Distinct Transcriptional Control Mechanisms of Killer Immunoglobulin-like Receptors in Natural Killer (NK) and in T Cells*

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Jing Xu‡†, Abbe N. Vallejo§, Yong Jiang§, Cornelia M. Weyand‡, and Jörg J. Goronzy‡||

From the ‡Department of Medicine, Lowance Center for Human Immunology, Emory School of Medicine, Atlanta, Georgia 30322, the §Departments of Pediatrics and Immunology, University of Pittsburgh School of Medicine, Children’s Hospital Rangos Research Center, Pittsburgh, Pennsylvania 15213, and the ¶Division of Physiological Imaging Research Laboratory, Mayo Clinic, Rochester, Minnesota 55905

Killer immunoglobulin-like receptors (KIR) are expressed by natural killer (NK) cells and by subsets of CD4+ and CD8+ T cells, which are therefore thought to be subject to similar regulatory mechanisms. Here, we show that the transcriptional machinery to express KIR is limited to NK and T cells; however, the KIR transcriptional control differs between these two types of lymphocytes. T cells selectively express transcriptional activators binding to positions −52 to −61 of the KIR promoter, whereas an AML site around position −98 is relevant for transcription in NK cells. Although KIR expression is restricted to subsets of memory T cells, our studies demonstrate that transcriptional activators for KIRs are not acquired during T cell differentiation but are already present in naive T cells, suggesting a basic role of KIRs in T cell biology. We suggest that the regulated expression of KIRs in T cells profoundly influences peripheral tolerance and antigen-specific immune responses.

The killer immunoglobulin-like receptors (KIR)¹ comprise a family of structurally related receptors that recognize major histocompatibility complex (MHC) class I molecules and are preferentially expressed on natural killer (NK) cells (1, 2). Members of the KIR family include inhibitory and activating receptors. Their ability to recognize MHC class I alleles enables them to survey tissue changes in MHC expression patterns. It is thought that this function is central to the role of NK cells in preventing tumor outgrowth (2–5). Inhibitory KIRs may play an important role in immune regulation by actively promoting peripheral tolerance, by enhancing survival of effector cells, and by dampening immune cascades (6–8), whereas stimulatory KIRs have been implicated in active host defense against infectious organisms (9, 10).

KIRs represent the major mechanism of how the NK cell repertoire is diversified (11, 12). Diversity is achieved through different mechanisms. First, the family of KIR receptors has extreme genetic polymorphism, derived from gene duplications and from allelic variations (13). In addition, the expression patterns of these genes apparently follow stochastic rules, and an individual NK cell can express different possible combinations of KIR genes (12). KIR expression in NK cells occurs during NK cell development, but the exact mechanisms that control KIR diversification are unknown (14, 15). The expression patterns of KIRs on NK cells are determined at the transcriptional level, with the contribution of regulatory promoter elements binding different transcription factors and different degrees of promoter demethylation (16–18).

Expression of KIR molecules is tissue-specific, a phenomenon recapitulated in transgenic mice that were constructed using a P1 artificial chromosome that encompasses the KIR region, including 10 KIR genes (19). In addition to NK cells, KIRs are only expressed on small subsets of T cells, including γδ T cells and small subsets of CD4+ and CD8+ T cells (20, 21). It appears that KIR expression increases with age and is exclusively found in T cells with a memory phenotype (22, 23). Why and how T cells gain the expression of KIRs remains unclear. Expression of inhibitory KIRs on T cells has been associated with defective immune function (4). In these studies, KIRs transmit an inhibitory signal that interferes with T cell receptor (TCR)-mediated activation and down-regulates cytokine production by T cells (24). Thus, it has been postulated that the expression of inhibitory KIRs facilitates reactivation of chronic infection and impairs responses to tumor antigens (4). In contrast, expression of stimulatory KIRs has been correlated with autoimmunity. Stimulatory KIRs provide costimulatory signals in the absence of professional antigen-presenting cells and, therefore, circumvent peripheral tolerance mechanisms (25). Such aberrant costimulation appears to play a key pathogenetic role in rheumatoid arthritis where CD4+ T cells have been found to express several regulatory molecules such as stimulatory KIRs and NKG2D that costimulate interferon-γ production and T cell proliferation (26, 27). These observations have led to a model that the gain of KIR expression on CD4+ T cells is a risk factor for autoimmunity by lowering the threshold for the induction of CD4+ T cell responses in a microenvironment that lacks professional costimulatory ligands (28). In further support of this model, stimulatory KIR genes have been associated with an increased risk for rheumatoid vasculitis, diabetes mellitus, and psoriatic arthritis (29–33). In acute coronary syndromes, cytotoxicity mediated by KIR-expressing T cells is in part responsible for smooth muscle cell apoptosis.

