

# The Ontogeny and Fate of NK Cells Marked by Permanent DNA Rearrangements<sup>1</sup>

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A subset of NK cells bears incomplete V(D)J rearrangements, but neither the consequence to cell activities nor the precise developmental stages in which recombination occurs is known. These are important issues, as recombination errors cause cancers of the B and T lineages. Using transgenic recombination reporter mice to examine NK cell dynamics in vivo, we show that recombination<sup>+</sup> NK cells have distinct developmental patterns in the BM, including reduced homeostatic proliferation and diminished Stat5 phosphorylation. In the periphery, both recombination<sup>+</sup> and recombination<sup>-</sup> NK cells mediate robust functional responses including IFN- $\gamma$  production, cytotoxicity, and tumor homing, suggesting that NK cells with distinct developmental histories can be found together in the periphery. We also show that V(D)J rearrangement marks both human cytolytic (CD56<sup>dim</sup>) and immunoregulatory (CD56<sup>bright</sup>) populations, demonstrating the distribution of permanent DNA rearrangements across major NK cell subsets in man. Finally, direct quantification of *rag* transcripts throughout NK cell differentiation in both mouse and man establishes the specific developmental stages that are susceptible to V(D)J rearrangement. Together, these data demonstrate that multipotent progenitors rather than lineage-specified NK progenitors are targets of V(D)J recombination and that NK cells bearing the relics of earlier V(D)J rearrangements have different developmental dynamics but robust biological capabilities in vivo. *The Journal of Immunology*, 2008, 180: 1432–1441.

Variable diversity joining V(D)J<sup>3</sup> recombination, the process of Ag receptor rearrangement, first initiates in uncommitted hemopoietic progenitors before NK/DC/B/T divergence (1, 2). Thus, a proportion of murine and human NK cells, as well as a subset of murine dendritic cells, harbor so-called incomplete V(D)J rearrangements (1, 3). That is, NK cells perform the initial steps in this process but do not generate a completely rearranged Ag receptor. Approximately 5% of murine NK cells (1, 4) and 3.2–36% of human NK cells in the periphery (5) possess

incomplete rearrangements at the Ig H chain (IgH) or *TCRD* or *TCRG* gene loci. These observations may not be attributed to contamination by T cells or NKT cells, because such rearrangements are detectable in NK cells from *TCR $\beta\delta$ <sup>-/-</sup>* mice (1, 4) as well as from an athymic patient with complete DiGeorge syndrome (5). V(D)J recombination is associated with fatal leukemias of the B and T lineages, and the implication for NK cell biology in normal or disease circumstances remains unknown (6).

How or whether the permanent DNA modifications mediated by V(D)J recombination impact normal NK cell activities has been debated for the last 20 years, and the recent suggestion that a minor subset of neutrophils may rearrange and express products from the TCR locus rekindles this debate (7). The detection of novel transcripts produced by rearranged loci in NK cells (4, 8) is a contentious issue, because the presence of functional rearrangements raises the possibility that V(D)J rearrangements can contribute to NK target cell recognition (9–11). NK cell activity has been examined in two murine models of SCID to understand the contribution of V(D)J recombination to NK cell function. Mice deficient in the *rag2* component of the V(D)J recombinase or mice defective in DNA repair have normal to elevated numbers of NK cells that possess cytotoxic activity, suggesting that recombination is not required for the production of most murine NK cells (12–15). *rag2* activity is also dispensable for the recently described ability of NK cells to mediate hapten-specific recall responses (13). Moreover, NK cells do not detectably express variable immune receptors on their surface. Thus, it is difficult to understand how V(D)J recombination could directly contribute to target recognition or target-specific killing activities (16).

Given that classical NK cells do not express surface Ig or TCR proteins, incomplete rearrangements at Ag receptor loci might be expected to be biologically neutral during normal NK cell development. However, the frequency of recombination<sup>+</sup> NK cells in the bone marrow (BM) vs periphery is disproportionate. Approximately 50% of murine common lymphoid progenitors (CLPs),

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<sup>3</sup> Abbreviations used in this paper: V(D)J, variable diversity joining; IgH, Ig H chain; BM, bone marrow; BEX, blue excited; CLP, common lymphoid progenitor; DAPI, 4',6'-diamidino-2-phenylindole; pNK, NK precursor; SLT, secondary lymphoid tissue; VEX, violet light excited.

BM precursors to NK cells, have D-J rearrangements at IgH loci (17), but only ~5% of splenic NK cells carry these rearrangements (1, 4). Likewise, in a transgenic mouse model in which V(D)J recombinase activity is indicated by the permanent expression of violet light-excited (VEX) fluorescence, >70% of BM precursor NK cells but only 20–30% of peripheral NK cells are VEX<sup>+</sup> (1). Errors in the DNA cleavage or joining steps of V(D)J recombination lead to apoptosis in B and T lymphocytes, suggesting the possibility of NK cell attrition due to recombination failures. Additionally, recombination<sup>+</sup> NK cells could be undesirable developmental intermediaries derived from the failed progression of common lymphoid progenitor subsets to the B or T cell fates.

Because the analysis of DNA rearrangement status is a destructive process that precludes subsequent functional studies, virtually nothing is known about the developmental dynamics, persistence, or functional competence of recombination<sup>+</sup> vs recombination<sup>-</sup> NK cells. In this study, we exploit a fluorescent V(D)J recombination reporter substrate in which murine cells expressing V(D)J recombinase activity are permanently marked by VEX fluorescence. This approach enables us to compare the *in vivo* developmental dynamics and functional activities of murine NK cells that lack vs possess a history of V(D)J recombinase activity. We also extend our studies to examine the occurrence of V(D)J rearrangements across major functional subsets of human NK cells. Finally, NK precursors have recently been resolved in both mouse and man, and examination of *rag1/2* expression explicitly defines the stages of NK lineage development that are potential targets of V(D)J recombination and, hence, recombination-mediated oncogenesis.

## Materials and Methods

### Mice

H2-SVEX founder lines have been extensively characterized elsewhere (1, 18, 19). Tissues from the two independent H2-BEX (where BEX is blue-excited fluorescence) founders were provided by Rachel Gerstein. Mice were treated humanely in accordance with federal and state government guidelines and the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA).

### Flow cytometry and cell sorting

Freshly isolated murine BM, spleen, or lymph node cells were resuspended at  $3 \times 10^7$ /ml in staining medium containing PBS (pH 7.2), 0.02% sodium azide, 1 mM EDTA, and 3% newborn calf serum and treated with 2.4G2 Fc block for 10 min on ice. Murine lymphoid tissues were isolated as previously described (1, 18, 19). Hepatic lymphocytes were enriched using a discontinuous Percoll gradient (20). Before the sorting of specific NK cell subsets, BM or spleen cells were enriched using the NK cell isolation kit (Miltenyi Biotec). Cells were incubated with primary Abs for 20 min, washed three times, incubated with streptavidin reagents for 15 min, and then washed three more times. After the final wash, samples were resuspended in 1  $\mu$ g/ml propidium iodide or 4',6'-diamidino-2-phenylindole (DAPI) to exclude dead cells. VEX was detected using 407 nm excitation (21). Primary anti-mouse Abs included NK1.1 FITC or PE or biotin, TCR $\beta$  allophycocyanin or FITC or biotin, CD122 PE, CD3 biotin, CD4 PE, CD8 PE, IFN allophycocyanin, and phospho-Stat5 A488. Secondary reagents were streptavidin-Cy5-PE, streptavidin-Cy7-PE, or streptavidin-Pacific Blue (BD Pharmingen, eBioscience, or Molecular Probes). Flow cytometry was performed on a three-laser, nine-detector LSR II or a three-laser, 10-detector FACSAria device (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

Human peripheral blood buffy coats were obtained from the Pittsburgh Blood Bank (Pittsburgh, PA) and human tonsillar tissue was from the Health Sciences Tissue Bank at the University of Pittsburgh as approved by the University of Pittsburgh School of Medicine Institutional Review Board. Buffy coats and tonsillar tissue were enriched using lymphocyte separation medium (Cellgro) per the manufacturer's instructions. NK cells were further enriched using the NK cell isolation kit (Miltenyi Biotec) before staining with Abs to CD56 FITC or PE, CD3 PE or PerCP, CD34 FITC, CD94 PE, CD19 allophycocyanin, and CD117 biotin for cell sorting. Flow cytometry and cell sorting were performed as described for mouse cells except that Fc blocking was performed with 10% human AB serum.

