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*J Immunol* 2011; 186:4590-4598; Prepublished online 14 March 2011; doi: 10.4049/jimmunol.1002732
http://www.jimmunol.org/content/186/8/4590

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/03/14/jimmunol.1002732.DC1

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Human NK Cells Proliferate and Die In Vivo More Rapidly than T Cells in Healthy Young and Elderly Adults

Charles T. Lutz,* Anush Karapetyan,*+ Ahmad Al-Attar,* Brent J. Shelton,† Kimberly J. Holt,* Jason H. Tucker,*+ and Steven R. Presnell*

NK cells are essential for health, yet little is known about human NK turnover in vivo. In both young and elderly women, all NK subsets proliferated and died more rapidly than T cells. CD56
bright
NK cells proliferated rapidly but died relatively slowly, suggesting that proliferating CD56
bright
cells differentiate into CD56
dim
NK cells in vivo. The relationship between CD56
dim
and CD56
bright
proliferating cells indicates that proliferating CD56
dim
cells both self-renew and are derived from proliferating CD56
bright
NK cells. Our data suggest that some dying CD56
dim
cells become CD16
−CD56
−
NK cells and that CD16
−CD56
low
NK cells respond rapidly to cellular and cytokine stimulation. We propose a model in which all NK cell subsets are in dynamic flux. About half of CD56
dim
NK cells expressed CD57, which was weakly associated with low proliferation. Surprisingly, CD57 expression was associated with higher proliferation rates in both CD8
+ and CD8
−
T cells. Therefore, CD57 is not a reliable marker of senescent, nonproliferative T cells in vivo. NKG2A expression declined with age on both NK cells and T cells. Killer cell Ig-like receptor expression increased with age on T cells but not on NK cells. Although the percentage of CD56
bright
NK cells declined with age and the percentage of CD56
dim
NK cells increased with age, there were no significant age-related proliferation or apoptosis differences for these two populations or for total NK cells. In vivo human NK cell turnover is rapid in both young and elderly adults. The Journal of Immunology, 2011, 186: 4590–4598.

Natural killer cells rapidly kill infected, damaged, and transformed cells. NK cells also secrete cytokines and chemokines that directly affect abnormal cells and shape subsequent adaptive immunity (1). Human NK cells can be divided into four subsets based on CD16 and CD56 expression. CD56
bright
(CD56++CD16low/−) cells compose a small fraction of blood NK cells but are highly enriched in some tissues (1). They have limited cytotoxic activity but secrete cytokines and chemokines upon stimulation. CD56
dim
(CD56+CD16+) are the major blood NK subset, spontaneously kill susceptible tumor cells, and rapidly secrete cytokines and chemokines (1, 2). At least some CD56
dim
NK cells are derived from differentiating CD56
bright
cells (1, 3–6). The poorly characterized CD16
−CD56
low
population may include recently activated CD56
dim
NK cells, because CD16 is downregulated by various stimuli, but this population has not been thoroughly studied (7–9). CD16
−CD56
−
NK cells are heterogeneous. These cells increase in HIV infection and are poorly functional, consistent with senescence (10, 11). However, NK cells with the CD16
−CD56
−
cell surface phenotype are prevalent in neonates and differentiate into functional NK cells in vitro (12, 13). We know little of the stability of and flux between human NK subpopulations in vivo.

Unlike T and B lymphocytes, NK cell number is maintained in healthy aging, and NK function is affected only modestly on a per cell basis (8, 14–16). Compared with the healthy elderly, frail elderly people are NK deficient and are very susceptible to infections and cancer deaths, suggesting that NK cells are critical for healthy aging (14). At all ages, NK cell deficiency is associated with infectious diseases and with noninfectious diseases of several organs (1, 17–19).

Although important for health, relatively little is known about NK cell turnover. DNA labeling and cell cycle analysis showed that adult mouse splenic NK cells proliferate faster than total splenic T cells but similar to NK1.1
+ T cells (20). In comparison, 
[2H]
DNA labeling studies in humans and primates suggested that NK cells proliferate more slowly (21, 22). Because human NK turnover is poorly characterized, we investigated proliferation and apoptosis among four NK subsets in relation to aging.

Materials and Methods

Donor recruitment

As approved by the human subjects institution review board, we deferred subjects with conditions that affect NK cells, acute illness, or abnormal vital signs, as described (23). Most studies were conducted on 38 young (median age 26.5 y, range 21–30 y) and 34 elderly (median age 81.2 y, range 74–96 y) women. CD57
+ cell proliferation was assessed in five female and seven male healthy subjects, ages 28–72 y. NK cell function was studied in three healthy males, ages 37–57 y. Blood from one to three subjects was collected into heparin by standard venipuncture and analyzed the same day.

