Epigenetic regulation of killer immunoglobulin–like receptor expression in T cells

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With increasing age, T cells gain expression of killer immunoglobulin–like receptors (KIRs) that transmit negative signals and dampen the immune response. KIR expression is induced in CD4 and CD8 T cells by CpG DNA demethylation suggesting epigenetic control. To define the mechanisms that underlie the age-associated preferential KIR expression in CD8 T cells, we examined *KIR2DL3* promoter methylation patterns. With age, CD8 T cells developed a patchy and stochastic promoter demethylation even in cells that did not express the *KIR2DL3*-encoded CD158b protein; complete demethylation of the minimal *KIR2DL3* promoter was characteristic for CD158b-expressing cells. In contrast, the promoter in CD4 T cells was fully methylated irrespective of age. The selectivity for CD8 T cells correlated with lower DNMT1 recruitment to the *KIR2DL3* promoter which further diminished with age. In contrast, binding of the polycomb protein EZH2 known to be involved in DNMT1 recruitment was not different. Our data suggest that CD8 T cells endure increasing displacement of DNMT1 from the KIR promoter with age, possibly because of an active histone signature. The ensuing partial demethylation lowers the threshold for transcriptional activation and renders CD8 T cells more susceptible to express KIR, thereby contributing to the immune defect in the elderly. (Blood. 2009;114: 3422-3430)

Introduction

With increasing age, the ability of the immune system to protect against new antigenic challenges or to control chronic infection erodes.[1] Elderly persons are more likely to develop bacterial infections in lungs, skin, and the urinary tract.[2] The incidence and severity of viral infections increases, and the responses to prophylactic vaccinations decline.[3-7] A dwindling in thymic production of naive T cells, resulting in a decreasing size and diversity of the naive cell compartment, contributes to the defective adaptive immune response.[8-10] However, the immune defect is not limited to naive T cells; memory T cells and, in particular, CD8 T cells are also affected by aging. Central memory CD8 T cells decline at the expense of end-differentiated CD8 effector T cells.[11] In parallel, CD8 T cells undergo phenotypic changes that are of functional importance.[12] CD8 T cells tend to lose the expression of the CD28 molecule and are, therefore, less responsive to stimulation by antigen-presenting cells. Because cross-presentation of antigen by dendritic cells is an important pathway to elicit CD8 responses to viral infections, the CD28 loss has a negative effect.[13,14] More important than the CD28 loss may be the de novo expression of negative regulatory receptors that occurs on many CD8 T cells.[15,16] The best-studied receptors so far are the killer immunoglobulin–like receptors (KIRs) that recognize MHC class I variants. KIRs are a multigene family encoded within the leukocyte receptor cluster on chromosome 19 and comprise inhibitory and, less frequently, stimulatory receptors.[17,18] Inhibitory receptors have ITIM motifs in their cytoplasmic domains and recruit SHP-1 that dephosphorylates various tyrosine kinases in the early T-cell receptor (TCR) signaling pathway.[19,20] On antigenic stimulation of the TCR, the KIR receptor recognizes an MHC class I ligand on the very same target cell, is recruited to the TCR recognition complex, and enters the central supramolecular activation cluster with a delay of approximately 30 minutes.[21] This delay is sufficient to not compromise effector cell functions such as cytotoxicity; however, the eventual recruitment of KIRs to the antigen-recognition complex prematurely terminates TCR signaling and inhibits activation-induced transcription that requires sustained stimulation such as the initiation of T-cell proliferation and cytokine transcription.

The mechanisms that drive increasing KIR expression on T cells with age are unclear. KIRs are primarily expressed on natural killer (NK) cells where they are clonally distributed.[22] The clonal distribution pattern is entirely maintained by CpG DNA methylation. Inhibition of the DNA methyltransferase (DNMT) by 5-aza-2′-deoxycytidine (5-Aza-dC) leads to a global expression of KIRs on all NK cells.[23,24] Previous studies have shown that transcriptional control of KIR expression differs in T cells compared with NK cells; however, even naive CD4 T cells have the transcriptional machinery to support the activation of the minimal KIR promoter in reporter gene assays.[25] Epigenetic mechanisms may therefore also determine the increased frequency of KIR expression on T cells with age.

DNA CpG demethylation has been shown to be very important in fate decisions in T-cell differentiation. A classical example is the differentiation into T helper type 1 and T helper type 2 T cells that are characterized by different cytokine patterns imprinted by CpG demethylation of enhancer-like regions or locus control regions in the cytokine genes.[26,27] It is possible that expression of KIR genes is part of normal T-cell differentiation into effectors cells. However, KIR expression is usually not found on T-effector cells in the young adult; even in the elderly, KIR expression is preferentially found on terminally differentiated T cells that have lost the expression of CD28 and have an extensive replicative history.[28,29] Rather than being a directed process, KIR expression could, therefore, be a

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Table 1. Demographics of study population

	Young	Elderly
Number	70	44
Age, y, mean ± SD	25.3 ± 3.3	77.1 ± 4.4
Ethnicity		
White	70	42
Black		2
Sex, F/M	39/31	24/20
Medication		
Antihypertensives	0	17
Aspirin/NSAID	1	22
Biphosphonate	0	2
Birth control/hormone replacement	4	8
Proton pump inhibitor	0	5
Statin	0	12

consequence of cumulative passive promoter demethylation because of antigen-stimulated proliferation or homeostatic turnover.

To prevent KIR expression on T cells with age, a better understanding of the regulatory mechanism is needed. The current study was designed to identify the mechanisms that lead to DNA demethylation of the KIR promoter in T cells. Representative for the different KIR genes, we examined the *KIR2DL3* gene, which has a high population frequency and whose promoter closely resembles the sequence of other KIR promoters. Here, we show that the *KIR2DL3* promoter is progressively demethylated in CD8, but not in CD4 T cells, even before KIR transcription and protein expression is noted. Increased KIR promoter demethylation with age correlates with reduced recruitment or increased replacement of DNMT1 to the promoter. Our data suggest a model in which cumulative passive demethylation lowers the quantitative threshold for KIR transcription and leads to an increased probability of gene expression in older age that impairs CD8 T-cell responses.