### Analysis of proliferation and apoptosis

For analysis of proliferation by BrdU staining, mice were injected *i.p.* with 200  $\mu$ g of BrdU in PBS or PBS alone as a control at 12-h intervals as previously described (22). Forty-eight hours after the first injection, BM staining was performed with the BrdU flow kit (BD Pharmingen). For analysis of apoptosis, BM populations from unmanipulated mice were stained for surface markers and subsequently incubated with annexin-V according to manufacturer's instructions (BD Pharmingen).

### Effector activity

Cytokine production and cytolytic activity were measured directly *ex vivo* as well as after cytokine-mediated expansion of FACS-sorted NK cells. For direct *ex vivo* analysis of IFN- $\gamma$  production,  $3 \times 10^4$  FACS-sorted splenic NK cells were stimulated with 2 ng/ml recombinant murine IL-2 (BD Biosciences) and 1 ng/ml recombinant murine IL-12 (eBioscience) in 200  $\mu$ l of RPMI 1640 complete medium for 5 h. Brefeldin A (eBioscience) was added during the last 3 h of culture per manufacturer's instructions. Cells were then harvested and then stained with Abs to intracellular IFN- $\gamma$  or the isotype control. For analysis of IFN- $\gamma$  secreted into cell supernatants, sorted NK cells expanded in 6,000 IU/ml IL-2 for 6 days and were then replated at  $3 \times 10^5$  cells/well and stimulated with IL-2 plus IL-12 for 48 h, after which supernatants were harvested (23). The cytolytic potential of freshly isolated FACS-sorted NK cells was examined by CD107 expression. Sorted NK cells (6,000 cells) were cultured at a 1:1 E:T ratio with YAC-1 tumor targets for 5 h in the presence of Abs to CD107a and CD107b. Monensin (eBioscience) was added for the entire culture period per manufacturer's instruction. At the end of culture, cells were restained with Abs to NK 1.1 analyzed for CD107 expression by flow cytometry. For direct examination of cytotoxic activity, sorted NK cells that expanded for 6 days in 6,000 IU/ml IL-2 were cocultured with YAC-1 targets at the indicated E:T ratios. Cytotoxicity was measured using chromium-labeled YAC-1 targets (24), and the percentage of lysis was calculated as: [(sample cpm - spontaneous release cpm)/(maximum release cpm - spontaneous release cpm)]  $\times$  100.

For *in vivo* analysis of tumor infiltration, sorted NK cells from Thy 1.2<sup>+</sup> donors cells were adoptively transferred to congenic Thy1.1<sup>+</sup> recipients bearing established B16 lung tumors (F10 subline) as previously described (25). In brief,  $2 \times 10^5$  B6 lung tumor cells were administered via tail vein injection and 7 days later  $1-5 \times 10^6$  IL-2 expanded VEX<sup>+</sup> or VEX<sup>-</sup> NK cells were adoptively transferred. To support the transferred NK cells, animals received *i.p.* injections of 0.5 ml of RPMI 1640 containing 60,000 IU of IL-2 complexed to polyethylene glycol at 12-h intervals. Mice were sacrificed after 5 days, and tissue sections were examined by a blinded observer for the presence of NK cells at 30–45 tumor foci per mouse.

### Stat5 phosphorylation

Freshly sorted NK cells were preincubated in RPMI 1640 complete medium for two hours at 37°C in 5% CO<sub>2</sub>, and IL-2 (6,000 U/ml) or PBS was added for 15 min as previously described (26). The cells were fixed with formaldehyde, permeabilized with methanol, and immediately stained with Abs to phosphorylated Stat5.

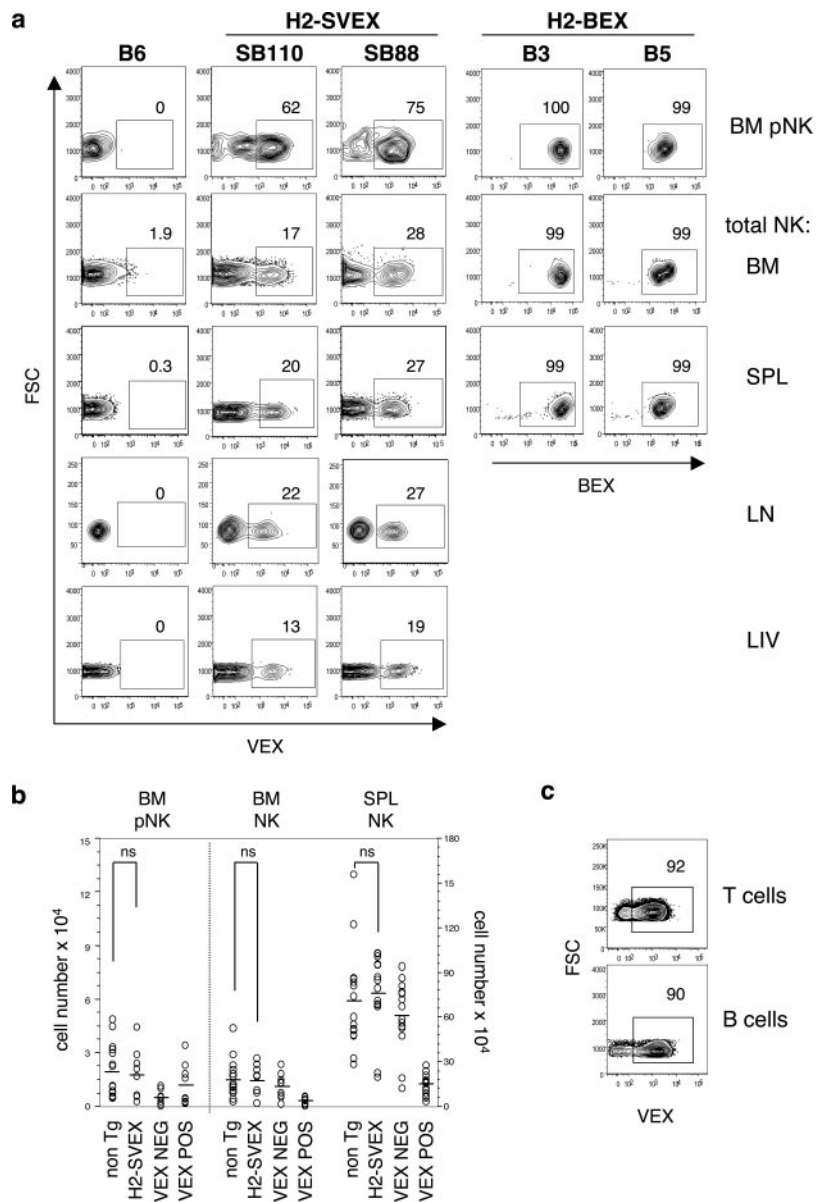
### RT-PCR

Total RNA for quantitative RT-PCR was prepared from sorted cells using the RNeasy micro or mini kit (Qiagen) and reverse transcribed. Probes and primers sets for murine *rag1* and *rag2* were from Applied Biosystems, and sequences for  $\beta$ -actin-specific probes and primers were generously provided by R. Hardy (Fox Chase Cancer Center, Philadelphia, PA). Probe and primer sets for human *rag2* and human 18S RNA were from Applied Biosystems. Samples were treated with DNase before reverse transcription to removing contaminating genomic DNA.

