes and have significant implications in medicine and cellular therapy.

**Materials and Methods**

**Cell purification and flow cytometry**
Heathly donor peripheral blood was obtained after informed consent approved by the St. Jude Children's Research Hospital Institutional Review Board. Lymphocyte subsets were sorted with FACSariaII (BD Biosciences) and FlowJo 8.8.6 software (Tree Star, Ashland, OR). The purity of CD3"CD56+" T cells, CD3"CD56-" NK cells, CD3"CD56+" T cells, CD3"CD56+"KIR+ T cells, and CD3"CD56-"KIR+ T cells was >98% in all experiments. InKt cells were cloned from PBMCs using a modified protocol (46). Briefly, the PBMCs were cultured with 100 ng/ml of galacatosylceramide (OαG) and 50 ng/ml IL-2 for 3 wk. The InKt cells were flow sorted for TCRVα24 (clone 6B11). Then, the cells were restimulated with allogeneic PBMCs, fresh IL-2, and OαG every week. Before each experiment, cells were resorted for TCRVα24. The purities of InKt for TCRVα24 and CD1d-tetramer were 98% and respectively.

**Phenotypes of cell subsets were analyzed by flow cytometry with LSRII (BD Biosciences) and FlowJo 8.8.6 software (Tree Star, Ashland, OR).** The following clones of Abs were used: anti-KIR2DL1 (EB6.B), anti-KIR2DL2/3 (CH-L and GL183), anti-KIR3DL1 (DX9), anti-CD95 (UB2), anti–DNAM-1 (BD Biosciences) and FlowJo 8.8.6 software (Tree Star, Ashland, OR). The expression of HLA-ABC was monitored by the St. Jude Children's Research Hospital Institutional Review Board. Lymphocyte subsets were sorted with FACSAriaII (BD Biosciences, San Jose, CA) using anti-CD3, anti-CD56, anti-CD14, anti-KIR2DL1, anti-KIR3DL1, anti-CD95 (UB2), anti–DNAM-1 (BD Biosciences), and anti–CD56 (BD Biosciences). The purity of CD3"CD56+" T cells, CD3"CD56-" NK cells, CD3"CD56+" T cells, CD3"CD56+"KIR+ T cells, and CD3"CD56-"KIR+ T cells was >98% in all experiments. InKt cells were cloned from PBMCs using a modified protocol (46). Briefly, the PBMCs were cultured with 100 ng/ml of galacatosylceramide (OαG) and 50 ng/ml IL-2 for 3 wk. The InKt cells were flow sorted for TCRVα24 (clone 6B11). Then, the cells were restimulated with allogeneic PBMCs, fresh IL-2, and OαG every week. Before each experiment, cells were resorted for TCRVα24. The purities of InKt for TCRVα24 and CD1d-tetramer were 98% and respectively.

**Multiplex cytokine measurement**

The production of Th1, Th2, regulatory T cell, and Th17 cytokines, after stimulation with 1 of the 20 stimuli for 3 d, was measured by the Luminex system using a Milliplex MAP 7-plex Panel I kit for IFN-γ, IL-1, IL-5, IL-6, IL-10, IL-13, and IL-17; Panel II kit for IL-21; and Panel III kit for TGF-β. Cells were plated at a density of 5 × 10^5 cells/well. Stimuli included TLR ligands 1–9 (Human TLR1–9 agonist kit; InvivoGen, San Diego, CA), EBV (B95-8 type 1 purified viral lysate), HSV-1 (MRC-5 (from ATCC, Rockville, MD), and 5% human AB serum (Lonza, Basel, Switzerland) for 5 d. The cells were allowed to proliferate for 7 more days with the addition of low-dose IL-2 (10 ng/ml; R&D Systems) and IL-23 (20 ng/ml; R&D Systems).

**CD4+ T cells from various lymphocyte subsets were sorted by flow cytometry (Aria II, BD Biosciences) and cultured under Th17 differentiation conditions as described previously (53). Cells plated at 5 × 10^5 per well were primed with immobilized CD3 and CD28 Abs for 3 d and then incubated with IL-2 (20 U/ml; R&D Systems, Minneapolis, MN), IL-1β (10 ng/ml; R&D Systems), IL-6 (50 ng/ml; R&D Systems), IL-23 (20 ng/ml; R&D Systems), neutralizing anti–IFN-γ (10 μg/ml; BD Biosciences), and anti–IL-4 (10 μg/ml; BD Biosciences) for 5 d. The cells were allowed to proliferate for 7 more days with the addition of low-dose IL-2 (10 U/ml; R&D Systems) and IL-23 (20 ng/ml; R&D Systems).