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† Deceased February 19, 2004.

‡ To whom correspondence should be addressed: Kathleen B. and Mason I. Lowance Center for Human Immunology, Emory University School of Medicine, Rm. 1005; Woodruff Memorial Research Bldg., 101 Woodruff Circle, Atlanta, GA 30322. Tel.: 404-727-7310; Fax: 404-727-7371; E-mail: jgoronzy@emory.edu.

§ Departments of Pediatrics and Immunology, University of Pittsburgh School of Medicine, Children’s Hospital Rangos Research Center, Pittsburgh, Pennsylvania 15213.

¶ Division of Physiological Imaging Research Laboratory, Mayo Clinic, Rochester, Minnesota 55905.

1 The abbreviations used are: KIR, killer immunoglobulin-like receptors; MHC, major histocompatibility complex; NK, natural killer cells; SEAP, soluble alkaline phosphatase; EGFP, enhanced green fluorescence protein; RACE, rapid amplification of cDNA ends; TCR, T cell receptor; UTR, untranslated region; PBMC, peripheral blood mononuclear cell; IL, interleukin.
and plaque instability (34). Thus, KIR expression on CD4+ and CD8+ T cells appears to have mostly unwanted consequences. Depending on the molecule expressed, KIR expression can compromise tumor- or virus-specific T cell responses, provide co-stimulatory signals leading to autoimmune disease, or even mediate direct tissue injury if there is a coordinated expression of stimulatory KIRs and their adapter molecule, DAP12 (35, 36).

KIRs are not expressed on naïve T cells or the vast majority of memory cells. KIR-expressing T cells are usually negative for CD28 expression, are monoclonally or oligoclonally expanded (37), and have additional features reminiscent of senescent cells, such as telomere shortening (38). Thus, it has been postulated that KIR expression occurs as a consequence of chronic immune stimulation. Diversification of the KIR repertoire occurs following clonal expansion because KIR expression is stochastic and cumulative on clonal progeny (39–41). The repertoire of the clonal T cell population is therefore more or less stochastic and cumulative on clonal progeny (39–41). The resulting KIR repertoire is generated from the clonal T cell population. Nevertheless, KIR repertoire diversity is sometimes observed even within a clonally expanded population. This resulting KIR repertoire of the clonal T cell population is therefore more or less diverse depending on whether KIR expression occurred early or late during the expansion period. This resulting KIR repertoire generates diversity in the immune response pattern even within a clonally expanded T cell population.

Despite similarities in expression patterns between NK and T cells, it should be noted that KIR expression occurs at very different developmental stages in these two cell lineages. In NK cells, it occurs early in development (14, 15), whereas KIR expression in T cells is a feature of end-differentiated, and possibly, senescent cells (42, 43). It is unclear whether KIR expression occurs as a programmed step in T cell differentiation. Recent studies have shown that T cell differentiation involves the de novo expression of transcription factors and epigenetic control mechanisms (44). Induction of T cell subset-specific transcription factors, such as GATA3, T-bet, and c-Maf, controls the differentiation into Th1 and Th2 helper cells (45–47). It is currently unknown whether KIR expression is similarly part of the normal T cell differentiation program, regulated by a selective set of transcription factors. Alternatively, replicatively stressed T cells may default to regulatory control mechanisms that are characteristic of NK cells. The finding that KIR+ T cells have numerous NK-like features (43) may favor the latter hypothesis. Here, we examined the transcriptional control of KIR expression in T cells to address whether regulatory elements in NK and T cells are identical and whether the expression of relevant transcription factors correlates with differentiation.

EXPERIMENTAL PROCEDURES

Cell Lines—T cell lines (Jurkat, 6TCEM20, HUT78), EBV-transformed B cell lines (Ck, Tab), and the monocytic cell line U937 were cultured in RPMI 1640 medium (Cambrex, Walkersville, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT, 2 mM l-glutamine (Invitrogen), and, in the case of HUT78, 20 units/ml human IL-2 (Chiron, Emeryville, CA). NK cell lines NK92 and NKL were cultured in RPMI 1640 medium containing 10% human plasma, 2% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, and 200 units/ml human IL-2. T cell lines and NK cell lines were obtained from the American Type Culture Collection (Manassas, VA). The B cell lines were generated in the laboratory by standard cell transformation procedures with EBV. Peripheral blood mononuclear cells (PBMC) were collected from healthy volunteers after obtaining informed consent. The protocol was approved by the Mayo Institutional Review Board.