### Single cell PCR and bulk PCR

For single cell PCR, MACS-depleted human peripheral blood lymphocytes were subject to two rounds of FACS sorting with Abs to CD56 and CD3. In the second sort, single NK cells were sorted directly into 96-well plates as described (1, 19). PCR was performed using previously described nested primers for *TCRD* or, as a positive control, CD95 (27–29). First round primers were V $\delta$ 2-5'N (forward) and D $\delta$ 3-3'N (reverse) specific for TCR, and CD95 death domain external forward and reverse primers. Separate second round reactions were performed using primers V $\delta$ 2-3 (forward) and D $\delta$ 3-3 (reverse) for TCR rearrangements or death domain internal forward and reverse primers for CD95. The first round cycling conditions were 94°C for 1 min, 58°C for 1.5 min, and 72°C for 2.5 min for 35 cycles, with a 5-min end extension at 72°C. The second round cycling conditions were identical except that a 60°C annealing temperature was used with TCR

**FIGURE 1.** A subset of NK cells is marked by a history of V(D)J recombination activity. *a*, VEX expression, a permanent marker of V(D)J recombination activity, was examined throughout sequential stages of NK cell maturation in BM, spleen (SPL), lymph node (LN), and liver (LIV). H2-SVEX is a transgenic V(D)J recombination reporter substrate that expresses VEX-GFP (a spectral variant of GFP) after recombination. H2-BEX uses the same transgenic backbone to express BEX-GFP (another variant of GFP) constitutively. The two transgenes differ in that V(D)J recombination is required for VEX but not BEX expression (i.e., BEX expression is constitutive). SB88 and SB110 are two independent H2-SVEX founders, whereas B3 and B5 are two independent H2-BEX founders. B6 mice are shown as a control. NK cells were identified as BM pNK (CD122<sup>+</sup>NK1.1<sup>-</sup>TCRβ<sup>-</sup>), BM NK (NK1.1<sup>+</sup>TCRβ<sup>-</sup>), or spleen NK (NK1.1<sup>+</sup>TCRβ<sup>-</sup>). *b*, Absolute number of pNK, BM NK, or spleen NK in nontransgenic (non Tg) or H2-SVEX mice. Each point represents an independent animal. The number of each NK subset in the VEX<sup>+</sup> (VEX POS) and VEX<sup>-</sup> (VEX NEG) population is shown; H2-SVEX reflects the sum of these populations. *c*, VEX expression in spleen TCR αβ<sup>+</sup> T cells and CD19<sup>+</sup> B cells. The data are representative analyses of 2–50 independent mice. FSC, Forward scatter.



primers. Rearranged TCR products were confirmed by direct sequencing in both directions (29). Bulk PCRs were performed exactly the same way except that PCR products were cloned into TA vectors for sequence determination.

#### Statistical analysis

A Student's *t* Test was used for statistical analysis of cell numbers in B6 vs H2-SVEX mice. For analysis of paired data (i.e., VEX<sup>-</sup> vs VEX<sup>+</sup> samples), a Wilcoxon signed-rank test was used. Differences were regarded as significant at *p* < 0.05. Analyses were performed using the JMP version 5.1 statistical software package (SAS Institute).

## Results

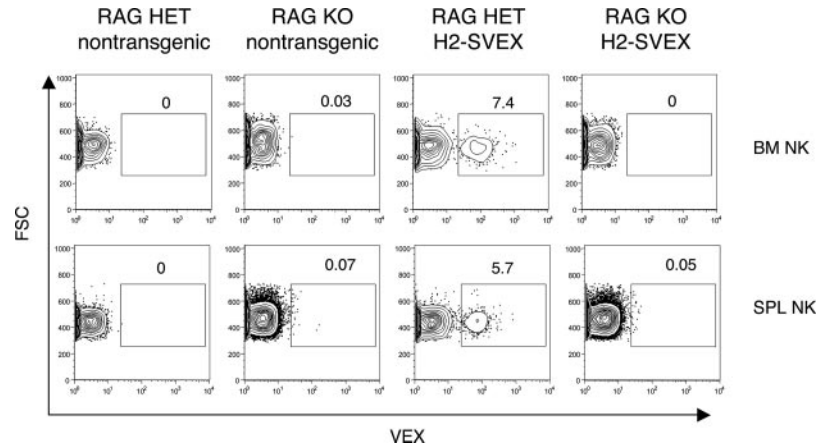
### Developmental dynamics of murine NK cells bearing V(D)J rearrangements

Approaches to studying germline rearrangements generally rely on DNA isolation, an inherently destructive process that precludes comparative functional analysis of V(D)J recombination<sup>+</sup> and V(D)J recombination<sup>-</sup> cell subsets. To examine the developmental and functional dynamics of viable NK cells with V(D)J recombination activity, we exploited transgenic H2-SVEX mice (1, 19) that bear a fluorescent recombination substrate based on VEX, a type of

GFP (21). In these mice, the transgenic VEX substrate is in the antisense orientation and cannot be expressed. V(D)J recombination activity directs inversion of VEX to the correct orientation for transcription (1). VEX expression in the rearranged transgene is driven by an active H2K promoter that has robust activity throughout hemopoietic development (30–32). VEX expression is therefore a permanent developmental tag that allows us to trace the *in vivo* fate of NK cells derived from precursors that lack vs express a history of V(D)J recombination activity.

Evidence of V(D)J recombination activity is detectable in the majority of NK precursors (pNK; CD122<sup>+</sup>NK1.1<sup>-</sup>TCRβ<sup>-</sup>) in murine BM. Fig. 1*a* shows that 62–75% of pNK are VEX<sup>+</sup> in either of two independent H2-SVEX founders, SB88 and SB110 (*top row, left side*). These data are consistent with our previous findings that ~50% of BM CLPs, common progenitors to the lymphoid lineages, are VEX<sup>+</sup> and have D-J joins at IgH loci (1, 19). VEX expression in the NK lineage strictly depends on V(D)J recombination, because mice deficient in *rag1*, an essential component of the V(D)J recombination, lack detectable VEX (Fig. 2). Specificity to the NK lineage is associated with the acquisition of NK1.1,

**FIGURE 2.** V(D)J recombination in NK cells requires *rag1* activity. NK1.1<sup>+</sup> BM cells or splenocytes (SPL) lacking CD3 and CD19 were examined for VEX expression in *rag1*<sup>+</sup> heterozygous (RAG HET) or *rag1* null animals (RAG KO) crossed to the H2-SVEX background (SB110). The data are representative of at least two independent experiments.



and VEX expression is detectable in 17–28% of BM-derived NK cells (NK1.1<sup>+</sup>TCR $\beta$ <sup>-</sup>) (Fig. 1a, second row from top). VEX is permanently expressed from the rearranged transgene (1, 19) and, like total BM NK, an appreciable proportion of NK cells in the periphery are VEX<sup>+</sup>. The frequency of VEX<sup>+</sup> NK cells in the spleen is ~17%  $\pm$  6.2% ( $n = 18$  mice) with a range from 10 to 28% (Fig. 1a, third row from the top and data not shown). Comparable frequencies of VEX expression are detectable in NK1.1<sup>+</sup>TCR $\beta$ <sup>-</sup> NK cells in the spleen, liver, and lymph node (Fig. 2a, lower three rows). Virtually identical results were observed at each stage of development in two independent founders, SB88 and SB110, highlighting the position-independent regulation of recombination in NK cells (Fig. 1a).

The presence of the H2-SVEX transgene does not negatively impact NK cell development. Fig. 1b depicts the absolute numbers of BM pNK, total BM NK, and splenic NK in nontransgenic vs H2-SVEX mice, as well as the individual VEX<sup>+</sup> and VEX<sup>-</sup> subsets. The absolute number of NK cells at each stage is comparable in nontransgenic controls vs H2-SVEX mice: BM pNK, 19,991  $\pm$  15,101 ( $n = 15$ ) vs 17,682  $\pm$  13,741 ( $n = 9$ ); total BM NK, 181,146  $\pm$  132,920 ( $n = 15$ ) vs 179,168  $\pm$  101,941 ( $n = 9$ ); spleen NK, 711,644  $\pm$  337,975 ( $n = 15$ ) vs 764,304  $\pm$  266,095 ( $n = 14$ ),  $p > 0.05$ .