**CD107 degranulation assay**
Lymphocyte subsets were activated for 3 d with either 500 U/ml recombinant human IL-2 and 10 ng/ml IL-15 (R&D Systems) or anti-CD3 (OKT3) and CD28 (CD28.2) Abs (BioLegend) and then used in a CD107 mobilization assay with K562 cells as the target (4). A redirected assay was performed with PB15 cells (American Type Culture Collection, Manassas, VA) that were preincubated with the indicated Abs (5 μg/ml) and washed twice before being used as the target cells.

**Statistical analysis**

The exact Wilcoxon signed-rank test was used to compare samples from the same donor, and the exact Wilcoxon rank-sum test was used for independent
samples. The relationships of various lymphocyte subsets were revealed using dendrograms by cluster analysis with the complete linkage method based on surface phenotype similarity. All analyses were performed using R software (version 2.10). No adjustments were made for multiple testing.

**Results**

**KIR+ T cells primarily reside in the CD56+ T cell population with limited TCRβ repertoire, TCR excision circle, and telomere length**

In adult blood, about half of the NK cells were KIR+; in contrast, only ~1% of CD3+ T cells were KIR+, and they primarily resided in the CD56+ rather than the CD56- fractions (Fig. 1A, 1B). CD56+ T cells accounted for ~4.8% of the lymphocytes in adult blood (Supplemental Fig. 1A), but they were rare in cord blood (0.41% on average; Supplemental Fig. 1B). In iNKT cells, a substantial percentage was CD56+ (average, 36.9 ± 21.7%; Supplemental Fig. 1C), but they were all KIR-.

Compared with CD56- T cells, CD56+ T cells had a less diverse TCRβ repertoire and a skewed Gaussian pattern (Supplemental Fig. 1D). As expected, iNKT cells were solely TCRβ11+, and NK cells did not express any TCRβ. Thus, CD56+ T cells have a lower TCRβ complexity score than CD56- T cells but higher than that of NK and iNKT cells (Fig. 1C). CD56+ T cells also had lower copy numbers of the signal joint TCR excision circle (TREC) than CD56- T cells (Fig. 1D), indicating that CD56+ T cells had undergone extensive postthymic proliferation. This finding supported the observation that the telomeres of CD56+ T cells were shorter than those of CD56- T and NK cells (Fig. 1E).

CD56+ T cells have distinct surface phenotype and genome-wide transcriptional pattern

To distinguish CD56+ T cells from CD56- T, NK, and iNKT cells, we performed an immunophenotyping array using 41 NK and T biomarkers related to activation, costimulation, inhibition, cytotoxicity, adhesion, and development (Fig. 2A, Supplemental Table I). Compared with CD56- T cells, CD56+ T cells expressed higher levels of NK markers including CD16, CD94/NKG2a, NKG2D, CD122, DNAM-1, and granzyme B as well as TCRγδ and CD8. In contrast, CD56- T cells expressed more CD4, CD27, and TCRαβ than CD56+ T cells. Compared with NK cells, CD56+ T cells expressed more CD4, CD8, and activation markers CD27 and CD44 but less NK-associated molecules including CD16, Nkp30, Nkp46, 2B4, and CD122. Remarkably, no marker was expressed more in both CD56+ T and NK cells than in CD56- T cells. Compared with iNKT cells, CD56+ T cells expressed more CD22, CD8, CD45RA, CD27, and CD62L, as well as NK receptors CD16, NKT-A, NKG2D, DNAM-1, Nkp46, 2B4, NKG2a, and CD94, but less CD4, CD38, CD25, CD45RO, and CD69. Notably, CD56+ T cells expressed the most DNAM-1, CD8, and TCRγδ among the four cell types. In a dendrogram generated based on data from all 41 biomarkers using cluster analysis with the complete linkage method (Fig. 2B), CD56+ T and NK cells were in the same branch but were disparate from CD56- T cells and farthest from iNKT cells in surface phenotype.