Methods

Cells

Peripheral blood was obtained from healthy young (aged 20-30 years) and elderly (aged 70-85 years) persons. Demographics and medication history are shown in Table 1. Persons who had an immune-mediated disease or history of cancer, except basal cell cancer, were excluded. Hypertension and hyperlipidemia were permitted if controlled on medication. Written informed consent was obtained in accordance with the Declaration of Helsinki, and the study was approved by the Emory Institutional Review Board. CD4, CD8, and NK cells were positively selected from peripheral blood mononuclear cells with the use of magnetic microbeads (Miltenyi Biotec Inc). To isolate CD158b/j$^+$ and CD158b/j$^-$ T cells, NK cells were depleted by anti-CD16 magnetic microbeads; the negative collection was incubated with phycoerythrin (PE)–conjugated CD158b mAb (BD Biosciences) and anti-PE magnetic microbeads (Miltenyi Biotec Inc). CD158b/j$^+$ T cells, CD4$^+$CD158b/j$^-$ and CD8$^+$CD158b/j$^-$ cells were then positively selected. To isolate CD8$^+$ CD45RO$^+$ CD158b/j$^-$ and CD8$^+$ CCR7$^-$CD45RA$^+$ T cells, negatively isolated CD8 T cells were depleted with PE-conjugated anti-CD158b/j mAb (for CD8$^+$ CD45RO$^+$ CD158b/j$^-$ isolation) or PE-conjugated anti-CCR7 mAb (R&D Systems; for CD8$^+$ CCR7$^-$CD45RA$^+$ isolation) and anti-PE magnetic microbeads. CD45RO$^+$ T cells and CD45RA$^+$ T cells were then positively selected. Jurkat and HUT78 T-cell lines were maintained as previously described.[14]

Flow cytometry, real-time polymerase chain reaction, and cell culture

Surface expression of CD158b/j was analyzed by flow cytometry on a LSRII cytometer (BD Biosciences). The following antibodies were used: FITC-conjugated anti-CD158b/j, allophycocyanin (APC)–Cy7 anti-CD3, PerCP anti-CD4, APC anti-CD8, PE anti-CD28, PE-Cy7 anti-CD45RA, and PE anti-CD16 (all from BD Biosciences). Total RNA from sorted cells was isolated with the use of TRIzol (Invitrogen Life Technologies). *KIR2DL3* and *β-actin* transcription levels were assayed by SYBR quantitative reverse transcription polymerase chain reaction (PCR) with the use of primers listed in Table 2. Serial dilutions of a plasmid-expressing gene-specific sequence were amplified in parallel. Transcript numbers were determined by interpolation on the standard curve. Results are expressed as the number of *KIR2DL3* transcripts per 10^7 *β-actin* transcripts. For *KIR2DL3* induction, cells were cultured with rIL-2 (50 U/mL) and Dynabeads CD3/CD28 T-cell expander (Invitrogen Life Technologies). On day 3, DNMT inhibitor 5-Aza-dC (Sigma-Aldrich) was added at a final concentration of 1 μM. The cells were cultured for an additional 24 to 72 hours before being analyzed for CD158b/j surface expression and *KIR2DL3* transcription.

Bisulfite sequencing and methylation-specific PCR

Genomic DNA was isolated from cells by proteinase K digestion followed by phenol/chloroform extraction and RNase digestion. DNA (0.5 ~ 1 μg)

Table 2. PCR primers

Name	Sense	Antisense
KIR2DL3 mRNA	CCACTGAACCAAGCTCCG	CAGGAGACAACTTTGGATCA
β-actin	ATGGCCACGGCTGCTTCCAGC	CATGGTGGTGCCGCCAGACAG
Methylation-specific primers		
KIR2DL3		
1st round	TTATTTTGATGTTTTTGGTTTAGTAG	CCTATATCTCCAACTCTAAACC
2nd round	CGTGTATGAGAGGTTGGATTTGAG	CCTTCCAAAACTCACCAAC
CpG −170/−160	TATGTTTCGTTTTTGAGC (M) TATGTTTTGTTTTTGAGT (U)	TTCCAAAACTCACCAACACA (M and U)
CpG −120/−116	TAGAAAGAGTTTGCGTAC (M) TAGAAAGAGTTTGTGTAT (U)	TTCCAAAACTCACCAACACA (M and U)
CpG −50	GGTTTTATGTAAGGTAGAAAGAGT (M and U)	AACTCAACTCAACAACG (M) AACTCAACTCAACAACA (U)
CpG −27/−23	GGTTTTATGTAAGGTAGAAAGAGT (M and U)	ATACAAACAAACGACCG (M) ATACAAACAAACAACCA (U)
ChIP		
KIR2DL3 (−96 to +11)	GGTCAACATGTAAACTGCATG	ATGAGCGACATGGTGCTGTCT
CD70 (−481 to −395)	GCTCAGACAGGAGAATCGCTTG	TCTGTCACCCAGGCTGGAGT
CD11a (−1206 to −1101)	AACTCTTGACCTCAGGTGATC	ACTGGTTGTGCTCCATTAGT

M indicates methylated; and U, unmethylated.

was treated with sodium bisulfite overnight, and 100 ng of bisulfite-modified DNA was amplified with the use of nested *KIR2DL3* promoter-specific primers listed in Table 2. The PCR conditions were 95°C for 4 minutes, followed by 35 cycles of 95°C for 45 seconds, 52°C for 1 minute, 72°C for 2 minutes, and 72°C for 30 minutes. The products from the second-round PCR were cloned with the use of a TOPO TA cloning kit, subcloned, and sequenced (Invitrogen Life Technologies). Methylation status at specific CpG sites in the *KIR2DL3* promoter was assayed with the use of quantitative methylation-specific PCR with 3′-locked nucleic acid methylation- or unmethylation-specific primers (Table 2). Primers for unmethylation and methylation were chosen to yield similar PCR efficiencies. PCR (25 μL) was assembled with the use of 0.5 U of PlatinumTaq DNA polymerase (Invitrogen Life Technologies), 100 nM of each primer, 5 mM $MgCl_2$, 3% DMSO, 200 μM dNTPs, and 2 μL of 1 000 000-fold diluted second-round PCR products as just described. The reactions were cycled with 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 52°C for 15 seconds, and 72°C for 1 minute. Standard curves were generated with the use of serial dilutions of plasmids containing unmethylated or methylated PCR products.