The decrease in the frequency of VEX<sup>+</sup> cells during maturation is unique to the NK lineage. As previously described (1, 19), 90–95% of splenic B and T lymphocytes are VEX<sup>+</sup> (Fig. 1c). Conceivably, the reduction in the frequency of VEX<sup>+</sup> cells after the pNK stage of development from ~70% to ~25% (Fig. 1a, compare first and second rows from the top) could have been due to down-regulation of transgene expression during maturation. To address this possibility, we exploited H2-BEX transgenic mice (R. Gerstein, unpublished observations) that use the identical core H2K transgenic construct to drive constitutive expression of BEX (blue excited), another variant of GFP (21). The seminal difference between H2-SVEX and H2-BEX mice is that V(D)J recombination is required for fluorescence expression in the former but not the latter transgenic line. Although VEX expression decreases from ~70% in pNK to ~25% in BM and spleen NK cells, BEX expression is >99% in each of these subsets (Fig. 1a, first and second rows from the top, right side), indicating robust H2K-dependent transgene expression. BEX expression is maintained in the periphery, as >99% of splenic NK cells are BEX<sup>+</sup> (Fig. 1a, third row from the top, right side). Identical results were observed in two independent H2-BEX founder lines, B3 and B5 (Fig. 1a). Thus, H2K expression remains robust throughout NK cell development, suggesting that the loss of VEX<sup>+</sup> cells during maturation is not an artifact of transgene activity. Together, these data dem-

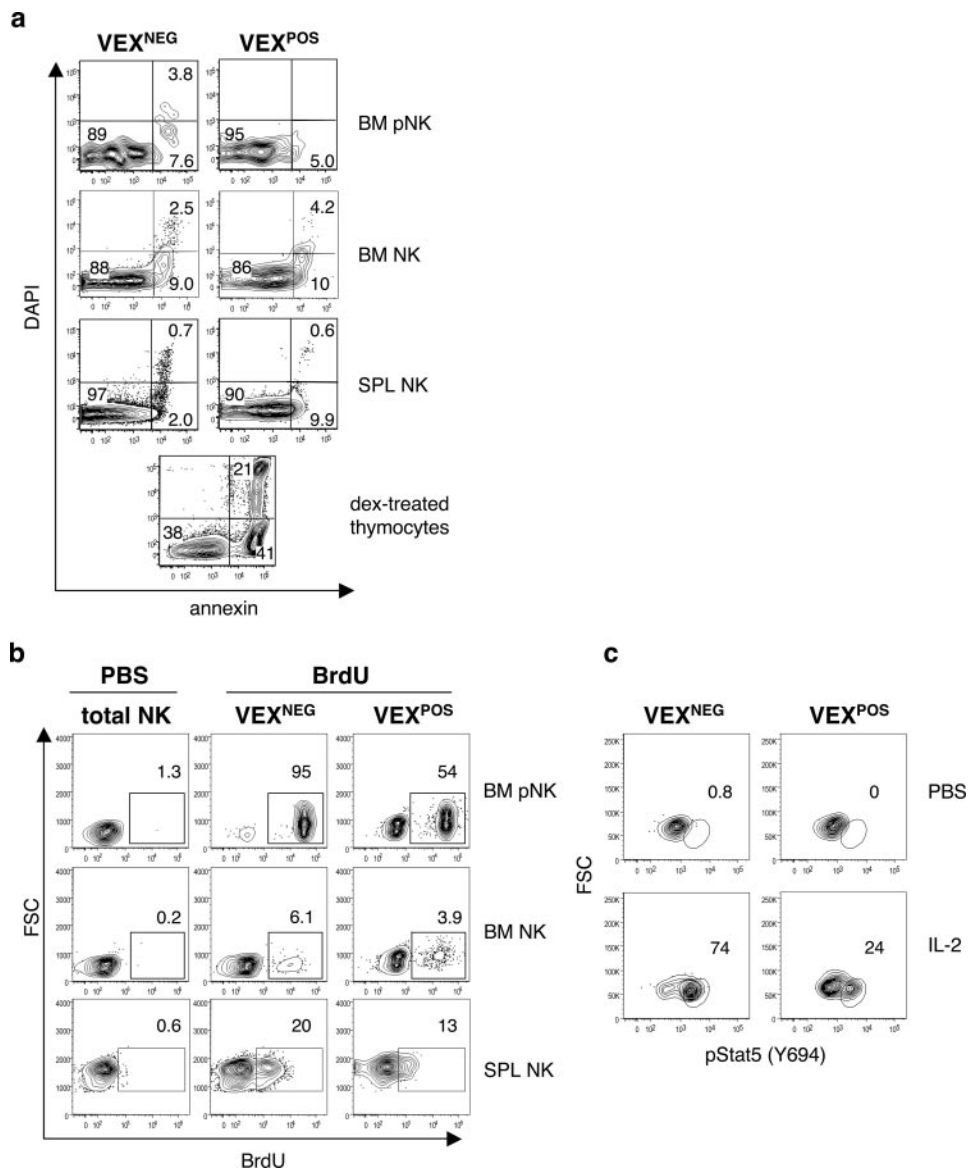
onstrate that V(D)J recombinase activity marks a distinct developmental subset of murine NK cells in vivo.

#### *Distinct in vivo developmental profiles of recombination<sup>+</sup> vs recombination<sup>-</sup> NK cells*

The decrease in the frequency of VEX<sup>+</sup> NK cells after the pNK stage (Fig. 1a) could be due to alterations in cell survival or proliferation, and we examined both parameters. We first characterized the levels of apoptosis in freshly isolated VEX<sup>+</sup> vs VEX<sup>-</sup> NK cells. Fig. 3a shows that the percentage of annexin-V<sup>+</sup>DAPI<sup>-</sup> cells is <10% in all three developmental subsets examined (BM pNK, total BM NK, or spleen NK) regardless of VEX expression. In a broader analysis of three independent mice, the frequency of total annexin-V<sup>+</sup> cells in VEX<sup>+</sup> vs VEX<sup>-</sup> subsets was: pNK, 3.3%  $\pm$  1.2 vs 12.7%  $\pm$  5.6; total BM NK, 8.7%  $\pm$  5.8 vs 12.7%  $\pm$  1.0; spleen NK, 5.6%  $\pm$  2.2% vs 3.5%  $\pm$  1% ( $p > 0.05$ , Wilcoxon test for paired data); suggesting comparable levels of apoptosis. Viability of both VEX<sup>+</sup> and VEX<sup>-</sup> NK cells is >95% at each stage as assessed by DAPI exclusion (Fig. 3a), indicating that VEX expression does not promote cell death. Because rapid clearance of dying cells in vivo may lead to underestimation of apoptosis, we examined the expression of *bcl-2*, an anti-apoptotic factor critical for NK cell persistence (33). Consistent with our observations, freshly isolated VEX<sup>+</sup> and VEX<sup>-</sup> NK cells had uniformly high levels of *bcl-2* expression as assessed by intracellular staining and flow cytometry. Greater than 95% of cell in both subsets were *bcl2*<sup>+</sup> with comparable mean fluorescence intensity of expression (data not shown). VEX<sup>+</sup> NK cells were not preferentially sensitive to experimentally induced apoptosis in vitro either (data not shown). Together, these data suggest that VEX<sup>+</sup> NK cells are not targeted for apoptosis as defective lymphocytes.

We then examined homeostatic proliferation in vivo. We injected H2-SVEX mice with BrdU and quantified the percent of BrdU<sup>+</sup> cells by intracellular staining and flow cytometry. At each stage of NK development in the BM and spleen, VEX<sup>+</sup> NK cells had a 25–50% reduction in the percentage of BrdU<sup>+</sup> cells as compared with VEX<sup>-</sup> NK cells (Fig. 3b). BrdU incorporation in VEX<sup>-</sup> vs VEX<sup>+</sup> cells was reduced from 95 to 54% in pNK, from 6.1 to 3.9% in total BM NK, and from 20 to 15% in spleen NK. Identical results were observed in both the SB110 and SB88 founder lines (Fig. 3b and data not shown). A similar reduction in proliferative capacity was observed following challenge with the viral mimic polyinosinic/polycytidylic acid (poly(I:C)). Two days after poly(I:C) administration, 38  $\pm$  6.0% of VEX<sup>-</sup> NK cells were BrdU<sup>+</sup> vs 18  $\pm$  8.0% of VEX<sup>+</sup> NK cells ( $n = 3$  mice; data not shown). These data suggest that NK cells marked by V(D)J recombinase activity have reduced proliferation in vivo.