Because phenotypic analyses using Abs and flow cytometry in this and prior studies could provide only limited data, we further characterized the CD56+ T cells at a genome-wide level by comparing the transcriptome in the four cell types using microarray profiling. A large number of genes (9366) were differentially transcribed ($p = 0.0006$, FDR < 0.01). Pairwise comparisons between CD56+ T cells and each other cell type revealed 101 CD56+ T "signature genes" that were differentially expressed with $p < 0.0024$ and FDR < 0.3 (Fig. 2C). Among those transcripts, nine genes were unidentified, and 30% were enriched compared with CD56- T. NK, and iNKT cells, especially those involved in transcription repression in MAPK pathways, such as znf594, gdpd5, tcfp221, and zn4g12. Genes that were preferentially downregulated in CD56+ T cells were those involved in metabolism, such as stx6, nnt, galnt2, and hvenl; in DNA replication, such as tums, rpa1, mif1, and escop; and in adhesion, such as cd47 and tsap3. Analysis using the Kyoto Encyclopedia of Genes and Genomes canonical pathway database (49) showed that the activity of 29 pathways was significantly suppressed in CD56+ T cells compared with the other subgroups. Among the top pathways were those associated with cell division and metabolic activity, including cell cycle, DNA and nucleotide synthesis, oxidative phosphorylation, fatty acid biosynthesis, amino acid metabolism, and allograft rejection. Notably, no pathways were significantly enriched in CD56- T cells, indicating definitively their relative transcriptional quiescence and defining ultimately their molecular identity apart from other lymphocyte subsets.

CD56+ T cells are cytotoxic in proinflammatory milieu through the KIR+ subset

Although CD56+ T cells have been called "NK-like," surprisingly, we found that highly purified resting CD56+ T cells were tolerant to standard NK-susceptible targets, such as K562, THP-1, U937, Jurkat, and 721.221 cells (Supplemental Fig. 1E), compatible with our genome-wide hypoaactive transcriptome data. In addition, they are tolerant to standard iNKT-susceptible targets such as CD1d-restricted α-GC-loaded Jurkat cells (Supplemental Fig. 1F). To confirm that the nonreactivity of CD56+ T cells to α-GC-loaded Jurkat cells was not a unique target cell–line effect, we generated a CD1d-transduced cell line using NK-resistant RS4;11 cells to avoid any false readout by contaminating NK cells. We found that the α-GC-loaded RS4;11-CD1d mutant was indeed susceptible to killing by iNKT cells but not by CD56+ T cells.

On the basis of the genome-wide quiescent transcriptional pattern, we hypothesized that CD56+ T cells required a proinflammatory milieu to exhibit their NK-like activity. Indeed, CD56+ T cells stimulated with IL-2 and IL-15 or IL-12 and IL-18 could kill all NK-susceptible targets (Fig. 3A). Because the KIR+ rather than KIR- subset of NK cells is primarily cytotoxic, we further hypothesized that a similar distinction existed in CD56+ T cells. In a single-cell degranulation assay, significantly more K562-reactive cells were found in KIR+ than in KIR-CD56+ T cells (Fig. 3B). Similarly, a significantly higher percentage of KIR+CD56+ T cells degranulated in the presence of FcγRIIa+ murine target cells coated with Abs specific to CD3/CD28 (Fig. 3C), even though both subsets were CD3+, and the KIR-CD56+ T cells expressed significantly less CD28 and CD90 (Supplemental Fig. 2A). Using anti-CD16 Ab–coated P815 cells, we found that CD107a+ cells were confined to the KIR+ CD56+ T subset, whereas <5% of the KIR+CD56+ T had degranulation (Fig. 3D). In CD56- T cells, the TCRγδ+ rather than the TCRαβ+ population is naturally cytotoxic (54). In CD56+ T cells, we found that the TCRγδ+ population was enriched with the KIR2DL2/ 3+ and KIR3DL1+ cytotoxic subset (Supplemental Fig. 2B).