Chromatin immunoprecipitation assay

$CD4^+CD158b/j^-$ and $CD8^+CD158b/j^-$ T cells were crosslinked with 1% formaldehyde for 15 minutes at room temperature; the reaction was stopped by glycine at a final concentration of 125 mM. The cells were washed twice with ice-cold PBS, lysed with lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, (pH 8.1)], and sonicated. The samples were 10-fold diluted with dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, (pH 8.1), and 167 mM NaCl], precleared with salmon sperm DNA/protein A agarose (Millipore Corporation), and then precipitated with the following antibodies overnight at 4°C: anti-DNMT1 (Imgenex Corp), anti-DNMT3a (Imgenex Corp), anti-DNMT3b (Abcam Inc), anti-EZH2 (Millipore Corporation), anti–H3-Trimethyl-Lys27 (Millipore Corporation), and anti-UHRF1 (gift from Dr C. Bronner, Université Louis Pasteur and Proteogenix); rabbit IgG was used as negative control. Immune complexes were collected, washed, and eluted. The DNA was purified with proteinase K digestion and phenol/chloroform extraction and then used for real-time PCR. Primers selected for hypermethylated regions of the *KIR2DL3*, *CD70* and *CD11a* promoters are listed in Table 2. Immunoprecipitated (IP) DNA was calculated using the following equation: $(DNA_{\text{specific IP}} - DNA_{\text{control IP}})/DNA_{\text{input}}$ with $DNA_{\text{specific IP}}$ = amount of DNA IP using a specific antibody; $DNA_{\text{control IP}}$ = amount of DNA IP with rabbit IgG; DNA_{input} = sheared chromatin before IP.

Results

Age-associated expression of CD158b/j correlates with increased transcription activity

With increasing age, T cells gain the expression of KIR receptors. This phenomenon is essentially limited to CD8 T cells and only marginally involves CD4 T cells. The expression of CD158b/j encoded by *KIR2DS2*, *KIR2DL2*, or *KIR2DL3* is shown in Figure 1A. Approximately 30% to 40% of NK cells are positive for these receptors, independent of age. CD4 T cells are essentially negative. The frequency of CD158b/j-positive cells in CD8 cells increases from a median of 1.4% in the age group between 20 and 30 years to 6.4% in 70- to 85-year-old healthy persons ($P < .001$). The frequency of expression in the elderly is highly variable and can reach up to 32%. Increased protein expression highly correlates with transcript numbers; results are shown for the *KIR2DL3* transcripts that account for the majority of CD158b/j expression (Figure 1B). Transcriptional activity is essentially absent in CD4 T cells. CD8 T cells even in the young persons have detectable *KIR2DL3* transcripts which increase with age ($P = .02$). The transcript numbers in the 70- to 85-year-old persons in CD8 T cells reaches approximately half of that present in NK cells.

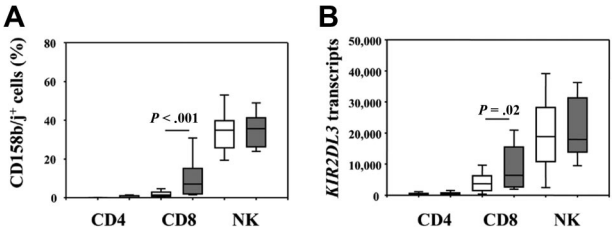

Figure 1. Age-associated expression of *KIR2DL3*. Cell-surface expression of CD158b/j on CD4 T cells, CD8 T cells, and NK cells in 30 persons in the 20- to 30-year-old group (□) and 30 persons in the 70- to 85-year-old group (▨) is shown as box plots with medians, 25th and 75th percentiles as boxes and 10th and 90th percentile as whiskers (A). *KIR2DL3* transcription was measured by real-time PCR in purified T-cell subsets and NK cells in 20- to 30-year-old person (n = 22; □) and 70- to 85-year-old persons (n = 21; ▨) (B). Transcript numbers are expressed relative to 10^7 *β-actin* transcripts.

KIR expression in T cells is controlled by DNA methylation

Recent studies that used reporter gene assays of the minimal KIR promoter have shown that most, if not all, T cells have the transcriptional machinery to support KIR expression, suggesting that KIR transcription in T cells is epigenetically controlled. In NK cells, the single most important mechanism to control expression is promoter demethylation. Inhibition of DNA methylation is sufficient to convert from the stochastic KIR expression in individual NK-cell clones to global expression in all NK cells. We, therefore, examined whether KIR expression in T cells is sensitive to DNA demethylation. T cells from 20- to 30-year-old and 70- to 85-year-old persons were stimulated with anti-CD3 and anti-CD28 antibodies for 72 hours, cultured in the presence or absence of 5-Aza-dC for an additional 72 hours, and examined for *KIR2DL3* transcripts and the cell-surface expression of CD158b/j. Cells naturally expressing CD158b/j were lost under these culture conditions, and the frequencies of $CD158b/j^+$ cells in the culture were very low even in the elderly. DNMT inhibition induced expression in approximately 40% of all cells (Figure 2A). In contrast to naturally occurring CD158b/j expression, no difference was observed between CD4 and CD8 T cells. Protein expression data correlated with transcriptional activity.

To identify relevant DNA methylation sites in the *KIR2DL3* promoter, we compared 2 T-cell tumor lines that differed in KIR expression. The results from bisulfite sequencing of the *KIR2DL3* promoter in $CD158b/j^+$ HUT78 and $CD158b/j^-$ Jurkat cells are shown in Figure 2C. *KIR2DL3* has 12 CpG sites in the first 250 base pairs upstream of the translation initiation site. With the exception of more extensive demethylation at positions +16 and −246, Jurkat cells only showed some scattered demethylation at most other positions. In contrast, the *KIR2DL3* promoter in HUT78 cells was globally unmethylated. To obtain a quantitative assessment of demethylation, we designed 3′-locked nucleic acid primer sets specific for methylated and unmethylated sequences; quantitative PCR (qPCR) results are shown in Figure 2D. Jurkat cells were essentially fully methylated at positions −23 and −27; in all other positions, a high degree of methylation was seen. In contrast, methylation levels in HUT78 cells were 20% or less at all 4 sites.