Cloning of KIR Promoters and Construction of Reporter Plasmids—Genomic DNA was obtained from PBMCs of a healthy donor who typed KIR2DS1* KIR2DS2* KIR2DL3* KIR2DL2* KIR2DL1* KIR2DL4* KIR3DL1* KIR3DL2*. KIR genotyping was performed as originally described by Uhrberg et al. (48). A 1,984-bp fragment of the KIR2DL2 5'-untranslated region (UTR) was amplified by PCR using primers corresponding to the poly(A) region of the upstream KIR2DS2 gene and to the consensus leader sequence. The KIR2DS2 full-length promoter was amplified using a primer corresponding to a sequence motif in the distal 5'-UTR of KIR2DS2 and the leader sequence primer. Sequences of the primers are given in Table 1. PCR-amplified DNA was cloned into the pSEAP (Clontech, Palo Alto, CA) reporter gene vector. All clones were authenticated by DNA sequencing.

Truncation variants were generated by PCR using the primers shown in Fig. 1 and the cloned full-length 5'-UTR (SB8). For each construct, the bp numbers were designated beginning with the translation codon. The 500- and 208-bp fragments were subcloned from the full-length KIR2DL2 promoter, the 121-, 81-, 61-, and 41-bp fragments from the 208-bp fragment.

Mutation variants were constructed by overlap extension PCR. Mutagenesis followed the concept of A → C and G → T exchange, except for the mutation for the AML (also referred to as 5'-UTR) site (SB8). All mutated constructs were generated from the 208-bp template.

To generate enhanced green fluorescence protein (EGFP) reporter constructs, the SEAP gene was replaced by the EGFP gene using EcoRI at the multiple cloning site and XbaI at the 3'-terminal of the SEAP gene. The EGFP reporters from 500 to 1,964 bp of the KIR2DL2 5'-UTR served as the negative control.

Reporter Gene Bioassays—All plasmids used in reporter bioassays were purified by cesium chloride density centrifugation. The plasmid preparations were then dialyzed against 20 volumes of Tris-EDTA, desalted by column chromatography (NAP-10 columns, Amersham Biosciences), and stored at −20 °C. At least two independent preparations were used for all reporter gene assays. Reporter plasmids (25 μg) were transiently transfected by electroporation. 5 μg of plasmid (25 μg of EGFP reporter construct) and the control EGFP reporter were used as a binding probe. As a control, the cotransfected transfection efficiency. 1 × 105 cells were resuspended in 250 μl of RPMI 1640 containing 2% fetal calf serum, mixed with plasmids in 50 μl of serum-free RPMI 1640 in electroporation cuvettes (4-mm gap Cuvette Plus, Btx, San Diego, CA), and incubated for 10 min. Electroporation was performed for 20 ms at 300 V for 6TCEM20 cells and at 275 V for all other cell lines (ElectroCell 600, Btx, Holliston, MA). Cuvettes were immediately transferred onto ice, incubated for 10 min, and then transferred to a final volume of 2 ml of complete culture medium in sterile tubes or plates. After 24 h of culture, EGFP expression was analyzed by flow cytometry, and soluble placental alkaline phosphatase activity (SEAP) in the supernatant was measured using a SEAP detection kit (Clontech). Photoemissions were measured with a luminometer (Lumat LB9501, Berthold Analytical, Nashua, NH). As a system control, parallel transfections were done using SEAP or EGFP constructs under the control of a SV40 promoter/enhancer (Clontech). All results were corrected for transfection efficiency. Data are shown as the mean of triplicates relative to the positive control (e.g. wild-type promoter sequence or SV40 promoter/enhancer).

Mapping of the Transcription Start Site—Total RNA was purified from HUT78 T cells using TRIzol (Invitrogen). Rapid amplification of cDNA ends (RACE) for KIR2DL2 transcripts was performed with a GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. Primers corresponding to sequences in exon 3 of the KIR2DL2 gene were used for reverse transcription (gcctcgagacacaatac, and PCR (ccagctggagaaagttggct). PCR products were cloned into pcRii (Invitrogen) and sequenced using the M13 reverse primer.

T Cell Subsets and Reporter Gene Activity—PBMCs were transiently transfected with EGFP reporter constructs of the 61- or 121-bp fragments using the Nucleofector system (Amaxa, Gaithersburg, MD). 50 μg of plasmid and 5 × 106 cells were used for each transfection. After transfection, cells were cultured for 24 h. Transfected cells were stained with fluorochrome-labeled anti-CD3, -CD4, -CD8, -CD28, and -CD45RA antibodies and analyzed by flow cytometry.

Gel Shift Assays—Preparation of nuclear extracts and conditions of DNA binding reactions were as described previously (49). For the present studies, the cloned 61-bp KIR2DL2 was used as a binding probe. As competitors, synthetic double-stranded oligonucleotides corresponding to the consensus SPI and Ets binding sequences, and positions −51 to −61 and −61 to −85 of the cloned KIR2DL2 promoter were used at 5–300 nM excess compared with that of the 61-bp binding probe. In Ets supershift assays, either the 61-bp KIR2DL2 promoter or the synthetic Ets sequence was used as a binding probe, and 5 μg/ml anti-Ets1 antibody or IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reactions. All binding reactions were carried out in a final volume of 60 μl containing 15 μg of nuclear extracts and 40 fmol of radiolabeled binding probe. DNA-protein complexes were resolved in 6% non-denaturing polyacrylamide gels and autoradiography.