KIR+CD56+ T cells are phenotypically distinct and more NK-like than KIR-CD56+ T cells

In comparison with KIR+CD56+ T cells, TCR and flow analyses revealed considerably restricted TCRβ spectrum (Supplemental Fig. 2C) and higher expression of Nkp46, NKG2D, 2B4, CD16, CD38, CD122, CD11a, and granzyme B in KIR+CD56+ T cells (Supplemental Fig. 2D). We then extended the comparison with genome-wide level by expression profiling of KIR+ and KIR-CD56+ T cells in the context of those in NK, CD56- T, and iNKT cells. As shown in the principal component analysis plot (Fig. 3E), the KIR+ subset clustered closer to NK cells, whereas the KIR- subset...
was closer to CD56– T cells. Among the 146 genes differentially expressed between the KIR+ and KIR– subsets of CD56+ T cells \((p < 0.001, \text{FDR} < 0.28; \text{Fig. 3F})\), genes in glycolysis, glutaminolysis, and ATP synthesis were enriched in the KIR– subset. NADP+-dependent cytosolic malic enzyme \((me1)\), which converts malic acid to pyruvate during fatty acid biosynthesis, had the highest fold difference between the two subsets with 42.37-fold higher expression in KIR– population. Other genes differentially expressed in the KIR– subset regulated lipid metabolism (e.g., \(elov4\) and \(cyp2e1\)), solute/nutrient transport (e.g., \(clc5\), \(kcnk6\), and \(slc4a7\)), and differentiation (e.g., \(rorc\), \(il4i1\), \(ltk\), and \(runx2\)). In contrast, the KIR+ subset was characterized by the lack of transcripts for glycolysis and glutaminolysis but increased expression of genes related to cytotoxicity regulation (e.g., \(arrb\)) and viral infection (e.g., \(ctbp2\), \(golm1\), and \(trim6-34\)). It is known that during T cell activation and memory development, metabolic transcription program could alter cell fate and differentiation (55); thus, although activated T cells increase nutrient uptake, ATP synthesis, and energy utilization mainly through glycolysis and glutaminolysis, memory T cells by contrast maintain housekeeping energy conversion process through lipid oxidation (56, 57). Collectively, our gene expression data support the hypothesis that KIR–CD56+ T cells are metabolically active T subset poised to effector differentiation and reprogramming, whereas KIR+CD56+ T cells are metabolically quiescent memory cells equipped with cytotoxic pathways set for long-term pathogen control.

**KIR+CD56+ T cells expand in human CMV infection**

Previous studies have shown that some KIR+ T cells are CMV specific (15, 18, 24, 25), but the kinetics of their response to CMV
FIGURE 2. CD56+ T cells are distinct from NK, CD56− T, and iNKT cells in surface phenotype. (A) Box plots summarize the percentage of cells testing positive in CD56− T, NK, CD56+ T, and iNKT populations. Markers without substantial differences are not shown in the figure: NKp44, NTB-A, FasL, TRAIL-R1, TRAIL-R2, TRAIL, Fas, CD48, PVR, 4-1BB, ICAM-1, CD69, CLTA-4, CD11a, and CD90. (B) Dendrogram shows the relationship among CD56− T, CD56+ T, NK, and iNKT cells in surface phenotype using all 41 biomarkers. (C) Hierarchical clustering of 101 transcripts with differential expression between CD56+ T cells and other cell types. Red indicates relative high expression, and green indicates relative low expression across the data set. Each column represents a sample from different individuals. Names of the genes significantly upregulated or downregulated in CD56+ T cells are listed on the right. Data in (A)–(C) are from five to six individuals.
reactivation is unclear. Therefore, we examined a cohort of donors and recipients of hematopoietic stem cell transplantation. We found that almost half of the CD56+ T cells were KIR+ in healthy asymptomatically CMV-seropositive donors, which was significantly more than in CMV-seronegative donors ($p = 0.0018$; Fig. 4A).

Notably, KIR2DL2/3 was overrepresented in CMV+ donors but not KIR2DL1 or KIR3DL1; thus, the majority of KIR positivity in CD56+ T cells was related solely to KIR2DL2/3 expression (Fig. 4B). Furthermore, in CMV+ donors, expression of CD57 in KIR+CD56+ T cells was higher than that in KIR–CD56+ T cells (Fig. 4C), whereas nearly all NKG2C+CD56+ T cells resided in the KIR+ rather than the KIR– population (median, 37.7 versus 5%; Fig. 4D). Thus, KIR2DL2/3CD57NKG2C+ was a signature phenotype of the KIR+CD56+ T cells.