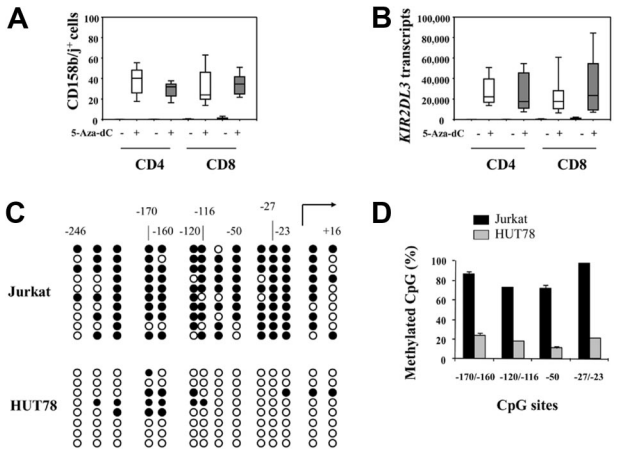

Figure 2. *KIR2DL3* expression in T cells is controlled by DNA methylation. CD4 and CD8 T cells were isolated from 20- to 30-year-old persons (n = 13; □) and 70- to 85-year-old persons (n = 12; ▨), activated by CD3/CD28 beads for 72 hours, and then cultured in the absence or presence of 1 μM 5-Aza-dC for an additional 72 hours. CD158b/j surface expression was analyzed by flow cytometry (A) and *KIR2DL3* transcripts were quantified by real-time PCR and are given relative to 10^7 *β-actin* transcripts (B). Methylation of CpG sites in the *KIR2DL3* core promoter in CD158b/j$^-$ Jurkat cells and CD158b/j$^+$ HUT78 cells were analyzed by bisulfite sequencing. Each row represents an individual subclone; nucleotide positions are relative to the translation initiation codons. Closed symbols indicate methylated, open symbols unmethylated, CpG (C). DNA methylation levels at CpG −170/−160, −120/−116, −50, and −27/−23 were quantified for Jurkat and HUT78 cells by methylation-specific real-time PCR with the use of 3′-locked nucleic acid primers (D). Results are shown as mean ratio ± SD of methylated DNA versus total copies and are representative of 3 experiments.

Demethylation of the *KIR2DL3* promoter is age-associated and T-cell subset specific

CD4 T cells, CD8 T cells, and NK cells were purified from 13 persons in the 20- to 30-year-old group and 13 persons in the 70- to 85-year-old group, and demethylation of CpG islands in the *KIR2DL3* promoter was quantified by qPCR (Figure 3). With the exception of position −50, which showed a trend toward higher demethylation in the older subjects ($P = .07$), age did not have an affect on demethylation of the *KIR2DL3* promoter in CD4 T cells. In contrast, age-associated demethylation was seen in CD8 cells for all sites analyzed. In fact, at all sites the demethylation status reached approximately the same level that was seen in NK cells. No influence of age on the methylation status was noted for NK cells.

KIR promoter analysis in CD158b/j$^-$ CD8$^+$ T cells suggests age-associated cumulative DNA demethylation

The finding that KIR promoter demethylation in CD8 T cells approached the levels seen in NK cells was surprising because CD158b/j-expressing CD8 T cells are less frequent than NK cells and usually do not exceed 10% to 20%, preferentially in the subset of terminally differentiated effector cells (Figure 1). We therefore examined whether CD8$^+$CD158b/j$^-$ T cells already developed evidence of progressive KIR promoter demethylation with age. Results are shown in Figure 4. T cells from 20- to 30-year-old and 70- to 85-year-old persons were separated into CD4 and CD8 T cells negative for CD158b/j and T cells that express CD158b/j. Compared with cells expressing CD158b/j, transcript levels in CD158b/j$^-$ T cells were minimal to absent (Figure 4A). However, although CD4 T cells did not have *KIR2DL3* transcripts at all, a low transcript number was found in CD8 T cells lacking CD158b/j protein, in particular in the older age group. Still, this transcriptional activity constituted less than 5% of what was seen in CD158b/j$^+$ cells. T-cell subsets were examined for CpG demethylation in the KIR promoter by qPCR. For most CpG sites examined, an age-associated increase in *KIR2DL3* promoter demethylation was observed in CD8 T cells that lacked CD158b/j protein expression. The only exceptions were the CpG motifs at −27/−23 which remain methylated in CD8$^+$CD158b/j$^-$ T cells irrespective of age (Figure 4B). Terminally differentiated CD45RA$^+$CD8$^+$ T cells exhibited a higher degree of promoter demethylation, in particular in the elderly; however, even CD8$^+$CD45RO$^+$CD158b/j$^-$ T cells display an age-associated increase in promoter demethylation at positions −170, −160, −120, −116, and −50 (Figure 4C). These data suggest that demethylation of the *KIR2DL3* promoter in CD8 T cells is scattered and cumulative. A similar progressive demethylation is not found in CD4 T cells. Efficient transcription and protein expression requires full demethylation, including positions −27/−23. This model was supported by bisulfite sequencing that showed a scattered *KIR2DL3* demethylation in elderly CD8$^+$CD158b/j$^-$ T cells with a trend toward allelic clustering (Figure 4D).

Age-associated promoter demethylation predisposes to CD158b/j expression

As described in Figure 2, both CD4 and CD8 cells in young persons are able to express CD158b/j protein on 72-hour DNMT inhibition. We wanted to determine whether the scattered demethylation seen in the *KIR2DL3* promoter with age renders T cells more susceptible to full KIR expression. T cells were activated and cultured in the presence or absence of 5-Aza-dC. CD158b/j$^+$ cells emerged as early as 24 hours after DNMT inhibition (Figure 5A). CD8 T cells from elderly persons were more susceptible to DNMT inhibition and displayed an earlier expression of CD158b/j in the presence of 5-Aza-dC compared with CD8 T cells from young persons ($P = .001$) or to CD4 T cells from elderly persons ($P < .001$). At 72 hours, CD4 and CD8 T cells reached similar CD158b/j expression plateaus independent from the age of the donor with approximately a 40% positive rate.