Flow cytometry analysis

PBMCs were preincubated with human IgG (Sigma-Aldrich, St. Louis, MO) to block nonspecific mAb uptake and then stained with mAb to CD14 (Invitrogen, Carlsbad, CA), CD3 (Abcam, Cambridge, MA), CD16 (Invitrogen), CD56 (BioLegend, San Diego, CA), CD57 (BioLegend), and either NKG2A (BD Biosciences, San Jose, CA) or killer cell Ig-like receptor (KIR). The anti-KIR mAb mixture consisted of NKYFS1 (Abcam), specific

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for KIR2DL1, KIR2DS1, KIR2DS2, and KIR2DS4, and DX9 (BD Biosciences), specific for KIR3DL1. After staining, washed cells were fixed with 2% paraformaldehyde and analyzed on a flow cytometer (LSRII; BD Biosciences). Live and apoptotic cells were distinguished by standard light scatter criteria (24). NK cells were identified as CD3+ cells that expressed CD16, CD56, or both markers. NK subsets were identified as illustrated (Fig. 1). NK-like T (TNK) cells were defined as CD3+ cells that expressed CD16, CD56, or both markers, whereas CD3+ conventional T (T Con) cells expressed neither marker.

For TUNEL and Ki67 assays, a separate aliquot of cells were stained as above, but without mAb to CD14 or CD57. Stained cells were fixed, washed, and then permeabilized in 70% ethanol on ice for 0.5 h and followed by −20°C overnight. For the TUNEL assay (24), washed cells were labeled for 1.25 h at 37°C with BrdU and terminal deoxynucleotransferase (Invitrogen). Washed cells were incubated with FITC anti-BrdU or control mAb (Invitrogen). For the Ki67 assay, washed cells were incubated with FITC anti-Ki67 or control mAb (BD Biosciences). For both assays, cell clumps and debris were excluded from flow cytometry analysis using DAPI width, DAPI area, and light scatter characteristics by a scientist (A.K.) who was blinded to subject ages. Four elderly and two young donor samples were not analyzed for Ki67 and TUNEL either because of flow cytometer UV toxicity and debris were excluded from flow cytometry analysis using DAPI width, DAPI area, and light scatter characteristics by a scientist (A.K.) who was blinded to subject ages. Four elderly and two young donor samples were not analyzed for Ki67 and TUNEL either because of flow cytometer UV laser malfunction or because of mAb quality on the day of the experiment. An additional elderly sample was excluded as an outlier (54% Ki67+ among CD56dim NK cells compared with the elderly mean ± SD of 3.3 ± 2.1%). Representative examples of cell gating are shown in Fig. 1. The range and median number of TUNEL+ events used to calculate proliferation in T and NK subsets were as follows: total NK cells (60–20,602; 2,597), total T cells (210–82,617; 2,597), CD56dim NK cells (16–9,674; 646), CD56bright NK cells (0–561; 21), CD16+CD56+ NK cells (30–19,166; 2,907), CD16+CD56dim NK cells (4–3,847; 263), T Con cells (167–83,736; 1,919), and T NK cells (26–9,868; 571). For Ki67+ events; total NK cells (28–2,756; 382), total T cells (24–4,121; 792), CD56dim NK cells (15–1,297; 161), CD56bright NK cells (3–506; 61), CD16+CD56+ NK cells (2–754; 110), CD16+CD56dim NK cells (1–60; 49), T Con cells (20–3,829; 680), and T NK cells (3–656; 72). For CD57 proliferation studies, mAbs to CD57 were used to detect CD57+ events for CD57+ NK cells (12 subjects) and CD57+ T cells (11 subjects; BioLegend) were added, whereas mAbs to KIR and NKG2A were omitted.

For functional studies, NK cells were enriched using RosetteSep (StemCell Technologies, Vancouver, BC, Canada) to 92–98% purity with ±1% T cells. NK cells (0.1 × 10^6/well in 0.2 ml in 96-well V-bottom plates) were stimulated for 2 h with K562 cells (0.3 × 10^6/well), IL-15 (100 ng/ml), or IL-12 (10 U/ml) plus IL-18 (100 ng/ml) in media containing RPMI 1640, 10% FBS, amino acids, sodium pyruvate, and antibiotics. Cells were processed, stained with mAbs to CD3, CD16, CD56, and CD69 (BioLegend), and analyzed by flow cytometry as described above, except that NK subset gates were adjusted to accommodate stimulation-induced changes in marker expression (Supplemental Fig. 1). For the cytotoxic granule exocytosis assay, anti-CD107a (BioLegend) and 0.2 μl monensin solution (GolgiStop; BD Biosciences) were included in the culture medium, and anti-CD69 was deleted from staining. Alternatively, cells were surface stained, fixed, permeabilized, and stained with anti–IFN-γ mAb (Beckman Coulter, Miami, FL) as described (25), except using permeabilization wash buffer (BioLegend).

Descriptive statistics included means and 95% confidence limits and least squares analysis. Age group and lymphocyte subset differences were analyzed by a non-parametric Wilcoxon rank sum test. The p values greater than the overall α level of 5% were considered non-significant. Relationship between groups was assessed using the non-parametric Spearman’s rank

**FIGURE 1.** Flow cytometry gating and representative results. Shown are sample gating and Ki67 data for CD56bright and CD56dim NK cells from (A) a 24-yr-old subject and (B) a 77-yr-old subject. Cells were gated using DNA content (DAPI area) and pulse width (DAPI width) to exclude cell aggregates and debris (panel 1). Forward and side scatter were used to select lymphocytes (panel 2). After selection of CD3+ cells (panel 3), NK cells were selected as cells that expressed either CD16 or CD56 or both, in the four boxed areas, excluding the left lower quadrant (panel 4). The CD56bright and CD56dim NK subpopulations and the percentage among total NK cells are denoted. Also shown in panel 4 are CD16+CD56dim cells (dashed-line box) and CD16+CD56dim cells (dotted-line box). Ki67 results are show for CD56bright and CD56dim populations (panels 5 and 6). Identical Ki67 gating was used for all NK and T cell populations.
correlation coefficient. Doubling time was calculated as $\log_2(2)$, where $x$ is proliferation. For NK cells, $\log_{1.0528}(2) = 13.5$ d.