Among the 30 stem cell transplant recipients, 12 (40%) had CMV reactivation detectable in blood by real-time PCR in the first 100 d after transplantation. Notably, all had an increase in the percentage of the KIR+ subset in CD56+ T cells at the time of CMV reactivation. When the CMV titers started to rise, both the percentage and absolute number of KIR+CD56+ T cells increased (Fig. 4E). Once the viremia was under control, the KIR+CD56+ T population gradually decreased but remained generally higher than before viral reactivation. In contrast, patients without CMV reactivation during transplant courses had constant KIR+CD56+ T cell numbers (Fig. 4F). Thus, overall, the median percentage of KIR+CD56+ T cells during the first 100 d after transplantation was significantly higher ($p = 0.0021$) in patients with CMV reactivation than in those without (Fig. 4G).

During CMV reactivation in the transplant recipients, we did not observe a similar response in the KIR+CD56– T cell or KIR+CD56+ NK cell populations. When these three cell populations from seropositive donors were investigated on their potential for memory...
response to CMV in vitro after 2 wk of activation using 1 μg/ml CMVpp65 peptide, the percentage of the KIR+ subset increased only in the CD56+ T cell population (from median 41.3–73%) and not in CD56– T cells (p, 0.05; Fig. 5A). Furthermore, expansion was not observed in KIR+CD56+ T cells obtained from CMV-seronegative donors, indicating that the expansion in seropositive donors was a memory response.

The median frequency of HLA-A*02:01 CMVpp65 tetramer+ cells was 0.39% in KIR+CD56+ T cells, which was 4-fold higher than that in KIR+CD56– T cells and 1 log higher than that in KIR+CD56+ T or KIR+CD56– T cells in seropositive donors (Fig. 5B). Notably, the mean fluorescence intensity was much higher in the KIR+ than in the KIR–CD56+ T cells (Fig. 5C). No binding was observed in either KIR– or KIR+ CD56+ T cells from seronegative donors.

Using IL-2, IL-15 and OKT3, we successfully expanded ex vivo and subsequently flow-sorted enough CMVpp65-specific KIR+ subsets of CD56+ T cells for further functional experiments. We tested whether KIR+CD56+ T cells, which were obtained from HLA-A*02:01+ CMV-seropositive donors and proliferated ex vivo
KIR+CD56+ T cells in human blood

for 2 wk in response to CMVpp65 peptide, had variable cytotoxicity to CMV-infected cells in a KIR-dependent manner. The NK cell–resistant HDLM-2 lymphoblastic cell line, which was HLA-A*02:01+ and HLA-C1+ (ligand for inhibitory KIR2DL2/3), was used as the target. For CMVpp65-tetramer+CD56+ T cells (Fig. 5D), the KIR+CD56+ T cells had only a modest effect on the CMVpp65-peptide–loaded HDLM-2 cells pretreated with pan-MHCI Ab (black bar) or untreated (white bar). Unloaded HDLM-2 cells with (gray bar) or without (dotted white bar) pan-MHCI Ab were used as control. In contrast, CMVpp65-peptide–specific CD56+ T cells were tested against CMVpp65-peptide–loaded HDLM-2 cells with or without pan-MHCI Ab. Data represent four (C–E) independent experiments.

Rorc was among the top 5 of 146 genes differentially expressed between KIR+ and KIR− subsets of CD56+ T cells in our genome-wide expression analysis (Fig. 3F). The KIR+ subset had a 27.7-fold higher expression (Fig. 6A), which was confirmed by real-time PCR (Fig. 6B). Rorc mRNA was found in CD56− T and iNKT cells as well, but the level of Rorc was significantly higher in KIR+CD56+ T cells than in CD56+ T, NK, and KIR−CD56+ T cells.
**Rorc** is the human homolog of murine transcription factor **Rorγt**, which is involved in IL-17 induction in subsets of αβ and γδ T cells (53, 58). **Rorc** also directs the production of IL-17 in iNKT cells (59) and IL-17 and IL-22 in lymphoid tissue–inducer cells and NK-like cells (52). In contrast, mature NK cells express little or no RORC (52). We questioned whether the expression of rorc in the KIR’CD56+ T subset was related to IL-17 production, similar to that in CD56− T lineages. We first investigated whether the RORC protein could be induced and maintained in CD56− T cells. Under Th17 differentiation conditions with IL-1β, IL-6, IL-2, and IL-23 (53), a prominent population of RORC+ cells was found in both the KIR’CD56− Th and KIR’CD56− Th populations.
but not in NK cells (Fig. 6C). Notably, the KIR⁺CD56⁺ T cell subset remained negative for RORC at both the transcript and protein level. Using CD56⁻ T cells as control, we found that KIR⁺CD56⁺ T cells were able to secrete IFN-γ and IL-13 upon PMA stimulation (Fig. 6D, 6E). In comparison with KIR⁺CD56⁺ T cells, KIR⁻CD56⁺ T cells produced significantly more IFN-γ and IL-13 (Fig. 6F).