RESULTS

Cell-Specific Expression of KIRs in T and NK Cells—KIRs are usually expressed on NK cells but can be acquired by a subset of CD4 and CD8 cells after extensive replicative stress (42).
Similar to NK cells, the expression of KIRs is stochastic in T cells (39, 41). In some cases, there appears to be preferential expression (50). We therefore examined whether the constitutive expression of KIR in NK cells and the acquired expression in T cells are under different transcriptional regulation.

We first aligned the entire promoter region of 10 known KIR genes and subjected them to phylogenetic analysis using the Molecular Evolutionary Genetics Analysis Software 2001. The data show significant reporter gene activity in the NK cell line NKL and in the T cell line 6TCEM20, indicating that both cell lines had transacting factors necessary to drive transcriptional activity of the upstream KIR2DL2 promoter gene activity was minimal in Clk, an EBV-transformed cell line. The full genomic region between the KIR2DL2 translation initiation codon and the upstream KIR2DS2 gene was cloned into the SEAP reporter plasmid and transfected into cell lines representative of different cell lineages. The NK cell line NKL served as positive control. Depicted in Fig. 2 are the KIR promoter-driven reporter activities, relative to a SV40 enhancer/promoter-driven SEAP reporter after normalization for transfection efficiency.

The list of oligonucleotides used in the construction of reporter plasmids (truncation and mutation) and gel shift experiments is provided in Table I. The complementary antisense strand was also synthesized, annealed with the sense strand, and used in the gel shift assay.

### Table I

**List of oligonucleotides**

| Primer no. | Primer design | Primer sequence (5′→3′) |
|------------|---------------|-------------------------|
| 1          | Antisense, 5′-Hind III | gggaagctttaccaaccacgcccctagct |
| 2          | Sense, 5′-Xho I | gggctcgagtaaccatgtctgcccctcatg |
| 3          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 4          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 5          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 6          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 7          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 8          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 9          | Sense, 5′-Bgl II | Gggagctttaccaaccacgcccctagct |
| 10         | Sense, PCR with no. 1 | gcgcgcATATTgtctgcacagacagca |
| 11         | Antisense, PCR with no. 5 | tgcagcaAAATAAgcgcccctagctca |
| 12         | Sense, PCR with no. 1 | caataaalAAAGtgctgctgtcagt |
| 13         | Antisense, PCR with no. 5 | cgcgcCTCCGttatttgccctgctg |
| 14         | Sense, PCR with no. 1 | gcgcgcCCCGCCcatcctgctgctgctg |
| 15         | Antisense, PCR with no. 35 | ggttcccctgctgctgctgctgctg |
| 16         | Sense, PCR with no. 1 | ctgccatTTTTAATTTTTAAtaagactctctgtgctg |
| 17         | Antisense, PCR with no. 5 | tttttTTTTAAAATTTAAAtgtcgattgtgctg |
| 18         | Sense, PCR with no. 1 | caacactGCACCttcgtgctgctgctg |
| 19         | Antisense, PCR with no. 5 | CatgcagGgGgcatctgctgctgctgctg |
| 20         | Sense, PCR with no. 1 | cggagcatcctgctgctgctgctgctg |
| 21         | Antisense, PCR with no. 5 | gttgcagctcactgctgctgctgctg |
| 22         | Sense, PCR with no. 1 | ctgcgtCTGACccctcctgcctgctg |
| 23         | Antisense, PCR with no. 5 | gggggctgctgctgctgctgctgctg |
| 24         | Sense, PCR with no. 1 | ggccctCTCCGttatttgccctgctg |
| 25         | Antisense, PCR with no. 5 | cttcttcctctctctctctctctct |
| 26         | Sense, PCR with no. 1 | ggccctCTCCGttatttgccctgctg |
| 27         | Sense, 5′-EcoRI | ccggagatcctgctgctgctgctgctg |
| 28         | Antisense, 5′-XbaI | actagacttctgctgctgctgctgctg |
| 29a        | Sense, 5′-Bgl II | tcgagctgtgtgtgtgtgtgtgtgt |
| 30a        | Sense, 5′-Bgl II | gcctccagcgggggggggggggggg |
| 31a        | Sense, 5′-Bgl II | Taaactcctctctctctctctctct |
| 32a        | Sense, 5′-Bgl II | Actgcatggcagcggggggggg |
| 33         | Sense, 5′-Bgl II | tccaagtactcactgctgctgagctgagctgagctgagctgagctg |

*a* Capital letters indicate the introduced nucleotide substitutions to generate mutant constructs.