**Results**

**Proliferation and apoptosis**

To estimate in vivo proliferation, we measured Ki67, which is expressed by cycling cells and which correlates well with in vivo DNA labeling (26). Unlike $[^3]$H$\mathrm{DNA}$ labeling, Ki67 allows proliferation rates to be measured in minor lymphocyte subsets from many research subjects. Fig. 1 shows sample flow cytometric plots from a young (Fig. 1A) and an elderly (Fig. 1B) subject. Fig. 2 shows data pooled from young and elderly subjects. NK cells proliferated fast (5.3% Ki67$^+$, Fig. 2A), with an estimated doubling time of 13.5 d. As a benchmark for NK proliferation, we compared these results with those of T cells in the same flow cytometry tube. Consistent with prior findings with CD4 T cells (27), total T cells were 1.2% Ki67$^+$. Thus, NK proliferation was 4-fold faster than that of total T cells. Cell death is another measure of turnover, and we quantified apoptosis based on a modified TUNEL assay (Fig. 2B) and on light scatter characteristics (Fig. 2C). Because they were performed several hours after phlebotomy, these assays measured combined in vivo and in vitro apoptosis. In both assays, apoptosis was 3- to 4-fold greater in NK cells than in T cells (Fig. 2B, 2C). NK and T cell proliferation and apoptosis differences also were highly significant within each age group analyzed separately (data not shown). Thus, proliferation and apoptosis assays gave consistent results, suggesting that human NK turnover is 3- to 4-fold greater than T cell turnover. The CD16 and CD56 markers that were used to identify NK subsets also allowed us to identify the small subset of CD3$^+$ T cells expressing one or both of these NK-associated markers. These CD3$^+$ “TNK” cells include a much broader set of T cells than the narrowly-defined “NKT” cells that have restricted TCR $\alpha_24$ gene expression or CD1d Ag specificity (28). TNK cell proliferation and apoptosis rates were considerably greater than those of CD16$^-$CD56$^-$ TCon cells and were similar to those of NK cells (Fig. 2). Thus, human NK cells resemble mouse splenic NK cells that proliferate much faster than total splenic T cells but similar to NK1.1$^+$ T cells (20).

**NK subsets**

CD56$^{bright}$ cells proliferated rapidly but had relatively little apoptosis (Fig. 2). Imbalanced proliferation and death rates might indicate a net migration of CD56$^{bright}$ cells from blood to tissues. Alternatively, dividing CD56$^{bright}$ cells might differentiate into CD56$^{dim}$ cells, consistent with results of xenogeneic adoptive transfer studies and in vitro experiments (1, 3–5). CD56$^{dim}$ cells proliferated less than other NK cells and had a relatively low death rate, suggesting that this was the most stable NK subset (Fig. 2). Compared with CD56$^{dim}$ cells, CD16$^-$CD56$^{low}$ NK cells had high rates of proliferation and apoptosis. CD16$^+$CD56$^{−}$ cells were highly proliferative and very highly apoptotic (Fig. 2). These results are interpreted in the Discussion. KIR$^+$ cells proliferated faster than did KIR$^-$ cells, but this was due to the fact that all three of the rapidly proliferating NK subsets expressed considerably less KIR than did the slower proliferating CD56$^{dim}$ subset (Table I). NK proliferation and apoptosis rates did not correlate significantly with NKG2A expression (Fig. 2).

### Table I. Age-related expression of MHC class I receptors on T and NK subsets

<table>
<thead>
<tr>
<th>Marker</th>
<th>Set</th>
<th>Young (%)</th>
<th>Elderly (%)</th>
<th>Age Effect$^a$</th>
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<tbody>
<tr>
<td>NKG2A</td>
<td>T</td>
<td>3.2</td>
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<td>T</td>
<td>TNK</td>
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<td>1.1</td>
<td>0.0003</td>
<td></td>
</tr>
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<td>NK</td>
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<td>45.4</td>
<td>0.0018</td>
<td></td>
</tr>
<tr>
<td>CD56$^{bright}$</td>
<td>93.3</td>
<td>88.5</td>
<td>0.0037</td>
<td></td>
</tr>
<tr>
<td>CD56$^{dim}$</td>
<td>53.7</td>
<td>45.7</td>
<td>0.0133</td>
<td></td>
</tr>
<tr>
<td>CD16$^-$CD56$^{low}$</td>
<td>38.3</td>
<td>44.9</td>
<td>NS</td>
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<td>23.6</td>
<td>0.0311</td>
<td></td>
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<td>2.4</td>
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<td>TNK</td>
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<td>18.4</td>
<td>NS</td>
</tr>
<tr>
<td>TCon</td>
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<td>1.3</td>
<td>0.0113</td>
<td></td>
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<tr>
<td>NK</td>
<td>42.0</td>
<td>42.0</td>
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<td>CD56$^{bright}$</td>
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<td>15.1</td>
<td>NS</td>
<td></td>
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<tr>
<td>CD56$^{dim}$</td>
<td>53.5</td>
<td>50.2</td>
<td>NS</td>
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<tr>
<td>CD16$^-$CD56$^{low}$</td>
<td>11.5</td>
<td>14.9</td>
<td>NS</td>
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<tr>
<td>CD16$^+$CD56$^{−}$</td>
<td>29.3</td>
<td>25.9</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant.