To further assess the global cytokine profile of KIR⁺CD56⁺ T cells in innate and adaptive immunity, we performed multiplex cytokine arrays using 200 stimulation–cytokine combinations challenging KIR⁺CD56⁺ T cells with 20 common stimuli, including TLR 1–9 agonists, viral lysates, mitogens, and cytokines that favor various Th polarizations. The production of 10 Th1, Th2, regulatory T cell, and Th17 signature cytokines was measured. One-way nonparametric ANOVA showed that 16 of the 200 stimulation–cytokine combinations were statistically different across T, NK, KIR⁻CD56⁺ T, and KIR⁺CD56⁺ T cells (Fig. 6G). As expected, CD56⁻ T cells contained all subpopulations capable of response to various stimuli by producing all types of Th cytokines. NK cells also, as expected, secreted IFN-γ (with PMA or IL-2) and IL-6 (with TLR agonists). However, KIR⁺CD56⁺ T cells did not produce IL-5, IL-6, IL-10, or IL-17 upon stimulation with any TLRs, viral lysates, mitogens, or cytokines tested. All these data suggest that KIR⁺CD56⁺ T cells are not broad cytokine secretors but rather a unique population of previously unknown RORC⁺ IL-13–producing cells.

Discussion

In this study, we confirmed prior findings that KIR⁺CD56⁺ T cells are rare (<1%) in umbilical cord blood and account for <20% of T cells in CMV-seronegative adults (60, 61). However, during CMV reactivation in an immunocompromised state such as that in transplant recipients, we found that the KIR⁺CD56⁺ T population markedly expanded in real time corresponding to viral replication. Notably, after expansion, the proportion of these cells declined as the viral copy number in blood decreased but remained higher than that in the prereactivation phase. Because CMV persists throughout life, it is conceivable that repeated subclinical reactivations may mechanistically account for the expansion of the KIR⁺ fraction over time (62). This growth may occur at the expense of naive T cells to maintain homeostasis in the total T cell number (62). In this regard, the KIR⁺CD56⁺ T subset had a more restricted TCR repertoire than the KIR⁺CD56⁺ cells. In CMV-seropositive donors, as much as 50% of the CD56⁺ T cells were KIR⁺. These cells have a unique signature of being CD57⁺ NKG2C⁺KIR2DL2/3⁻ but KIR2DL1/KIR3DL1⁺. Phenotypically similar CD57⁺NKG2C⁺ NK cells have been observed previously during CMV infection and reactivation (63, 64). Thus, both T cells and NK cells may preferentially use some shared activating receptors such as NKG2C to recognize HLA-E that is altered during immune escape through downregulation of MHC and simultaneously prevent overaggression toward normal cells by prematurely terminating the TCR immune synapse (70). Furthermore, the DNAM-1¹ and CD16¹ subsets of CD56⁺ T cells was predominately KIR⁺, thus allowing both natural cytotoxicity and Ab-dependent cytotoxicity to be self-modulated by KIR.

Another novel finding of this study is that KIR can uncouple the effector function of CD56⁺ T cells. Specifically, although the KIR⁺ subset is cytotoxic and responsive to CMV infection and cancer cells, the KIR⁻ subset is a previously unknown RORC⁺ IL-13 producer. The high level of RORC expression uncovered initially by the genome-wide array in the KIR⁺CD56⁺ T cell population was surprising, because RORC is a transcription factor typically present in Th17 cells and other IL-17–producing cells, including γδ T cells, iNKT cells, and innate lymphoid cells (71). Instead of producing IL-17, this novel RORC⁺ KIR⁻CD56⁺ T subset produces IL-13, which may paradoxically inhibit Th17 cells in production of IL-17 through STAT6 and GATA3 (72, 73). Thus, our finding discloses a potential, unexpected role of RORC in negative regulation of IL-17–associated pathways.