**FIGURE 3.** Selective defect in homeostatic proliferation. *a*, Bone marrow and splenic (SPL) NK subsets from H2-SVEX (SB110) mice identified as in Fig. 1 were stained with annexin-V and DAPI to identify live, apoptotic, and dead cells. As a positive control for annexin-V detection, thymocytes from B6 mice were cultured with 1  $\mu$ M dexamethasone (dex) to induce apoptosis. *b*, Bone marrow from H-SVEX (SB88) mice injected with BrdU was subsequently stained for surface markers to identify BM and splenic NK subsets followed by intracellular staining with anti-BrdU Abs. Background staining was determined by injecting mice with PBS followed by the identical staining procedures. *c*, Freshly sorted VEX<sup>+</sup> (VEX<sup>POS</sup>) and VEX<sup>-</sup> (VEX<sup>NEG</sup>) spleen NK cells from H2-SVEX (SB88) mice were stimulated with 6,000 U/ml IL-2 or PBS for 15 min. Cells were immediately fixed, permeabilized, and stained with Abs to pStat5. The data are representative of 2–5 independent experiments. FSC, Forward scatter.



NK cell proliferation depends in part on signals transduced through the IL-2R. No gross differences in the expression of the IL-2R $\alpha$  and  $\beta$ -chains were detectable (data not shown). However, the frequency of phospho-Stat5 was reduced from 74 to 24% in VEX<sup>-</sup> vs VEX<sup>+</sup> spleen NK cells following IL-2 stimulation (Fig. 3c). Signals delivered through the Jak3:Stat5 pathway are critical for NK cell development and maintenance, and the diminished capacity of VEX<sup>+</sup> NK cells to activate Stat5 likely contributes to decreased *in vivo* proliferation.

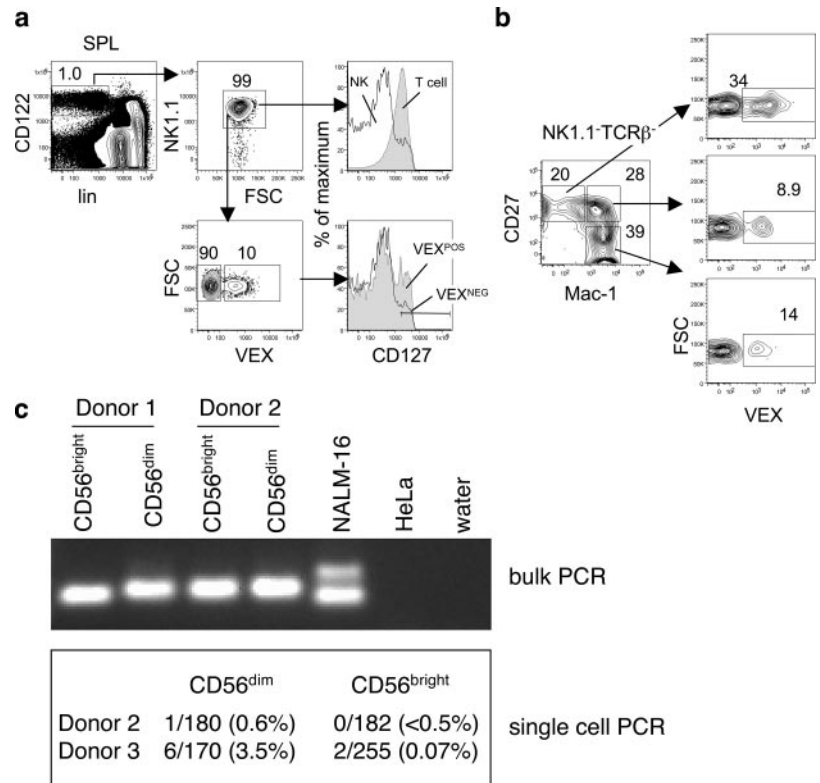
#### *V(D)J rearrangements mark universally mark major functional subsets of NK cells in mouse and man*

Recombination<sup>+</sup> NK cells represent a fraction of total NK cells, and we wondered whether the VEX indicator of V(D)J recombinase activity marks a specific functional subset. The markers CD127 and CD27 have been suggested to resolve distinct functional subsets of murine NK cells, and we determined the distribution of VEX expression across these subsets. CD127 has been used to distinguish thymic-derived NK cells in the periphery, a subset that has functional properties reminiscent of CD56<sup>bright</sup> human NK cells (34), while CD27 expression correlates with enhanced functional activity (35). Using the gating scheme described

by Vosshenrich et al. (34), CD127 expression is detectable within a minor subset of both VEX<sup>+</sup> and VEX<sup>-</sup> spleen NK cells, 10.2 and 5.2%, respectively (Fig. 4a, lower right panel). The CD27 marker distinguishes NK subsets with different cytotoxic potentials. CD27<sup>high</sup>CD11b<sup>low</sup> immature cells appear to give rise to CD27<sup>high</sup>CD11b<sup>high</sup> followed by CD27<sup>low</sup>CD11b<sup>high</sup> cells, of which the former population displays greater cytokine-producing and cytolytic capabilities. VEX expression was highest in the most immature subset, with 30% of CD27<sup>high</sup>CD11b<sup>low</sup> NK cells coexpressing VEX, and was expressed at similar levels, 8.9–14%, across the CD27<sup>high</sup>CD11b<sup>high</sup> and CD27<sup>low</sup>CD11b<sup>high</sup> subsets (Fig. 4b). Thus, VEX<sup>+</sup> NK cells are broadly distributed across functional subsets and are not confined to a specific population.

In man, differential expression of CD56 distinguishes NK cell subsets with distinct functions and tissue localization. To determine whether V(D)J recombination selectively marks a specific subset of human NK cells, we examined the occurrence of these permanent DNA rearrangements in CD56<sup>dim</sup> cytolytic NK cells vs CD56<sup>bright</sup> immunoregulatory NK cells. We sorted CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells from the peripheral blood of five healthy donors, and examined V $\delta$ 2-D $\delta$ 3 rearrangements at the *TCRD* locus by PCR. These primers detect incomplete but not complete *TCRD*

**FIGURE 4.** V(D)J rearrangements across major functional NK subsets. *a*, Splenic (SPL) CD127<sup>+</sup> NK cells in the periphery were resolved within the CD122<sup>+</sup>NK1.1<sup>+</sup> subset lacking lineage (lin) markers (CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD19<sup>-</sup>Gr-1<sup>-</sup>) and subsequently examined for VEX expression. Expression of CD127 on splenic T cells (TCRβ<sup>+</sup>) is shown for comparison. *b*, VEX expression was examined as a function of CD27 and Mac-1 expression in spleen NK cells. The data are representative of two to three independent experiments. FSC, Forward scatter. *c*, Human peripheral blood NK cells enriched by MACS depletion were subject to two rounds of cell sorting for CD56<sup>dim</sup>CD3<sup>-</sup> or CD56<sup>bright</sup>CD3<sup>-</sup> NK cells. Vδ2-Dδ3 rearrangements were analyzed by bulk PCR (*top panel*) or single cell PCR (*bottom panel*) as described in *Materials and Methods*. The NALM-16-positive control cell line occasionally amplifies as a doublet for unclear reasons. Data from three independent donors is shown (donor 1, LV68681; donor 2, LV70195; donor 3 KJ91815).



rearrangements (27). Fig. 4c depicts amplification of CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells from two representative donors, but not in the negative control cell line HeLa. Direct sequencing reveals extensive coding joint diversification in both CD56<sup>dim</sup> and CD56<sup>bright</sup> populations (Table I), a characteristic hallmark of V(D)J recombination. By single cell PCR, the frequency of detectable rearrangements in CD56<sup>dim</sup> NK cells from two independent donors was 1/180 (0.6%) and 6/170 (3.5%) single cells; PCR products were validated by sequencing (data not shown). The frequency of rearrangements in CD56<sup>bright</sup> NK cells from the same donors was 0/182 (<0.5%) and 2/255 (0.07%), respectively. In another study, 3.2–36% of total CD56<sup>+</sup> NK cells had incomplete V(D)J rearrangements at *TCRD* loci ( $n =$  four independent donors) (5). However, in our analysis of five independent donors the frequency of detectable rearrangements never exceeded that of donor 2 (Fig. 4c and data not shown). Together, these data indicate that V(D)J rearrangements are not restricted to a specific population of NK cells but rather universally mark distinct functional NK cell subsets in both mouse and man.