$^a$Wilcoxon signed-rank test p values.
Because CD16^−CD56^{low} and CD16^+CD56^{−} NK subsets in healthy subjects have received little attention, we studied function. Enriched blood NK cells were stimulated with K562 cells, IL-12/IL-18, and IL-15, and responses were assessed early (2 h) to limit modulation of NK subset markers. CD107a expression indicated cytotoxic granule exocytosis; CD69 and intracellular IFN-γ also were measured (Fig. 3). For all three parameters, CD16^−CD56^{low} NK cells responded more vigorously than the other NK subsets to K562 stimulator cells. Only K562 stimulation consistently elicited cytotoxic granule exocytosis (Fig. 3A). In addition, K562 stimulation induced much higher CD107a cell surface density on positive cells than did the other stimuli (Fig. 3B). IL-12/IL-18 stimulated varying amounts of IFN-γ production by all four subsets after 2 h, with CD16^−CD56^{low} cells being the most vigorous NK subset in all donors (Fig. 3C). In response to IL-12/IL-18, a few CD56^{bright} NK cells expressed CD69, which was much less than the CD69 expressed by the other three NK subsets (Fig. 3D). For all parameters and NK subsets, the response to IL-15 was typically less than the response to IL-12/IL-18. For both IFN-γ and CD69, staining intensities were similar for all positive NK cells and all stimuli (data not shown).

**NK homeostasis**

We investigated the relationship between cell number and proliferation in NK cell subsets. We found a strong linear correlation between the number of proliferating CD56^{bright} and proliferating CD56^{dim} NK cells. The relative number of proliferating CD56^{bright} and CD56^{dim} cells was calculated as (percentage of cells in subset × percentage Ki67^+)/100. The least squares line, Spearman correlation coefficient (r_s), and significance of association (p) are indicated. r_s = 1.0 or −1.0 denote perfect correlation; r_s = 0 denotes no correlation. Young (circles) and elderly (diamonds) subjects analyzed separately showed similar correlations, p < 0.0001.

**FIGURE 3.** CD16^−CD56^{low} NK cells rapidly respond to cellular and cytokine stimulation. NK cells were stimulated for 2 h as indicated and assayed for cell surface CD107a (A, B), intracellular IFN-γ (C), and cell surface CD69 (D). Results are presented as percentage positive cells in each indicated NK subset (A, C, D) or as geometric mean fluorescence intensity on the positive cells (B). Results shown of one subject are representative of three subjects, each analyzed in a separate experiment.

**FIGURE 4.** Number of proliferating CD56^{bright} cells is directly related to the number of proliferating CD56^{dim} NK cells. The relative number of proliferating CD56^{bright} and CD56^{dim} cells was calculated as (percentage of cells in subset × percentage Ki67^+)/100. The least squares line, Spearman correlation coefficient (r_s), and significance of association (p) are indicated. Young (circles) and elderly (diamonds) subjects analyzed separately showed similar correlations, p < 0.0001.

**FIGURE 5.** CD56^{dim} proliferation, but not CD56^{bright} proliferation, correlates with percentage CD56^{bright} NK cells. Ki67 expression in the CD56^{dim} subset (A) and the CD56^{bright} subset (B) are plotted against CD56^{bright} cells as a percentage of NK cells. A. The Spearman correlation coefficient (r_s) and significance of association are indicated; young (circles) and elderly (diamonds) subjects analyzed separately showed similar correlations and were significant. B. No significant correlations were obtained when young (circles) and elderly (diamonds) subjects were analyzed together or separately.
CD56\textsuperscript{dim} NK cells with a Y-intercept of 1.2 and slope of 1.0 (Fig. 4). Furthermore, the mean number of Ki67\textsuperscript{+} CD56\textsuperscript{dim} cells was 2.4 times greater than the mean number of Ki67\textsuperscript{+} CD56\textsuperscript{bright} cells (p < 0.0001, data not shown). The Y-intercept > 0 (Fig. 4) and the greater number of dividing CD56\textsuperscript{dim} cells indicate that some CD56\textsuperscript{dim} NK cells proliferated even when there were very few dividing CD56\textsuperscript{bright} NK cells. Clearly, not all proliferating CD56\textsuperscript{dim} NK cells were immediate products of dividing CD56\textsuperscript{bright} NK cells. However, the direct correlation between the number of proliferating CD56\textsuperscript{bright} and the number of proliferating CD56\textsuperscript{dim} NK cells (slope of 1.0) suggests that many Ki67\textsuperscript{+} CD56\textsuperscript{dim} NK cells were derived from dividing CD56\textsuperscript{bright} NK cells. Consistent with this conclusion, the proliferation rate of CD56\textsuperscript{dim} cells was directly related to percentage CD56\textsuperscript{bright} cells (Fig. 5A). In contrast, CD56\textsuperscript{bright} proliferation did not correlate significantly with CD56\textsuperscript{bright} percentage (Fig. 5B). Our data with human cells studied directly ex vivo support prior observations that CD56\textsuperscript{bright} NK cells differentiate into CD56\textsuperscript{dim} NK cells in culture and in xenografts (3–5).