In summary, the two populations of KIR⁺CD56⁺ and KIR⁺CD56⁻ T cells are molecularly, phenotypically, functionally, and metabolically distinct from the other well-characterized lymphocyte subsets. Whereas the KIR⁺CD56⁺ T cytotoxic effector cells may be valuable for immunotherapy of cancers and CMV infections in lymphopenic hosts, the KIR⁺CD56⁻ IL-13–producing cells may be useful for the treatment of IL-17–associated health conditions such as chronic inflammatory diseases and graft-versus-host disease (74, 75). Both populations can be easily isolated for future immunologic studies and clinical use.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.
References


**SUPPLEMENTAL TABLE**

Table S1 Summary of surface marker expression on CD56^+CD3^+ T cells compared with CD56^-CD3^+ T and CD56^-CD3^- NK cells.

<table>
<thead>
<tr>
<th></th>
<th>NK ≥ CD56^+ T ≥ CD56^- T</th>
<th>CD56^- T ≥ CD56^+ T ≥ NK</th>
<th>CD56^+ T &gt; CD56^- T&amp;NK</th>
<th>CD56^- T&amp;NK &gt; CD56^+ T</th>
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<tr>
<td><strong>NK</strong></td>
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<tr>
<td>Activation receptor/co-receptor</td>
<td>Nkp30, Nkp46, 2B4, CD16, CD38</td>
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<tr>
<td>Inhibitory receptor</td>
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<td><strong>NK/T</strong></td>
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<tr>
<td>Cytolysis</td>
<td>Granzyme B</td>
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<td>Cytokine receptor</td>
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<td>CD25, CD127</td>
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<td>CD27</td>
<td>DNAM-1</td>
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<tr>
<td>Adhesion molecule</td>
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<td>CD44, CD62L&lt;sup&gt;hi&lt;/sup&gt;</td>
<td></td>
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<tr>
<td><strong>T</strong></td>
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<tr>
<td>Receptor/co-receptor</td>
<td>CD8&lt;sup&gt;lo/+&lt;/sup&gt;</td>
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<td>TCRγδ, CD8&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CD45RO</td>
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Figure S1 Frequency, phenotype and function of CD56+ T cells. (A) Frequency of CD56+ T cells in adult peripheral blood and cord blood. (B) Percentage of indicated cell types in adult peripheral blood and cord blood. The bars represent the median percentage in 13 adults and 14 cord blood units. (C) Expression of CD56 on TCRVα24+Vβ11+CD3+ iNKT cells. Numbers indicate the percent of cells in each region. (D) CD56- T cells showed a diverse and Gaussian pattern. CD56+ T cells showed less diverse and skewed TVRVβ usages. (E) Lack of cytotoxicity by resting CD56- T cells against leukemic cell lines. NK cells were used as positive control. (F) Lack of CD1d-restricted cytotoxicity. CD56+ T cells were tested against αGC-loaded Jurkat cells or CD1d-expressing, NK-resistant RS4;11-CD1d transduced cells. iNKT cells were used as positive control. The results are mean and S.E.M. from three different experiments. The results shown are representative of five (C-E) and six (F) independent experiments. *** p<0.001
Figure S2 Phenotypes and functions of KIR+ and KIR− CD56+ T cells. (A) Surface expression density of CD3 and percentages of CD28+ and CD90+ on KIR− and KIR+ CD56+ T cells. (B) Box-plots comparing the expression of KIR2DL1, KIR2DL2/3 and KIR3DL1 on TCRαβ+ and TCRγδ+ CD56+ T cells (n=5). (C) TCRβ usage diversity in KIR− and KIR+ subsets of CD56+ T cells as shown by complexity score. The bars in the plot indicate the median (n=6). (D) Box-plots compare the expressions of NK, NK/T and T cell receptors on KIR+ and KIR− subsets of CD56+ T cells (n=5). (E) Cytotoxicity of KIR− and KIR+CD56+ T cells against HDLM-2 cells with or without pan-MHCl antibody (10 μg/mL) or anti-KIR2DL2/3 antibody (10 μg/mL). Effector-to-target ratio was 20:1. Data are from 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001