*b* The complementary antisense strand was also synthesized, annealed with the sense strand, and used in the gel shift assay.

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2 Molecular Evolutionary Genetics Analysis Software 2001. Kumar, S., Tamura, K., Jakobsen, I. B., and Nei, M., Arizona State University, Tempe, AZ; www.megasoftware.net.
these results suggest that the expressions of transacting factors that drive KIR transcription are tissue-specific and restricted to T cells and NK cells. These data parallel the known distribution of KIR cell surface expressions that are limited to NK cells and a subset of memory T cells (1, 22, 39).

Differences in expression patterns among different hematopoietic cell lineages were maintained with progressive 5′/H1032-deletion clones of the KIR2DL2 promoter construct. As shown in Fig. 2, promoter constructs including a 500- and 208-bp truncation segment of the promoter region upstream of the translation initiation codon were equally active in NK cells and T cells, but showed minimal activity in B cells and were virtually inactive in the monocytic cell line. Reproducibly, the 208-bp truncation fragment yielded 2-fold higher promoter activity compared with the full-length promoter in both T cells and NK cells, likely caused by the higher concentrations of putative enhancer/activator elements in this shorter KIR promoter. These data demonstrate that cell-specific expression is conferred by a core promoter situated in the first 208-bp upstream of the translation initiation site.

Divergent Regulation of KIR2DL2 Promoters in NK and T Cells—The 208-bp KIR2DL2 promoter gene construct was equally expressed in T and in NK cells. Further progressive 5′-deletions of this promoter construct, however, yielded differences in promoter regulation in these two cell types. Results are shown in Fig. 3A. The 121-bp construct was equally effective in NK and T cells; its activity equivalent with the 208-bp promoter construct. All truncations further toward the translation codon resulted in a significant diminution of KIR promoter activity in NK cells. The 80-bp KIR2DL2 promoter clone conferred only about 30% of full promoter activity, suggesting that critical regulatory elements mapped to the region between /H1002121 and /H100280. Promoter activity was essentially lost with the /H100241 bp construct, whereas there was some activity left with the /H100261-bp construct, suggesting there were additional functional regulatory limits in this region. For T cells, the results were completely different. Progressive 5′-truncations down to the length of the −61-bp promoter construct only marginally reduced promoter activity. The critical regulatory element in the KIR promoter for T cells mapped between positions −61 and −41 with minimal reporter activity seen in T cells with the −41-bp construct. These data suggest that although the expression of KIRs is restricted to NK and T cells, the regulatory elements governing KIR expression in these two cell types are different.

To obtain additional evidence that KIR regulation in T cells and NK cells is different, the −121- and −61-bp promoter

Fig. 1. Diversity of KIR promoter sequences. Published sequences of KIR promoter regions (in the GenBank™ data base) were aligned and subjected to phylogenetic analysis using the MEGA2 software. The dendrogram shown was constructed by the neighbor-joining method, with the Jukes-Cantor matrix distance estimation. The HLA-A2 sequence was used to establish root distance. The numbers in the dendrogram are confidence probability levels estimated by bootstrapping.

Fig. 2. Cell specificity of KIR promoter activity. Reporter gene assays with full-length KIR2DL2 promoter and 500- and 208-bp truncation fragments of the proximal promoter were performed in tumor lines representing the T cell (6TCEM20), NK cell (NKL), B cell (Clk), and myelomonocytic cell (U937) lines. Results shown are mean (and S.D.) of triplicate transfections that were corrected for transfection efficiency, and expressed as percent of maximum reporter activity of cells transfected with SEAP reporters under the control of the SV40 enhancer/promoter.

Fig. 3. T and NK cells differ in the minimal KIR2DL2 promoter. A, reporter gene assays were performed with progressively truncated KIR2DL3 promoter sequences, and SEAP reporter activities in T cell (6TCEM20) and NK cell (NKL) tumor lines were compared. Results shown are mean (and S.D.) of triplicate transfections that were corrected for transfection efficiency and expressed as SEAP activity relative to the reporter activity from cells transfected with the 208-bp promoter. B, freshly isolated PBMCs were transfected with a 61- and 121-bp truncated KIR2DL2 promoter construct cloned upstream of an EGFP reporter gene, and fluorescence activity in gated CD16+ NK cells and CD3+ T cells was determined by flow cytometry.
constructs were cloned into EGFP reporters and used in transient transfection experiments with freshly isolated PBMCs. Fig. 3B shows a representative flow cytometry analysis. Approximately equal numbers of CD3+ cells express EGFP after transfection with either the −121- or the −61-bp promoter constructs when cell populations were gated for CD3, thereby confirming the data in Fig. 3A that show the short promoter construct was active in T cells. The results were different when CD16+CD3+ cells were gated. The −121-bp promoter construct drove EGFP expression in ~20% of all NK cells, whereas the ability of the −61-bp construct to drive expression was reduced to ~5%.