**NK receptor expression**

We investigated MHC class I-specific receptor expression on NK and T cell subsets. NKG2A expression showed an age-related decline on total NK cells and on CD56\textsuperscript{bright}, CD56\textsuperscript{dim}, and CD16\textsuperscript{+}CD56\textsuperscript{−} NK cells. NKG2A also declined with age on total T cells, T\textsubscript{Con} cells, and T\textsubscript{NK} cells (Table I). Results with individual subsets demonstrate that age-related loss of NKG2A expression on total NK cells and total T cells was not solely due to different ratios of various NK and T cell subsets. This finding extends previous findings that NKG2A expression decreased with age on NK cells and T\textsubscript{NK} cells (23). NK cell KIR expression did not change significantly with age (Table I). Few T\textsubscript{Con} cells expressed KIR compared with T\textsubscript{NK} cells, but there was a significant age-related increase in T\textsubscript{Con} and total T cell KIR expression (Table I).

**CD57 expression and cell proliferation**

CD57 has been described as an in vitro T cell senescence marker and as a marker of CD56\textsuperscript{dim} late differentiation, so we wished to correlate CD57 expression with NK and T cell proliferation (29–31). About 50% of CD56\textsuperscript{dim} NK cells expressed CD57 in both young and elderly adults; other NK cells had significantly less CD57 expression. For each T cell subset (CD8\textsuperscript{+} predominately CD4\textsuperscript{+}) T cell subsets, CD57 expression was associated with 2-fold and nearly 5-fold higher proliferation, respectively (Fig. 6). Because our findings contrast with prior in vitro studies, we considered the possibility that most proliferating CD57\textsuperscript{+} cells were recently activated CD57\textsuperscript{+} cells that began to express CD57 after stimulation. We reasoned that cells newly synthesizing CD57 would have relatively low cell surface density. Opposite of this prediction, CD57 cell surface levels trended higher among proliferating cells in both the CD8\textsuperscript{+}CD57\textsuperscript{+} and the CD8\textsuperscript{−}CD57\textsuperscript{−} T cell subsets (Fig. 7). These data support the hypothesis that CD57\textsuperscript{+} T cells proliferate well in vivo. We conclude that CD57 is not a marker of T cell senescence in vivo.

**Age effects**

As expected, percentage T cells declined with age, but percentage NK cells increased (Table II). Consistent with prior reports (32, 33), young adults had a higher percentage of CD56\textsuperscript{bright} cells; reciprocally, elderly subjects had more CD56\textsuperscript{dim} cells (Table II). We show for the first time to our knowledge that the percentage of CD16\textsuperscript{−}CD56\textsuperscript{dim} NK cells declined with age but that the percentage of CD16\textsuperscript{+}CD56\textsuperscript{−} NK cells did not differ between young and elderly women (Table II). NK proliferation trended lower in elderly women, but apoptosis trended higher (Table III).

![FIGURE 6. CD57\textsuperscript{+} T and NK cells proliferate in vivo. The ratio of percentage Ki67 expression in CD57\textsuperscript{+} cells and CD57\textsuperscript{−} cells within each indicated lymphocyte subset is shown as mean ± 95% confidence limit. Differences from an equal ratio are indicated. *p = 0.001, **p = 0.0005.](http://www.jimmunol.org/)

We found that CD57\textsuperscript{+}CD56\textsuperscript{dim} proliferated significantly slower than CD57\textsuperscript{−}CD56\textsuperscript{dim} NK cells (Fig. 6). However, Ki67 expression was only 29% lower in CD57\textsuperscript{+} cells than in CD57\textsuperscript{−} cells.

CD57 was expressed by many T\textsubscript{NK} cells, but few T\textsubscript{Con} cells, in both elderly and young adult subjects (Table II). In the CD8\textsuperscript{+} and CD8\textsuperscript{−} (predominately CD4\textsuperscript{+}) T cell subsets, CD57 expression was associated with 2-fold and nearly 5-fold higher proliferation, respectively (Fig. 6). Because our findings contrast with prior in vitro studies, we considered the possibility that most proliferating CD57\textsuperscript{+} cells were recently activated CD57\textsuperscript{+} cells that began to express CD57 after stimulation. We reasoned that cells newly synthesizing CD57 would have relatively low cell surface density. Opposite of this prediction, CD57 cell surface levels trended higher among proliferating cells in both the CD8\textsuperscript{+}CD57\textsuperscript{+} and the CD8\textsuperscript{−}CD57\textsuperscript{−} T cell subsets (Fig. 7). These data support the hypothesis that CD57\textsuperscript{+} T cells proliferate well in vivo. We conclude that CD57 is not a marker of T cell senescence in vivo.

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**Table II.** Elderly and young adult subjects differ in T and NK populations

<table>
<thead>
<tr>
<th>Set</th>
<th>Subset</th>
<th>Young (%)</th>
<th>Elderly (%)</th>
<th>Age Effect*</th>
</tr>
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<tbody>
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<td>Lymphocytes</td>
<td>T</td>
<td>73.7</td>
<td>58.7</td>
<td>0.0002</td>
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<tr>
<td>T</td>
<td>T\textsubscript{NK}</td>
<td>3.6</td>
<td>7.4</td>
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<tr>
<td>T</td>
<td>T\textsubscript{Con}</td>
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<tr>
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<td>NS</td>
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<td>23.3</td>
<td>NS</td>
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</table>

NS, not significant.