A similar trend was seen when cells were activated with anti-CD3 and cultured without DNMT inhibition for 1 week in the presence of IL-15 (Figure 5B). CD158b/j$^-$ cells were purified; residual contamination was less than 0.1%. After 7 days, less than 0.2% of CD4 T cells expressed CD158b/j irrespective of the age of the donor. Expression of CD158b/j in CD8 T cells from young persons was only slightly higher. In contrast, 1% of CD8 T cells

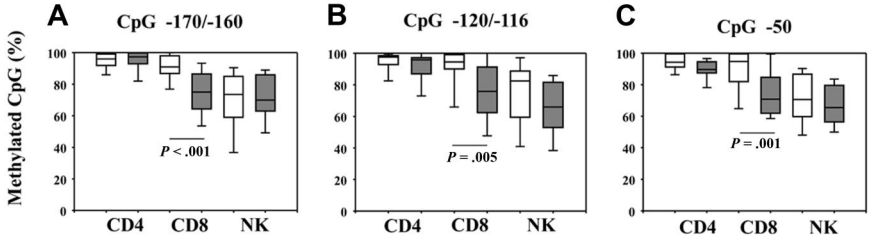

Figure 3. Age-associated decline in *KIR2DL3* core promoter methylation. CD4 T-cell, CD8 T-cell, and NK-cell DNA from 20- to 30-year-old persons (n = 13; □) and 70- to 85-year-old persons (n = 13; ▨) were treated with sodium bisulfite; methylation at CpG sites −170/−160 (A), −120/−116 (B), and −50 (C) were quantified by methylation-specific real-time PCR as described in Figure 2.

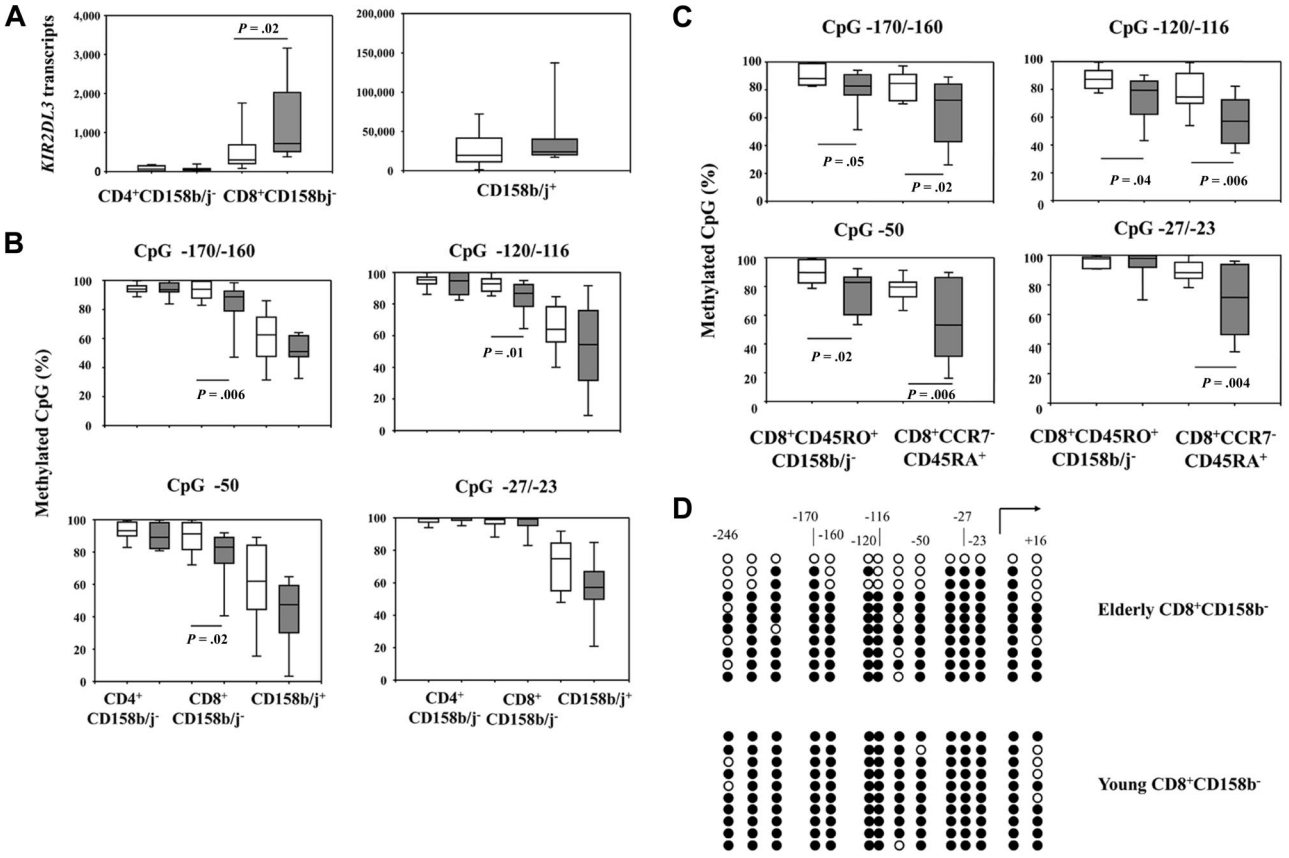

Figure 4. Age-associated demethylation of *KIR2DL3* core promoter precedes transcription. DNA methylation was quantified by qPCR in CD158b/j$^-$ CD4 and CD8 T cells and in CD158b/j$^+$ T cells. (A) *KIR2DL3* transcripts were low in CD158b/j$^-$ (18 young and 14 elderly persons) compared with CD158b/j$^+$ T cells (13 young and 12 elderly persons) irrespective of age, although a slight age-associated increase for CD8 T cells was seen. (B) Results for methylation-specific PCR of CpG sites −170/−160, −120/−116, −50, and −27/−23 in CD4 and CD8 T cells negative for CD158b/j cell-surface expression were compared with CD158b/j$^+$ T cells; results are shown as box plots of 15 young (□) and 15 elderly (▨) persons. (C) CD8$^+$CD45RO$^+$CD158b/j$^-$ and CD8$^+$CCR7$^-$CD45RA$^+$ from 10 young and 10 elderly persons were compared for demethylation of the *KIR2DL3* promoter. (D) The methylation of the *KIR2DL3* promoter in CD158b/j$^-$ CD8 T cells was examined by bisulfite sequencing. Closed symbols represent methylated, open symbols unmethylated, CpG. A representative example of an elderly and a young person is shown.

from elderly persons gained expression. CD158b/j acquisition in elderly CD8 T cells depended on IL-15 or, to a lesser effect, IL-7, whereas TCR stimulation was not sufficient (Figure 5C).