Putative Transcription Factor Binding Sites Controlling KIR Expression in NK and T Cells—To further understand what regulatory elements in the proximal 208-bp sequence of the KIR2DL2 control transcription, we examined its homology with the other KIR genes. Shown in Fig. 4A is a multiple alignment of the first 220 and 19 bp 5’ and 3’, respectively, of the translation codon. Consistent with the dendrogram of the full-length promoter sequences (shown in Fig. 1), this alignment shows the cladistic patterns of sequence homology of the 5’-proximal 220-bp segments. There is absolute identity of KIR2DL2, KIR2DS2, and KIR2DL3 sequences that comprise one clade. A near sequence identity exists between KIR2DS1, KIR2DS5, KIR2DL5, and KIR3DS1, which comprise the second clade. KIR3DL3 and KIR2DL4 sequences are significantly divergent, thereby representing distant branches. All of these 220-bp promoter segments, however, contain identical putative transcription factor-binding motifs that are also identicaly situated within the sequence stretch.

To determine the basis for the differential control of the KIR2DL2 promoter in NK and T cells, we mutated putative transcription factor binding sites within the 208-bp promoter gene construct. All mutations were either an A → C or G → T exchange except the mutation at −98 where a nonfunctional natural G → A variant has been described previously (51). The majority of mutations did not influence promoter activity in either T or NK cells. Representative results are shown in Fig. 4B. Mutation variants of the 208-bp KIR2DL2 promoter were generated for positions −25 (reverse Sp1), −66, −90 (TATA-like), −118 (cREB), −142 (Oct2), and −185 (Ets1). It might be noted that position −66 has been recently suggested to demarcate the minimal promoter region for KIR2DL4 (52). These mutated variants had either increased or unchanged activity; none of the mutations abrogated promoter activity regardless of whether these constructs were transfected into T cells or into NK cells. In contrast, significant reduction in promoter activity was found with mutations in position −61 and −98. Position −98 is in the center of a putative AML motif. As shown in Fig. 4C, mutation at this position of the promoter reduced reporter gene activity in NK cells by 30–40%. This was consistent with, albeit less impressive than, the expected loss of activity based on the truncation experiments (in Fig. 3A) where deletion of the region between −121 and −81 reduced more than 70% of promoter gene activity. Also, consistent with our truncation experiments, the mutation at position −98 did not affect promoter activity when transfected into T cell lines. The important regulatory motif for T cells centered at position −61, a putative reverse Ets1 motif. Mutations in position −61 reduced promoter activity in T cells by 70%.

T Cell-specific Promoter Element Coincides with the Transcription Initiation Site—To determine how position −61 relates to the transcription start site of the KIR gene, we conducted RACE experiments using mRNA from HUT78 or primary T cells that we had previously screened to express KIR2DL2 mRNA. We identified multiple products corresponding to the 5’-UTRs, all of them clustering in a small sequence stretch. The largest of these products included position −69 upstream of the translation initiation codon. The diagram in Fig. 5 shows the DNA sequence of the proximal 80 bp of the KIR2DL2 promoter. The 5’-ends of the sequenced products ranged from −56 to −69.

Transcriptional Regulators Binding to the Minimal KIR2DL2 Promoter Are T Cell-specific—The above results indicate that the minimal KIR2DL2 promoter in T cells is situated within the proximal 61-bp stretch upstream of the translation codon. We therefore examined whether the transcriptional regulators binding to this sequence motif are cell-specific. Fig. 6A shows representative gel shift assays with nuclear extracts from different cell lines with the 61-bp promoter sequence as binding probe. Parallel gel shifts with an Sp1 binding probe were included as controls. Results showed that 61-bp KIR2DL2 promoter binding activities were present in all three cell lines of the T cell lineage examined, namely 6TCEM20, HUT78, and Jurkat. There was also minimal binding activity with the NK cell line NK92. This minimal binding activity, however, had different mobility than the binding activities seen with nuclear extracts of T cells, suggesting that there may be a different protein complex in NK92 binding to this motif. There was no binding activity detected in the second NK cell line, NKL. Neither the monocytic cell line U937 nor two EBV-transformed B cell lines (Tab, Clk) examined had any DNA binding activities specific for this motif. Nuclear extracts from all cell lines had similar binding activities for the Sp1 consensus oligonucleotide (Fig. 6B).