*Wilcoxon signed-rank test p values.

![FIGURE 7. Proliferating CD57\textsuperscript{+} T cells do not have low-density CD57 expression. For each T cell subset (CD8\textsuperscript{+} top panel, CD8\textsuperscript{−} bottom panel), we defined CD57\textsuperscript{high} and CD57\textsuperscript{low} expression as above and below median CD57 surface staining intensity, respectively. Based on this median, we calculated a ratio of CD57\textsuperscript{high} to CD57\textsuperscript{low} cells among the Ki67\textsuperscript{+} proliferating population. A ratio >1 indicates that more proliferating cells were CD57\textsuperscript{high} than CD57\textsuperscript{low}.](http://www.jimmunol.org/)
suggested opposite inferences about the effect of age on NK turnover. No age-related change in proliferation or in apoptosis was statistically significant for total NK cells or for any of the NK subsets, with the sole exception that CD16^+CD56^- NK cells proliferated less rapidly in elderly women (Table III). This exception is addressed in the Discussion. We conclude that NK turnover does not change significantly in healthy aging women.

Elderly subjects had increased percentage T_NK cells, many of which expressed CD57. These results are consistent with prior findings that the elderly accumulate effector-memory T cells, which tend to express CD16, CD56, and CD57 (34). Total T cell and T_{Con} cell proliferation was ~1.5-fold higher in elderly subjects than in young subjects. This result is not surprising given the increasing memory/naive T cell ratio that is found during aging (27, 35–37). In contrast, T_{NK} cell proliferation was identical in the two age groups (Table III). Table III. Proliferation and apoptosis rates in elderly and young adult subjects by cell subset

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*Wilcoxon signed-rank test p values.

Discussion

Our major finding was that human blood NK cell proliferation is rapid with an estimated doubling time of 13.5 d. Both proliferation and apoptosis measurements indicated that NK cells turned over three to four times faster than did T cells. This result in healthy human subjects correlates well with the observation that NK cells rebound more rapidly than other blood lymphocytes after bone marrow and hematopoietic stem cell transplantation (38). NK turnover has been directly measured in only a few studies. Using in vivo labeling and cell cycle analysis, Jamieson et al. (20) found that mouse splenic NK cells had a rapid turnover rate, which was much faster than that of splenic T cells. In contrast to mouse NK cells and to our findings, [3H]DNA labeling studies suggested relatively slow human and rhesus monkey CD3^-CD16^- NK cell turnover (21, 22). We used the Ki67 technique because it allowed proliferation rates to be measured in minor lymphocyte subsets from many research subjects, unlike cumbersome isotope methods (36). We found major differences between NK subsets, with higher proliferation among CD56^{bright}, CD16^-CD56^{dim}, and CD16^-CD56^- NK cells than among the major CD56^{dim} NK subset. Given that results of Ki67 analysis usually correlate well with [3H]DNA labeling (26), it may seem puzzling why both techniques did not indicate a high NK turnover rate. However, the labeling studies excluded CD16^- NK cells, such as most human CD56^{bright} cells (Fig. 1) and their rhesus homologs (39). Failure to measure rapidly proliferating CD16^-CD56^{bright} and CD16^-CD56^{low} NK cells probably accounts for the relatively low proliferation rates observed in the [3H]DNA labeling studies. We conclude that human NK turnover is rapid. Rapid turnover of mouse and human NK cells raises the interesting question of how recently described “memory” NK cells are maintained for several weeks after priming (40, 41). Because some types of “memory” NK cells selectively home to the liver, it could be that “memory” NK cells compose a small fraction of total NK cells in spleen and blood but a larger fraction in other organs (42).

Our data show that CD56^{bright} NK cells proliferate rapidly but die slowly relative to other NK subsets. Imbalanced proliferation and death in a population at steady state is consistent either with differentiation into another subset or with a net migration out of the blood compartment. The former possibility is consistent with the known precursor–product relationship between CD56^{bright} and CD56^{dim} NK cells (3–5). In a [3H]DNA labeling study, a 10- to 21-d lag was found between isotope pulse and peak appearance of labeled human NK cells in the blood (21). This lag had been attributed to the time required for dividing bone marrow precursors to differentiate into NK cells and subsequently migrate into the blood, but our results suggest that a major part of the lag is due to the development of CD16^-CD56^{bright} cells into CD16^-CD56^{dim} NK cells. To reconcile our findings with prior DNA labeling studies, we examined the number of proliferating cells more closely. We found a direct correlation between the number of Ki67^-CD56^{bright} and the number of Ki67^-CD56^{dim} NK cells. This suggests that dividing CD56^{bright} NK cells gave rise to Ki67^-CD56^{dim} NK cells. In support of this idea, the proliferation rate of CD56^{dim} NK cells was directly related to the percentage CD56^{bright} cells. We conclude that many dividing CD56^{bright} NK cells differentiate into Ki67^-CD56^{dim} NK cells in vivo.