Our findings suggest that an appreciable proportion of murine NK cells in the periphery have a history of V(D)J recombinase activity. We wondered whether these recombination<sup>+</sup> NK cells were functionally competent or whether these cells represented failed developmental intermediaries during progression to the B cell lineage and, hence, were functionally anergic. We examined the effector responses of recombination<sup>+</sup> and recombination<sup>-</sup> NK cells ex vivo and in vivo. We first examined the cytokine-producing, freshly isolated VEX<sup>+</sup> and VEX<sup>-</sup> NK cells. As shown in Fig. 5a, 29–34% of both VEX<sup>+</sup> and VEX<sup>-</sup> NK cells produced IFN-γ after stimulation with IL-2 plus IL-12 as assessed by intracellular staining. Identical results were observed using independent approaches in which we examined IFN-γ secretion by ELISA. The total amount of IFN-γ released from FACS-purified, cytokine-expanded NK cells was only slightly increased in VEX<sup>+</sup> (54 ± 23 ng/ml) vs VEX<sup>-</sup> (45 ± 22 ng/ml) NK cells (Fig. 5b,  $n = 11$  independent mice;  $p < 0.05$  in a paired sample statistical analysis).

We then compared the effector activity of VEX<sup>+</sup> vs VEX<sup>-</sup> NK cells directly ex vivo. Freshly isolated VEX<sup>+</sup> and VEX<sup>-</sup> NK cells expressed comparable amounts of surface CD107a and CD107b, an indicator of lytic degranulation. After 5 h of coculture with YAC-1 targets, the proportion of VEX<sup>+</sup> and VEX<sup>-</sup> NK cell expressing CD107 was 31 and 32%, respectively, at a 1:1 effector to target (E:T) ratio (Fig. 5c). We also examined cytotoxicity directly by culturing FACS-sorted cytokine-expanded VEX<sup>+</sup> and VEX<sup>-</sup> spleen NK cells with YAC-1 targets at different E:T ratios. At every E:T ratio tested, 10:1, 5:1, and 1:1 VEX<sup>+</sup> and VEX<sup>-</sup> spleen NK cells had comparable cytolytic activity against YAC-1 targets (Fig. 5d and data not shown). Consistent with the integrity of functional responsiveness, VEX<sup>+</sup> NK cells expressed the Ly49 receptor repertoire, KLRG1, and CD94 surface molecules (data not shown). The uniformly low expression of CD127 in total splenocytes (36), combined with the similar representation of this marker (5–10%) across VEX<sup>+</sup> and VEX<sup>-</sup> spleen NK cells (Fig. 4a), suggests that any potential contamination by NK-like γδ T cells is minor and does not skew the results.

To establish the in vivo competence of VEX<sup>+</sup> and VEX<sup>-</sup> NK cells, we examined their ability to infiltrate B16 lung tumors in vivo. One week after tumor induction, Thy1.2<sup>+</sup> VEX<sup>+</sup> or VEX<sup>-</sup> donor NK cells were clearly detectable in the established tumors in Thy1.2<sup>-</sup> recipients as resolved by immunostaining with Thy1.2 (Fig. 5e). The number of infiltrating NK cells per square millimeter of tumor was comparable between the VEX<sup>+</sup> and VEX<sup>-</sup> subsets, 329 ± 71 and 272 ± 17, respectively ( $p > 0.05$ , Wilcoxon test for paired data; results representative of two independent experiments). Differences were not detectable in the number of tumors infiltrated either (data not shown), suggesting the functional competence of both VEX<sup>+</sup> and VEX<sup>-</sup> NK subsets. Thus, despite decreased homeostatic proliferation in vivo, VEX<sup>+</sup> NK cells have comparably robust functional properties. Together, these data suggest that NK cells with distinct developmental properties can be found together in the periphery.

Table I. Analysis of Vδ2-Dδ3 rearrangements by direct sequencing of single cell PCR products (\*) or cloning of nested PCR products (\*\*)<sup>a</sup>

Donor		Vδ2 Segment	Coding Joint (N or P)	Dδ3 Segment
		TACTGTGCCTGTGACACC		ACTGGGGGATACGCACAG
LV70195*	Dim	TACTGTGCCTGTGAC	CTTC	CTGGGGGATACGCACAG
KJ91815*	Dim	TACTGTGCCTGTGACACC	CCCACCGGCA	CACAG
	Dim	TACTACTGTGCCTGTGAC	TCCTTGG	G
	Dim	TACTACTGTGCCTGTGA	AA	TGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGT	CC	CTGGGGGATACGCACAG
	Dim	TACT	TGTGCCTGTG	
	Dim	TACTACTGTGCCTGTGACACC	TGGG	GATACGCACAG
	Bright (2) <sup>b</sup>	TA	TTGCGC	GGGATACGCTCAG
LV68681**	Dim	TACTGTGCCTGTGAC		TGGGGGATACGCACAG
	Dim	TACTGTGCCTG		TGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGACACC	GGG	TGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	T	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGA	TCACCCCGGGA	GGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGACACC	CGATT	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGACACC	GA	GGGGGATACGCACAG
	Dim (3)	TACTACTGTGCCTGTGACA	AGTGT	GGGATACGCACAG
	Bright	TACTACTGTGCCTGTGA	CCT	ACTGGGGGATACGCACAG
Bright (9)	TACTACTGTGCCTGTG	CCT	ACTGGGGGATACGCACAG	
LV70195**	Dim	TACTACTGTGCCTGTGAC	GGG	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGA	TCCA	GGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	GGTCCTGA	GGGATACGCACAG
	Dim	TACTACTGTGCCTGTGAC	CGGGGC	GGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	GGCAT	TGGGGGATACGCACAG
	Dim	TACTGTGCCTGTG	TCCT	ACTGGGGGATACGCACAG
	Dim	TACTGTGCC	CCCACCGGGAG	CTGGGGGATACGCACAG
	Dim	TACTGTGCCTGTGACACC	T	GGGGGATACGCACAG
	Dim	TACTGTGCCTGTG	CCGC	CTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	TCCTTT	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	TGGGGGGT	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGAC	CCC	CTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG	TCCGT	ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTG		ACTGGGGGATACGCACAG
	Dim	TACTACTGTGCCTGTGACAC	A	GGGGGATACGCACAG
	Dim (2)		AAGCC	ACTGGGGGATACGCACAG
	Bright	TACTACTGTGCCTGTG	GGGT	ACTGGGGGATACGCACAG
	Bright	TACTACTGTGCCTGTGACAC	A	ACTGGGGGATACGCACAG
	Bright	TACTACTGTGCCTGTGAC	GT	ACTGGGGGATACGCACAG
	Bright	TACTACTGTGCCTGTGAC	CCC	ACTGGGGGATACGCACAG
	Bright	TACTACTGTGCCTG	CCCAGGGGGG	TACGCACAG
	Bright	TACTACTGTGCCTG	CCCAGGGGGGT	TACGCACAG
	Bright	TACTACTGTGCCTGTGACACC	CTTAGGGT	CTGGGGGATACGCACAG
	Bright (2)	GTACTACTGTGCCTGTG	GGGT	ACTGGGGGATACGCACAG
	Bright (2)	TACTACTGTGCCTGTGAC	GT	ACTGGGGGATACGCACAG
	Bright (4)	TACTACTGTGCCT	CTTCTAGGA	GGATACGCACAG
	Bright (2)	TACTACTGTGCCT	C CGCCGG	GGGGGATACGCACAG
Bright (2)	TACTACTGTGCCTG	CCCA	GGGGGATACGCACAG	

<sup>a</sup> A single nucleotide substitution in the Dδ3 segment of one clone from donor LV70195\*\* is underlined.