Because position −61 of the KIR2DL2 promoter resembles a reversed Ets1 site, we examined whether the DNA binding activities in T cells included Ets. Competition gel shift assays were performed. Results are shown in Fig. 6C for competing oligonucleotides corresponding to the −51- to −61- and to the −61- to −85-bp stretches of the KIR2DL2 promoter. Only the −51 to −61 oligonucleotide competed for the binding of the nuclear protein complex. More importantly, a consensus Ets binding sequence also did not have any competitor activity for the 61-bp DNA binding probe. In addition, anti-Ets antibodies did not supershift the protein complex binding to the minimal 61-bp KIR2DL2 promoter, whereas these antibodies were successful in supershifting complexes binding to an Ets consensus sequence (data not shown).

Transacting Factors Regulating KIR Expression Are Not Restricted to Memory T Cells—Transcription of KIRs in T cells has a very limited and restricted pattern of expression. KIRs are only expressed on memory T cells and are not found on naive T cells. Moreover, even in the memory compartment, the expression is restricted to a small subset of cells. These cells have a characteristic phenotype with a loss of CD28, CD27, and CD7 (50, 53). KIR expression is seen in CD4+ and CD8+ T cells; however, in most individuals, more CD8+ T cells are able to express KIRs than CD4+CD28+ cells. We therefore examined whether the very restricted expression pattern in T cells correlates with the presence of transactivation activators binding to the minimal KIR2DL2 promoter. In these experiments, total PBMCs were transfected using the 121- and the 61-bp promoter constructs driving EGFP. Transfected cells were stained with antibodies to CD3, CD4, CD8, CD28, and CD45RA, and proteom gene activity in the different T cell subsets was determined. Results are shown in Fig. 7. EGFP was equally expressed in CD4+ and CD8+ T cells. Within CD4+ and CD8+ T cells, expression did not show an obvious restriction to a functional subset. Expression was not limited to CD4+CD28+ or CD8+CD28+ T cells. On the contrary, large populations of CD28+ T cells were able to express EGFP driven by the KIR2DL2 promoter (data not shown). Moreover,
the expression was not limited to memory T cells. EGFP was equally expressed in CD45RA naïve and CD45RA memory T cells. These data suggest that T cells constitutively have the transcriptional regulators to drive the expression of KIRs and that the restricted expression in T cell subsets is not a consequence of de novo transcription of transcription factors because of T cell differentiation.

**DISCUSSION**

Here we show that the transcriptional machinery of T cells is uniquely equipped to transcribe KIR genes. Naïve, memory, effector, and senescent T cells have sets of transcription factors that endow them with the ability to express KIRs. A significant finding is that the transcriptional control of KIRs in T cells is different from that in NK cells (Figs. 3, 4, and 6). These data
suggest that T cells are preprogrammed to express KIRs, implying that these genes serve important and discrete biological functions (Figs. 2, 3, and 6).

KIRs are nearly exclusively expressed on CD4+ and CD8+ T cells that have lost the expression of CD28 (22, 50). Loss of CD28 increases with age and is generally considered a consequence of replicative senescence (53–56). CD28+ T cells are oligoclonally expanded and have shorter telomeres than CD28− T cells from the same individual, which suggests an extensive replicative history (38–41). CD28+ KIR-expressing T cells are apoptosis-resistant, long-lived lymphocytes (7, 57). The gene expression profile of CD28+ T cells also includes many other gene types that are unrelated to this genetic region, including CD161, NKG2D, CD8α, and perforin (43). Given the close developmental relationship of T cells and NK cells (59, 60), we had speculated that senescent T cells assume an intermediate nature between T cells and NK cells. Our data here, however, clearly show that KIR-expressing T cells have not defaulted to transcriptional control mechanisms that are characteristic of NK cells but have their own unique way to express KIRs (Figs. 3, 4, and 6). The transcription initiation start site in T cells maps to about position −69 upstream of the translation initiation codon (Fig. 5), which is different from the transcription initiation site described for NK cells (18). The biochemical impact for the coincidence of the minimal promoter with the transcription start site of KIR2DL2 (Figs. 3–5) on the assembly of the basal transcription complex and the actual initiation of transcription remains to be examined.

Expression of KIRs in NK cells has been previously indicated to be controlled by an AML binding site (51, 52) centered at position −98. Mutations of this position or truncation reduced expression in NK cells but not in T cells (Fig. 4). This region has recently been described by Stewart et al. (18) to be occupied in a footprinting assay. Also, Vilches et al. (51) have described a natural variant with this polymorphism that abrogated expression of the variant KIR2DL5 gene. On the contrary, promoter truncation, mutational analysis, and gel shift assays mapped the regulatory region in T cells to a DNA sequence including position −61 (Figs. 3–6). These results are markedly distinct from the regulation of KIR2DL4 as described by Trompeter et al. (52). In NK cells, the minimal promoter of KIR2DL4 is localized within the proximal 65-bp region. Whereas positions −55 to −69 are generally conserved in all KIR isotypes, positions −62 to −67 are clearly distinct for KIR2DL4 and KIR3DL3 (Fig. 4A), the two most evolutionarily distant KIR promoters (Fig. 1).