CD56^{dim} NK cells had the lowest proliferation and apoptosis rates and therefore constitute the most stable NK subset in healthy people. [3H]DNA labeling showed that some label was found in CD3^-CD16^- NK cells at the first sampling point, 3 d after isotope pulse (21). This was interpreted as cell division among these NK cells, but instead could be interpreted as cell division by immediate precursors, such as CD56^{bright} NK cells. We found that the number of proliferating CD56^{dim} cells consistently exceeded the number of proliferating CD56^{bright} cells and that proliferating CD56^{dim} cells could be found even in subjects with very few proliferating CD56^{bright} NK cells. These data indicate that CD56^{dim} NK cells proliferated and were not all products of recently divided CD56^{bright} NK cells. Together these data indicate the CD56^{dim} proliferation rate is relatively low and that some Ki67 or [3H]labeled CD56^{dim} cells are immediate products of dividing precursors. Nonetheless, CD56^{dim} cells themselves divide at a low rate in healthy human subjects. In the setting of HIV infection, NK proliferation was vastly different—CD56^{dim} cells proliferated faster than CD56^{bright} cells (43).

Compared with CD56^{dim} cells, CD16^-CD56^{low} NK cells proliferated and died rapidly. We also found that CD16^-CD56^{low} NK cells vigorously released cytotoxic granules and made IFN-γ in response to a 2-h stimulation with K562 cells or IL-12/IL-18. Others showed that NK cells lost CD16 expression after a 2-wk TGF-β stimulation (9). NK cells also lost CD16 during cytotoxic attack on, or even binding to, target cells (7, 8). However, the reduction in CD16 was incomplete in stimulated CD56^{dim} cells after 4 h (7). We adjusted gating to minimize misclassification of CD56^{dim} NK cells in our functional studies (Supplemental Fig. 1). Nonetheless, some of the CD16^-CD56^{low} NK cells identified in this study might have been recently activated CD56^{dim} cells. Other CD16^-CD56^{low} NK cells might have been precursors that...
Activated CD56dim cells may temporarily assume a CD16+ phenotype. Some dying CD56dim cells that have lost cell surface CD56 expression, as occurs during apoptosis in vitro (46).

In a prior study of 15 elderly and 15 young mixed-gender subjects, we found an age-related decline in CD3+CD56 NK cell KIR expression (23). In our current study involving 34 young and 38 elderly adult women, NK cell KIR expression was identical in the two age groups. Furthermore, none of the NK subsets showed a significant age-related trend in KIR expression. The prior study design did not allow us to distinguish individual NK subsets. We speculate that the age-related KIR expression trend seen in the prior study may have been due to the elderly having a greater proportion of CD56dim NK cells, which have high KIR expression. Consistent with our current findings, two recent studies also found no significant trend in NK cell KIR expression in aging adults (47, 48).

More T cells and TCon cells expressed KIR in the elderly subjects than in young adult subjects. Although T NK cell KIR expression trended higher with age, differences were not statistically significant. A positive correlation between age and CD8 T cell KIR expression has been reported (47, 49). Van Bergen et al. found a similar trend in CD4 T cells (50), but that finding was not confirmed by others (47). To our knowledge, ours is the first report of an age-related increase in KIR expression on T cells that do not express CD16 or CD56 NK-associated markers.

We observed a significant age-related decline in NKG2A expression on NK cells and all NK subsets, except CD16+CD56low cells. This result confirms our previous finding that NK cell NKG2A expression declines with age (23). In contrast, Le Garff-Tavernier et al. did not find any change in CD56low NK cell NKG2A expression across adult age groups, although significantly more cord blood CD56low NK cells expressed NKG2A than did adult CD56low NK cells (48). The study design of Le Garff-Tavernier et al. (48) did not allow CD56dim NK cells to be distinguished from CD16+CD56low NK cells, which we found did not decrease NKG2A with age. We speculate that the differences between these two NK subsets accounts for the apparent discrepancy in findings.

With age, NKG2A expression decreased on T cells, including both TCon and T NK cells. This finding appears to contradict the lack of change in CD8 T cell NKG2A expression in a study of 11 young and 5 elderly adults subjects (49). However, the decline in T cell NKG2A expression was consistent with the age-related decline observed in our prior study of 15 young and 15 elderly subjects (23). It is not clear why NKG2A expression declines in both T and NK cells. NKG2A requires GATA-3 for transcription (51). GATA-3 responds to environmental cues in human hematopoietic cells (52, 53), and GATA family members show complicated changes in aging Caenorhabditis elegans (54). Therefore, changing environmental factors and aging itself might influence the expression of GATA-3 or other transcription factors needed for efficient NKG2A expression.