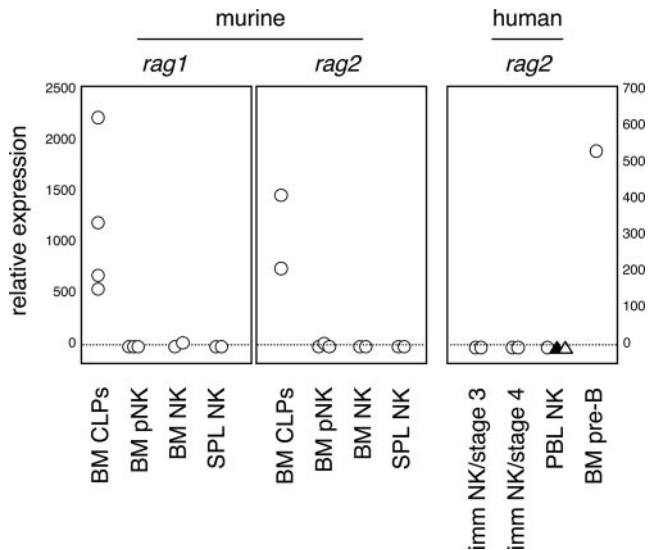
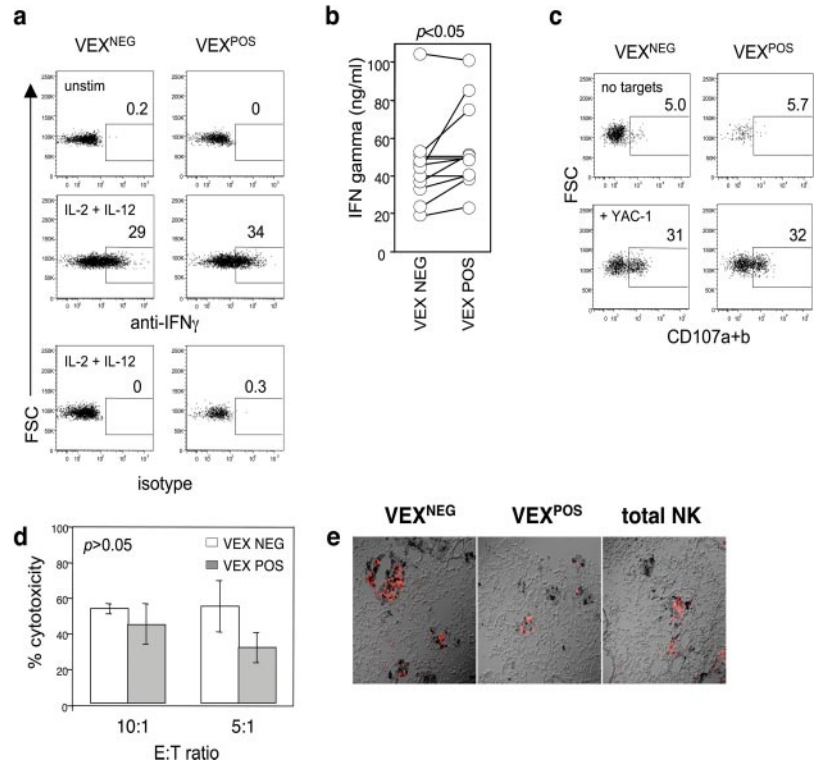
<sup>b</sup> The number in parentheses indicates the number of independent clones obtained.

#### Developmental timing of rag expression during NK lineage development

Recombination-dependent errors lead to leukemias of the B and T lineages, and it is important to define the precise stages at which NK progenitors are potentially susceptible to this oncogenic fate. Because expression from the rearranged H2-SVEX transgene is permanent (1, 19), the presence of VEX does not directly indicate the current presence of V(D)J recombinase activity, but rather that the V(D)J recombinase is or was active at some point in the developmental history of the particular NK cell. To explicitly characterize the active expression of V(D)J recombination machinery at specific stages of NK cell maturation, we directly quantified endogenous rag transcripts.

We sorted murine BM CLPs, pNK, BM NK, and spleen NK subsets and examined rag1 and rag2 mRNA by quantitative RT-PCR. Fig. 6 shows that rag1 and rag2 transcripts are detectable in BM CLPs, a stage of active V(D)J recombination (1, 2), but not in pNK, total BM NK, or mature spleen NK cells. Due to the low expression of CD122, it is not possible to resolve NK precursors in human BM. Therefore, we examined rag2 expression in a subset of NK cells that matures in secondary lymphoid tissue (SLT) (37). Within the CD3<sup>-</sup>CD19<sup>-</sup> compartment the sequential stages of NK-lineage specified development have recently been defined as CD34<sup>-</sup>CD94<sup>-</sup>CD117<sup>+</sup> (stage 3) and CD34<sup>-</sup>CD94<sup>+</sup>CD117<sup>+/-</sup> (stage 4) (38). Like murine BM NK cells, the rag2 transcript was not detectable at any stage of human NK cell development in SLT

**FIGURE 5.** Effector properties of VEX<sup>+</sup> NK cells. *a*, Freshly isolated splenocytes from H2-SVEX (SB88) mice were FACS sorted into VEX<sup>+</sup> (VEX<sup>POS</sup>) or VEX<sup>-</sup> (VEX<sup>NEG</sup>) NK1.1<sup>+</sup>TCRβ<sup>-</sup> subsets, after which 3 × 10<sup>4</sup> cells were stimulated with 2 ng/ml IL-2 and 1 ng/ml IL-12 for 6 h. Brefeldin A was added for the last 3 h of culture. Samples were then harvested and stained with Abs to detect intracellular IFN-γ by flow cytometry. FSC, Forward scatter; unstim, unstimulated. *b*, FACS-sorted VEX<sup>+</sup> or VEX<sup>-</sup> NK1.1<sup>+</sup>TCRβ<sup>-</sup> spleen cells from H2-SVEX (SB88) mice were expanded in the presence of 6,000 IU/ml IL-2 for 6 days and then 3 × 10<sup>5</sup> cells of each subset were stimulated with IL-2 plus IL-12 as above for 48 h. Supernatants were then collected for ELISA analysis. Each point in *b* represents an individual animal. Data from both SB88 and SB110 mice are included in each plot. *c*, Freshly sorted VEX<sup>+</sup> or VEX<sup>-</sup> NK1.1<sup>+</sup>TCRβ<sup>-</sup> splenic NK cells (6,000) from H2-SVEX (SB88) mice were cultured with IL-2 and IL-12 as above with or without YAC-1 targets at a 1:1 E:T ratio. After 5 h of coculture in the presence of Abs to CD107a and CD107b, total CD107 expression was examined on NK1.1<sup>+</sup> NK cells. *d*, H2-SVEX (SB110 mice) were cultured in the presence of 6,000 IU/ml IL-2 for 6 days after which cytolytic activity against chromium-labeled YAC-1 targets was examined at the indicated E:T ratios. *e*, Infiltration of B16 lung metastases by IL-2-expanded VEX<sup>+</sup> vs VEX<sup>-</sup> spleen NK cells. Donor NK cells are visualized by immunostaining with PE-conjugated anti-Thy1.2 Ab.