DNMT1 recruitment to the *KIR2DL3* promoter declines with age

DNA methylation has been described to decrease with age; however, CpG islands are usually spared from age-associated demethylation. In preliminary studies, we confirmed the age-associated decline, but CD158b/j$^+$ cells did not have a globally lower degree of DNA methylation compared with CD158b/j$^-$ T cells (data not shown). In addition, the selectivity of KIR promoter demethylation for CD8 T cells makes a global defect such as reduced expression of DNMT unlikely. To examine whether CD4 and CD8 T cells from donors of different ages differ in DNMT recruitment to the KIR promoters, chromatin immunoprecipitation (ChIP) assays on CD4$^+$ and CD8$^+$CD158b/j$^-$ T cells were performed. Results for DNMT1 and DNMT3a are shown in Figure 6A and B. Immunoprecipitation of DNMT3a did not show any cell-specific or age-associated effects. In contrast, DNMT1 binding to the *KIR2DL3* promoter was lower in CD8 than in CD4 T cells. This difference tended already to be present in cells from young persons and reached significance in elderly T cells ($P = .04$). DNMT1 recruitment to the *KIR2DL3* promoter decreased with age for both T-cell subsets and was virtually lost only in CD8 T cells from elderly persons ($P = .03$). The age-related decline in DNMT1 recruitment was selective for *KIR2DL3* and not seen for CD70 or *CD11a*. Of note, the decline in DNMT1 recruitment was seen in CD158b/j$^-$ CD8 T cells, suggesting that CpG demethylation and reduced recruitment of methyl-CpG–binding proteins are not the primary reasons. We also could not find any *KIR2DL3* promoter sequences associated with UHRF1, which is known to preferentially recruit DNMT1 in mammalian cells (data not shown). Polycomb proteins have recently been shown to be important in DNMT recruitment. Age did not influence KIR promoter binding of EZH2, a polycomb protein that binds all DNMT and methylates H3 at position Lys 27. Accordingly, we also did not find an age-associated decline in H3-trimethyl-Lys27 at the *KIR2DL3* promoter (Figure 6).

Discussion

KIR expression in CD4 and CD8 T cells is controlled by CpG DNA demethylation, resembling the epigenetic control mechanism that has been described for NK cells. With increasing age, CpG motifs in the *KIR2DL3* promoter are progressively demethylated in CD8 but not in CD4 T cells. The demethylation is patchy and appears to be stochastic with sparing of positions −27/−23 in CD158b/j$^-$

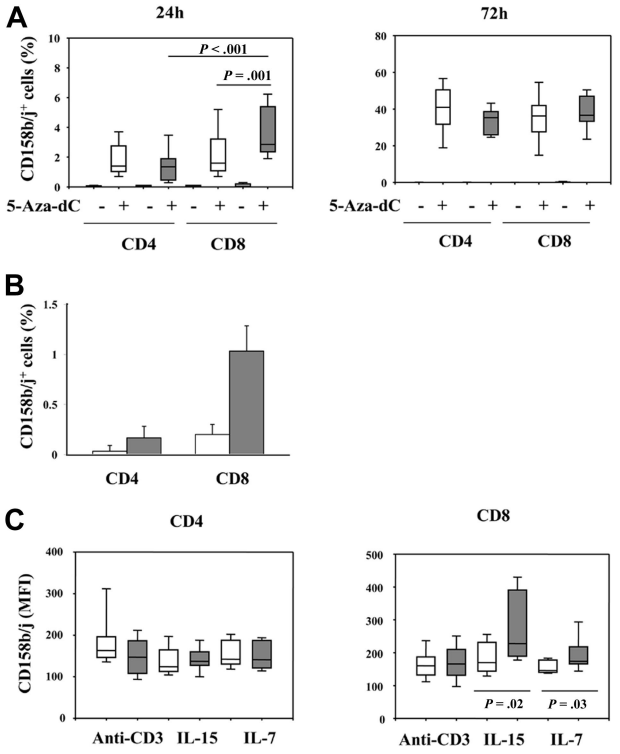

Figure 5. Decreased threshold for KIR transcription in the elderly. (A) CD158b/j$^-$ CD4 and CD8 T cells from 20- to 30-year-old persons (n = 15; □) and 70- to 85-year-old persons (n = 12; ▨) were activated with CD3/CD28 beads and cultured in the absence or presence of 1 μM 5-Aza-dC as described in Figure 2. Cells were examined at indicated time points by flow cytometry. At 24 hours, CD158b/j expression in CD8 T cells from elderly persons was higher than in CD4$^+$CD158b/j$^-$ ($P < .001$) and in young CD158b/j$^-$ CD8$^+$ T cells ($P = .001$). No difference among any of the 5-Aza-dC–cultured groups was seen at 72 hours. (B) CD158b/j$^-$ cells from 3 young and 3 elderly persons were cultured with anti-CD3 (0.1 ng/mL) and IL-15 (5 ng/mL); (C) cells from 7 young and 7 elderly persons were stimulated with anti-CD3, IL-15 (5 ng/mL), or IL-7 (10 ng/mL). CD158b/j expression was assayed after 7 days. Frequencies (B) and MFI (C) in young (□) and elderly (▨) persons are shown.