T cells are ideal substrates for examining transcriptional regulation of genes accompanying differentiation (44, 61, 62).
Naive T cells differentiate into two types of effector/memory T cells, Th1 and Th2 cells, which differ in their ability to produce cytokines and in their set of regulatory cell surface receptors (61). Lineage determination appears to follow an instructive model that is closely linked to the expression of unique transcription factors. Commitment to the Th1 pathway is correlated with the expression of the transcription factor T-bet, whereas expressions of the GATA-3 and c-MAF transcription factors are characteristic of Th2 cells (45–47). T-bet also regulates the terminal differentiation of NK and NKT cells (62). In addition to general changes in transcription factors with the differentiation of T cell effector function (63), epigenetic mechanisms have been shown to influence cytokine production (64), although their relative importance in determining differentiation or cell fate is less clear.

KIR expression has also been correlated with T cell differentiation. Studies of oligoclonal CD4+ and CD8+ T cell populations show a diverse KIR repertoire, suggesting that KIR expression and diversification may occur after TCR rearrangement (39–42). However, it is less clear at what differentiation stage KIRs become expressed. Culture conditions that induce KIR expression in vivo are unknown. It is also not known whether KIR expression follows an instructive or a stochastic model. Phenotypic studies have suggested that KIR expression in CD8+ T cells in vivo occurs at the transition from effector to memory T cells (57). In contrast, computer modeling of KIR diversity in CD4 T cells have suggested that KIRs are acquired early during clonal expansion, possibly already at the transition of naive to effector cells (39), a view consistent with our data that even naive CD45RA+ T cells already have active transcription factors necessary to drive KIR promoter activity (Fig. 7). However, it remains to be investigated whether KIRs are found only on particular subsets of memory/effector CD4+ and CD8+ T cells.

The finding that the minimal KIR promoter is active in CD45RA+ T cells (Fig. 7) was surprising. Expression of CD45RA is a reliable marker of naive CD4 T cells. In contrast, CD45RA can be expressed on a subset of CD8 memory/effector T cells that lack CD28 expression (65). However, such CD8 T cells were extremely infrequent in the PBMCs from young adults that were examined for the transcription studies, suggesting that naive T cells truly have the machinery to support the transcription of KIRs. KIR expression therefore fundamentally differs from the expression of genes that is gained during T cell differentiation into effector/memory cells. T cell-specific expression of KIRs is controlled by the minimal 61-bp promoter and is correlated to the presence of a protein complex binding to a DNA sequence around position −57. Based on the transcription experiments shown in Fig. 7, we cannot exclude that only a subset of T cells is able to support KIR expression; however, expression is certainly not restricted to memory/effector cells and does not correlate with T cell differentiation surface markers or with CD28 expression. We favor the interpretation that all T cells express the appropriate transcription factors and that restrictive expression of KIRs is controlled by epigenetic mechanisms, such as changes in DNA methylation (16, 17). This interpretation is consistent with our finding that the minimal KIR promoter is active in the Jurkat T cell line that naturally does not express KIRs (Fig. 2 and data not shown) (17). The importance of epigenetic control has been emphasized for NK cells where methylation patterns of CpG islands are responsible for the stochastic expression of KIR genes (16). Whether CpG demethylation of the KIR promoter is the decisive step in T cells is less certain. In contrast to NK cells, where demethylation with 5-aza-2’-deoxycytidin facilitated a global expression of KIRs, treatment of the Jurkat T cell line only induced a weak transcription of KIR3DL2 and not of other KIR genes (17). Given the physical proximity and the coordinated T cell subset-specific expression of several genes encoded on chromosome 19q13.4 (58), it is possible that a locus control region regulates the overall accessibility to this gene cluster, whereas individual expression patterns at the clonal level are determined by promoter demethylation.

Taken together, it is becoming increasingly clear that KIR expression in T cell subsets is predetermined and not an unwanted accidental side product of cellular senescence or T cell aging. Data presented here indicate that naive T cells are already prepared to initiate the transcription of KIR genes. These data support the idea that KIR expression in T cells is apparently regulated by epigenetic mechanisms and does not require complex changes in the array of transcription factors. The uniqueness of the transcriptional control mechanisms in T cells, differing substantially from those in NK cells, emphasizes the biological significance of KIR expression in T cells. The restriction of KIR expression to oligoclonal T cell populations provides important clues to understanding their purpose. One attractive idea is that expression of inhibitory KIRs can limit clonal expansion, thereby preventing clonal exhaustion and preserving TCR diversity during chronic infection and aging.

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