We were interested in measuring CD57 expression on NK cells because it was reported to be a reliable in vitro T cell senescence marker (29–31). Remarkably, nearly 50% of CD56dim NK cells expressed the CD57 “senescence marker” in young adult women. When we directly measured proliferation in a separate cohort, we found that CD57 was associated with 29% less proliferation among CD56dim NK cells. Our findings are quite consistent with past in vitro studies showing that CD57+ NK cell cytotoxicity and proliferation were good, but less vigorous than that of CD57− NK cells (55, 56). Recently, two groups showed that CD57 expression correlated with other markers of NK maturity on the CD56dim subset (57, 58). CD57+ NK cells were cytotoxic and made considerable IFN-γ in response to cross-linking of several cell surface receptors but had relatively poor IFN-γ responses to cytokines (57, 58). In both [3H]thymidine uptake assay (55) and CFSE dilution assay (57), proliferation by CD57+ CD56dim NK cells was variable and ranged from very low to 50% that of CD57− CD56dim NK cells. It might be relevant that CD57+ T cells are very prone to apoptosis and appear to be killed by CFSE labeling (59). In vivo, human CD57+ NK cells also proliferated much less than CD57− NK cells in two settings of high NK proliferation:

FIGURE 8. Model of human NK turnover. CD56dim cells are maintained by cell division (represented by curved arrow above the oval; arrow thickness is proportional percentage Ki67+). Ki67−CD56dim cells also are maintained by input from CD56bright cells and some input from immature CD16+CD56dim NK cells (represented by thin arrows between populations). CD56dim cells may temporarily lose CD16 expression after stimulation. At steady state, the number of new CD56dim cells is balanced by change of compartment is largely independent of turnover in another compartment (37).

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exogenous IL-15 transexpression in “humanized” mice and early after human hematopoietic stem cell transplantation (57). In contrast, proliferation of CD57+ and CD57- NK cells were much more similar 45 wk after transplantation (57). The latter finding was comparable with the moderate differences in Ki67 rates that we observed in CD57+ and CD57- NK cells from healthy subjects. The sum of the CD56dim NK cell data suggest that CD57 is associated with somewhat less robust proliferation in stable, healthy human subjects but is associated with markedly deficient proliferation under highly stimulatory conditions. CD57 was expressed on many T NK cells but few T Con cells. The high in vivo proliferation rate of T NK cells seemed inconsistent with cell senescence. Therefore, we directly tested how CD57 was related to proliferation. CD57 was associated with 2-fold more Ki67 expression among CD8 T cells and nearly 5-fold more among CD8+ (predominately CD4) T cells. Our findings appear to be at variance with observations that CD57 expression is associated with markers of T cell terminal differentiation and with poor replication in vitro (29–31, 60). We cannot exclude the possibility that Ki67+ CD57+ T cells are in cell cycle arrest. However, our results are consistent with the finding that although CD57+ T cells divided poorly in standard culture conditions, they divided well in media that contained human serum and IL-2 (59). In response to viral peptides, CD57+ CD8 T cells rapidly became cytotoxic, and both CD57+ CD4 and CD57- CD8 cells produced cytokines (30, 31). We propose that CD57 is not a reliable marker of T cell senescence in vivo.

Another major finding of our study is that NK cell proliferation and apoptosis did not significantly change with age, either among NK cells as a whole or in three subsets, CD56bright, CD56dim, and CD16+CD56dim. Only CD16+CD56- NK cells showed a significant age-related decline in proliferation, but not in apoptosis. As outlined above, the CD16+CD56- phenotype may comprise several distinct NK subsets, and it is not clear from our results why proliferation declined with age. We speculate that the age-related change in proliferation was due to a shift in the proportion of immature NK cells and senescent NK cells that express the CD16+CD56- cell surface phenotype. Prior [3H]DNA labeling data can be interpreted in light of our findings. Day 3 CD3+CD8+ NK cell labeling was not different between the 5 young and 8 elderly adults studied (21), consistent with our observation that CD56dim proliferation rates were similar in young and elderly adults. However, peak labeling was lower in CD3+CD16+ NK cells from elderly subjects than in those from young adult subjects. Some of this effect might be due to the lower proliferation among CD16+CD56- NK cells in the elderly, although these cells made up less than 20% of CD16+ NK cells in our elderly subjects. Lower peak isotope incorporation into mature NK cells could have been due to less incorporation by immature cells or lower percentage of immature cells. We favor the latter possibility because the elderly have a smaller percentage of CD56bright NK cells (our data and Refs. 32, 33) and because CD56bright NK cell proliferation and apoptosis in the young and elderly is similar (our data). Another contributing factor could be possible depressed bone marrow production of NK precursors in elderly adults.

In conclusion, most NK subsets and NK cells as a whole exhibit similar proliferation and apoptosis in healthy young and elderly adults. It will be important to examine NK turnover and activation in the frail elderly, who have relatively few NK cells and high mortality (14).

Acknowledgments
We thank Gregory Jicha for help with donor recruitment, Mikkel Moore, Jerold Woodward, Suzanne C. Segerstrom, and Donald Cohen for scientific discussions, Jennifer Strange and Greg Bauman for flow cytometry engineering, and the CR-DOC nurses for phlebotomy. We are grateful to the volunteer research subjects.

Disclosures
The authors have no financial conflicts of interest.

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8. Lighart, G. J., H. R. Schuit, and W. Hjimans. 1989. Natural killer cell function is not diminished in the healthy aged and is proportional to the number of NK cells in the peripheral blood. Immunology 68: 396–402.
Sun, J. C., J. N. Beilke, and L. L. Lanier. 2009. Adaptive immune features of
- No stimulation

- IL-12 and IL-18

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Supplemental Figure S1. Gating adjustment for stimulated cells. Cells were gated as described in the Materials and Methods, but CD16 and CD56 gates were adjusted to accommodate stimulation-induced changes. Shown are the same untreated and IL-12/IL-18-treated cells analyzed in Fig. 3A.