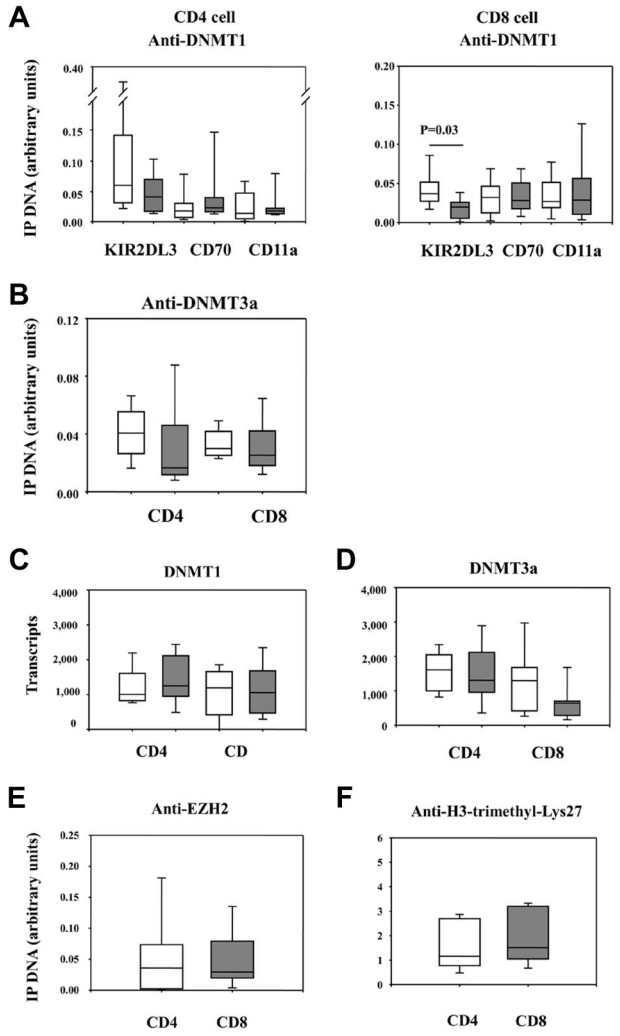

Figure 6. Age-associated decline in DNMT1 recruitment by the *KIR2DL3* promoter. Chromatin immunoprecipitation of CD158b/j$^-$ CD4 and CD8 T cells from 20- to 30-year-old persons (□) and 70- to 85-year-old persons (▨) was performed with antibodies to DNMT1 (A) and DNMT3a (B). Precipitates were analyzed for the presence of *KIR2DL3*, *CD11a*, and *CD70* promoter sequences. The ratios of $DNA_{specific}$ IP − $DNA_{control}$ IP to DNA_{Input} are shown as box plots of 7 young (□) and 7 elderly (▨) persons. Transcripts of DNMT1 (C) and DNMT3a (D) in the same donors were quantified by real-time PCR and are shown after normalization for *β-actin* transcripts. Recruitment of the polycomb protein EZH2 (E) and histone H3 trimethyl Lys27 (F) to the *KIR2DL3* promoter was assessed. ChIP assay results of CD158b/j$^-$ CD8 T cells are shown as box plots of 6 young (□) and 6 elderly (▨) persons.

cells, whereas complete promoter demethylation is characteristic for *KIR2DL3*-transcribing cells. The selectivity for CD8 T cells correlates with lower DNMT1 recruitment that further diminishes with age. Histone H3-Lys27 and H3-Lys9 methylation known to be involved in DNMT1 recruitment are not different in CD4 and CD8 T cells; however, CD8 T cells have a histone signature characteristic of transcriptional activation that may cause increased displacement of DNMT.[30] The reduced recruitment of DNMT1 and the partial demethylation of the KIR promoter seen with age lowers the threshold for transcriptional activation and renders CD8 T cells more susceptible to express KIR in response to environmental stressors and in particular to proliferative stress.

Epigenetic control mechanisms of KIR expression have been well documented in NK cells.[23,31] Individual NK cells express between 1 and 9 KIR genes in an apparently probabilistic distribution. In individual NK-cell clones, the pattern of KIR expression is stable; however, inhibition of DNMT by 5-Aza-dC, but not the histone deacetylase inhibitor Trichostatin A, induces global expression of all available KIR genes.[23] KIR promoters are densely methylated in silenced genes; demethylation strictly correlates with allelic KIR expression.[24] CpG methylation of the minimal KIR promoter inhibits transcription.[14,23] Demethylation is acquired during NK-cell development; the genetic region is fully methylated in hematopoietic stem cells.[30] Chan et al[31] have examined human NK cells and cell lines representing different lineages to address the question of whether histone modifications set the stage for DNA demethylation. That research identified a histone signature with acetylated histone H3 and H4 and trimethylated H3 lysine 4 which correlated with the competency to transcribe KIR genes but which was already present in NK cells that had methylated KIR promoter and lacked transcription. Santourlidis et al[30] extended these observations and described a repressive histone signature with H3-Lys9 demethylation and absence of H4-Lys8 acetylation in hematopoietic stem cells which was reversed in NK cells even before they transcribe KIR. These data are consistent with a model of histone activation preceding DNA demethylation. Chan et al[31] suggested that KIR promoter demethylation may be an active process by presumptive DNA demethylases recruited to the activated histone structure.

Less is known about the regulation of KIR expression on T cells. Initial studies suggested that KIR expression occurs in the transition to effector T cells; however, it is not a universal feature of all effector T cells.[32,33] T-cell stimulation in vitro is ineffective in inducing KIR. In contrast to NK cells, KIR expression on T cells in vivo is not random but shows hierarchical clustering in individual T-cell clones, consistent with successive activation of KIR genes during clonal expansion.[28] As in NK cells, epigenetic mechanisms control expression. In reporter gene assays with the minimal KIR promoter, even naive T cells supported transcription.[25] DNMT inhibition induces KIR expression equally in approximately 40% of CD4 and CD8 T cells; it is currently unclear whether the remaining T cells are refractory or whether the expression is incomplete because of tissue culture conditions. Of note, there was no obvious difference between CD4 and CD8 T cells, although KIR expression in vivo is largely restricted to CD8 T cells.

Cross-sectional studies in healthy persons of different ages supported the notion that KIR expression in T cells with aging is closely related to DNA demethylation. We examined 4 different CpG sites in the *KIR2DL3* promoter and found that all 4 sites are methylated in CD4 and CD8 T cells in the young adult, but demethylated in CD8 T cells of elderly persons. The preferential demethylation observed in CD8 T cells correlated with the observation that only CD8 T cells had an age-associated increase in KIR transcript and protein levels. However, surprisingly, the demethylation in CD8 T cells reached a similar extent as in NK cells, although transcript and protein expression were lower in CD8 T cells. We therefore explored whether demethylation already occurred with age in CD8 T cells that lack protein expression. This was indeed the case for 3 of the 4 sites examined; only demethylation at position −23/−27 appeared to be strictly correlated with expression.