**FIGURE 6.** Endogenous *rag1/2* transcripts throughout NK cell development. *Two left panels*, Murine *rag1* and *rag2* transcripts were examined throughout NK lineage development from BM to spleen (SPL) by quantitative RT-PCR. *Right panel*, Analysis of *rag2* expression in stage 3 and stage 4 NK lineage-specified developmental intermediates from human SLT or mature PBL. For SLT, the two independent donors were J043 and J076. For PBLs, total CD56<sup>+</sup> NK cells were from donor 4 (LV68267; circle) while the individual CD56<sup>bright</sup> (open triangle) and CD56<sup>dim</sup> (closed triangle) subsets were from donor 2 (LV70195). Detection of *rag* transcripts in human BM pre-B cells from a single donor is shown for comparison. The data were standardized to a reference gene (see *Materials and Methods*) and then normalized to mouse or human BM, respectively. The horizontal dotted line indicates the detection threshold. The data shown are a compilation of several independent experiments in which each point represents cells sorted from an independent mouse or human donor with the exception of the paired CD56<sup>bright</sup> and CD56<sup>dim</sup> samples.

or in the mature PBL subsets CD56<sup>bright</sup>, CD56<sup>dim</sup>, or total CD56<sup>+</sup> but was readily detectable in human pre-B cells (Fig. 6). Thus, the V(D)J rearrangements detectable in murine and human NK cells (1, 5) likely stem from DNA recombination at the uncommitted progenitor stages of development, a finding that emphasizes the potential for V(D)J recombination errors within CLP subsets rather than within NK lineage-specified precursors.

### Discussion

V(D)J rearrangements are detectable in both murine and human NK cells (1, 4, 5), yet the implications to NK cell biology remain unclear. Neither the stages of development at which these permanent DNA rearrangements are incurred nor their biological impact are known. Notably, V(D)J recombination errors are associated with fatal cancers of the B and T lineage, suggesting the possibility that such mistakes might contribute to cancers of NK lineage origin. In this study, we used a fluorescent tracer that permanently marks recombination<sup>+</sup> NK cells to examine the developmental and functional consequences of DNA rearrangements in this lineage. We show that NK cells with a history of V(D)J recombinase activity have different development dynamics in vivo. Regardless of their ontogeny, both recombination<sup>+</sup> and recombination<sup>-</sup> NK cells exhibit robust functional activity in the periphery, highlighting the developmental plasticity of this lineage. We also defined the precise stages of NK lineage fate specification during which cells are targeted for recombination, a result that has implication to the potential for V(D)J recombination-mediated oncogenesis in cancers of NK cell origin.

We found that NK cells with a developmental history of recombinase activity are present at reduced frequency in the periphery vs BM (Fig. 1). The developmental changes in the frequency of VEX<sup>+</sup> NK cells are associated with a reduction in cell proliferation in vivo and Stat5 activation ex vivo (Fig. 3). One plausible interpretation is that VEX expression may mark a distinct developmental subset with a different capacity for expansion than VEX<sup>-</sup> NK cells. A second possibility is that V(D)J rearrangement

may directly alter NK cell developmental dynamics. Intriguing observations that Ag receptor loci are not the only targets of V(D)J recombination suggest one potential mechanism by which this could occur. Cryptic recombination signal sequences are distributed every 40–50 bp throughout the genome, including both immune and nonimmune loci (39), and it is becoming increasingly clear that *rag*-mediated rearrangements at these cryptic sites can occur in the absence of gross translocations. Nonpathogenic rearrangements have been most extensively studied at the HPRT locus in which *rag* activity is associated with three different deletions in T cells from healthy newborns and young adults (40, 41). V(D)J rearrangements at cryptic recombination signal sequences are also detectable in  $\gamma$ -satellite DNA, a highly repetitive DNA element encompassing ~6% of the mouse genome (42). Whether rearrangements at these loci or other loci occur in NK cells remains to be established.

In our mouse studies, NK cells with a history of V(D)J recombinase activity are detected by the expression of VEX fluorescence while cells lacking recombinase expression do not express this fluorescence. Two lines of evidence argue against the possibility of transgene-dependent effects on NK cell dynamics. First, the decreased frequency of VEX<sup>+</sup> NK cells in spleen vs BM is observed in two independent H2-SVEX founders (Fig. 1). This precludes the possibility of position effects of the transgene; the transgene is Y-linked in SB88 founders, and is autosomal in SB110 founders (1). Second, VEX<sup>+</sup> NK cells possess normal functional activity as assessed by both cytokine production and cytotoxic potential (Fig. 5). Thus, the mere expression of the VEX fluorescent protein per se does not confer a general disadvantage. The possibility of contamination in VEX<sup>+</sup> vs VEX<sup>-</sup> NK cell subsets by irrelevant populations also seems unlikely, because identical results were observed after the specific exclusion of B cells, T cells, and myeloid cells by flow cytometric gating and/or MACS depletion (1). Moreover, freshly sorted VEX<sup>+</sup> vs VEX<sup>-</sup> NK displayed virtually identical IFN- $\gamma$  production and cytolytic capabilities directly ex vivo (Figs. 5, *a* and *c*), indicating a lack of contamination by immune cells not capable of these activities, and IL-2-expanded NK cells were universally NK1.1<sup>+</sup>TCR $\beta$ <sup>-</sup>, indicating the absence of T cell contamination following cell sorting. Finally, the uniformly low expression of CD127 across VEX<sup>+</sup> and VEX<sup>-</sup> NK, 5–10% (Fig. 4*a*), suggests minimal potential for contamination by NK-like  $\gamma\delta$  T cells in the spleen. Although we cannot completely exclude the possibility of transgene effects or the presence of minor contamination, the weight of the evidence suggests that this explanation is unlikely to account for the findings presented here.

A careful examination of *rag* expression constrains the specific hemopoietic subsets that are capable of undergoing V(D)J recombination during progression to the NK lineage. Endogenous *rag1* and *rag2* transcripts are detectable in murine BM CLPs but not in the pNK or downstream stages of NK cell development in the BM (Fig. 6). The *rag* transcript is not detectable in the intermediate stages of human NK cell development in SLT either. These data are consistent with the relic hypothesis (1) that BM NK cells are permanently marked by V(D)J rearrangements occurring in the multipotent CLP stages of development. In previous studies that relied on a GFP reporter of *rag2* expression, we showed that fluorescence was detectable in 92% of pNK (1). Although GFP reporters are a powerful tool that enable the detection of *rag* in very rare progenitors, concerns have been raised about the prolonged half-life of transgenic *rag2*GFP and even knock-in *rag1*GFP mice (2, 43). For example, 20–30% of common myeloid progenitors from multicopy *rag2*GFP NG-BAC transgenic animals were found to be GFP<sup>+</sup>, but endogenous *rag2* transcripts were not detectable (44). Moreover, unlike the endogenous *rag2* transcript that re-

quires the activity of the E47 transcription factor (18), *rag2*GFP expression is refractory to the ablation of E47, suggesting the possibility of position-dependent effects of the multicopy transgene that alleviate normal regulation (L. Borghesi, unpublished observations). For these reasons we directly analyzed endogenous *rag1/2* expression rather than relying on GFP reporters (Fig. 6). Further comparisons between the reporter models and endogenous *rag* will be helpful to ensure that GFP accurately reflects natural *rag* gene expression.

Detection of V(D)J recombination machinery in CLPs but not inNK cells places new emphasis on the potential for recombination-mediated oncogenesis at the uncommitted progenitor stages of development (6). Chromosomal translocations between Ag receptor loci and proto-oncogenes are widely recognized sources of oncogenesis in B and T cells (45). As far as we are aware, it is not known whether BM CLPs or multipotent thymic progenitors bearing *rag*-mediated chromosomal abnormalities can subsequently differentiate to multiple downstream lineages. Such transformation would be distinct from stem cell cancers because CLPs cannot self-renew but, like stem cell cancers, the progeny would be expected to bear a monoclonal translocation (46). Although NK and NK-like tumors have been identified, they remain a perplexing problem because their lineage origins are controversial and the underlying molecular defects, when resolved, are heterogeneous (47).

In summary, we demonstrate that NK cells bear V(D)J rearrangements stemming from *rag* expression in uncommitted hemopoietic progenitors and that these permanent DNA rearrangements universally mark major functional subsets of NK cells in both mouse and man. The presence of permanent DNA rearrangements may be a useful tool for establishing the precise lineage relationships of distinct functional subsets of NK cells and their unique developmental origins.

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## Disclosures

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