KIR expression is preferentially found in terminally differentiated CD45RA effector CD8 T cells that accumulate with age. To examine whether KIR promoter demethylation is restricted to these cells or whether it occurs in all CD8 T-cell subsets, purified subpopulations were analyzed. End-differentiated effector cells had a higher degree of promoter demethylation than normal effector T cells as one would expect from the preferential KIR expression in these cells. However, an age-related increase in CpG demethylation was seen for both CD8 CD45RA and CD45RO memory T-cell subsets. End-differentiated CD8 effector T cells have a longer replicative history than normal T cells. Our findings are therefore consistent with a model of progressive passive demethylation with age that may occur with clonal expansion or homeostatic proliferation. In contrast to NK cells in which KIR promoters are methylated in an all-or-none pattern with strict correlation to transcription, the demethylation is incomplete and patchy in many T cells.

The partial DNA demethylation observed for the KIR promoter may be gene specific or part of a global DNA demethylation that has been observed with aging.[34] In our own studies, the fraction of CpG-methylated DNA drops in T cells from 50% in young adults to 35% in elderly persons (data not shown). This demethylation appears to be directly related to age and not secondary to other cofactors such as comorbidities or medication usage that are frequently distinct in an elderly population. In the present study, the elderly patients were frequently on statins, NSAIDs, and antihypertensives. Although a contribution of these medications cannot be excluded, none of them has been implicated in DNA demethylation or DNMT1 inhibition.

Previous studies have suggested that the global DNA demethylation preferentially involves transposons and spares typical CpG islands; in fact, CpG islands are frequently hypermethylated with age.[35-37] CpG sites in the KIR promoter are not densely clustered and do not qualify as typical CpG islands. A similar increased gene expression with age and in particular in effector cells has also been reported for other demethylation-sensitive genes such as CD11a and *CD70*.[38-40]

If KIR promoter demethylation in T cells is the local sequel of a global defect, how can it be CD8 T cell–subset specific? CD4 and CD8 T cells have several similarities in regulating KIR transcription. Reporter gene assays with the KIR promoter show that the promoter is supported in CD4 T cells.[25] CD4 and CD8 T cells have a similar response pattern to DNMT inhibition with KIR expression in approximately 40% of all cells. Homeostatic turnover is known to be approximately 2-fold higher in CD8 cells than in CD4 T cells, and CD8 T cells have impaired homeostatic mechanisms with age, leading to the expansion of effector cells and accumulation of terminally differentiated cells.[41-44] The increased replicative stress may increase the susceptibility of these cells to passive demethylation. Alternatively, the selective KIR promoter demethylation may be gene and cell specific. In support of this latter hypothesis, recruitment of DNMT1 to the *KIR2DL3* promoter was lower in CD8 T cells than in CD4 T cells in young and old persons. DNMT1 recruitment declined in both subsets with age but reached very low levels and possibly limiting concentrations only in CD8 T cells. The reduced DNMT1 recruitment was selective for *KIR2DL3* and not seen for other methylation-sensitive genes such as *CD11a* and CD70. In conjunction with the constitutive proliferative activity of CD8 effector cells, this may be sufficient to accumulate passive demethylation.

Several molecular mediators have been implicated in DNMT recruitment. During proliferation, DNMT1 associates with nuclear antigen of proliferating cells and recognizes hemimethylated CpG dinucleotides in maternal and daughter DNA strands. In ex vivo T cells, less than 1% to 2% of all T cells are in cell cycle in young adults and approximately 2-fold as many in the elderly, suggesting that this mechanism does not account for the decreased DNMT 1 recruitment with age or in CD8 T cells. H3-Lys9 methylation, which is linked with gene silencing, is essential for DNA methylation in fungi and plants.[45,46] However, Santourlidis et al[30] have described a reduced H3-Lys9 methylation at the KIR promoter equally in CD4 and CD8 T cells, although the promoters were still fully methylated. A member of ubiquitin-like protein containing PHD and RING domains (UHRF1) is known to directly recruit DNMT1 to the target gene[47]; however, we could not demonstrate any UHRF1 protein associated with the *KIR2DL3* promoter in our ChIP assays, neither for CD4 nor for CD8 T cells (data not shown). Vire et al[48] have identified EZH2 as an important recruitment platform for all 3 DNMTs in vitro and in vivo. EZH2 is a histone methyltransferase that functions as part of Polycomb repressive complexes and methylates H3-Lys27. Depletion of EZH2 disturbs DNMT recruitment, decreases H3-Lys27 methylation, and derepresses genes.[48] KIR appears to be an EZH2 target gene (data not shown); however, we found equal amounts of EZH2 and H3-Lys27 methylation in our ChIP assays of CD4 and CD8 T cells of different age groups, suggesting that this mechanism is not causing the differences in DNMT1 recruitment or KIR promoter methylation. Other histone modifications may influence DNMT1 recruitment or, in conjunction with transcription factor binding, may lead to DNMT replacement. It is of interest that Santourlidis et al[30]

described a difference in histone signatures in T-cell subsets. Only CD8 but not CD4 T cells had acetylated H4-Lys8 associated with the *KIR2DL3* promoter. This active histone signature was observed in silent KIR genes that were densely methylated.[30] It is possible that this partial histone activation predisposes for DNMT 1 replacement.

Expression of KIR has important implications for T-cell immunity. Inhibitory KIRs recognize MHC class I molecules that are present on all somatic cells. On TCR stimulation, KIRs are recruited to the T-cell activation platform and attenuate signaling by recruiting the phosphatases SHP-1[19,20,49] and SHP-2.[49] In contrast to other negative regulatory receptors such as PD-1 that are transiently active and have been implicated in the failure to clear chronic infections, the constitutive expression of KIR and their ligands exerts an even more profound effect that is present at early and late stages of T-cell activation. Our observation that the KIR promoter is partially demethylated with age even in CD8 T cells that lack transcription raises the possibility of a widespread negative regulatory role of KIR in CD8 T cells. It is currently unclear when during an immune response KIRs are physiologically expressed on T cells; however, they probably function as a negative feedback mechanism to limit effector cell function. The partial demethylation may lower the threshold for transcriptional activation, increase the probability of KIR expression during antigen-induced clonal expansion, and thereby limit effector cell function. Indeed, CD8 T cells from elderly persons respond more rapidly to suboptimal DNMT1 inhibition with KIR expression and have a faster rate of KIR acquisition in cytokine-driven cultures (Figure 5).

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Authorship

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