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NK Cell Maturation and Peripheral Homeostasis Is Associated with KLRG1 Up-Regulation[1]

Nicholas D. Huntington,[2,3]*[†] Hy Tabarias,[§¶] Kirsten Fairfax,*[†] Jason Brady,* Yoshihiro Hayakawa,[‡] Mariapia A. Degli-Esposti,[§¶] Mark J. Smyth,[‡] David M. Tarlinton,* and Stephen L. Nutt*

NK cells are important for the clearance of tumors, parasites, and virus-infected cells. Thus, factors that control NK cell numbers and function are critical for the innate immune response. A subset of NK cells express the inhibitory killer cell lectin-like receptor G1 (KLRG1). In this study, we identify that KLRG1 expression is acquired during periods of NK cell division such as development and homeostatic proliferation. KLRG1+ NK cells are mature in phenotype, and we show for the first time that these cells have a slower in vivo turnover rate, reduced proliferative response to IL-15, and poorer homeostatic expansion potential compared with mature NK cells lacking KLRG1. Transfer into lymphopenic recipients indicate that KLRG1− NK cells are precursors of KLRG1+ NK cells and KLRG1 expression accumulates following cell division. Furthermore, KLRG1+ NK cells represent a significantly greater proportion of NK cells in mice with enhanced NK cell numbers such as Cd45−/− mice. These data indicate that NK cells acquire KLRG1 on their surface during development, and this expression correlates with functional distinctions from other peripheral NK cells in vivo. *The Journal of Immunology,* **2007, 178: 4764–4770.**

Natural killer cells represent the third largest lymphoid cell population in mammals and are critical in innate immune responses. NK cells express an array of activating and inhibitory receptors that are capable of recognizing stressed, virus-infected or transformed cells, and responding rapidly, without the need for immunization or preactivation (1). Given the unique functions of NK cells, identifying factors that control their development/maturation, homeostasis, and activation status is central to understanding innate immune regulation and to devising strategies to improve immunotherapy of cancers and infectious diseases.

Immature (Imm)[4] NK cells express NK1.1, NKG2D, CD49b, and 2B4, markers that classically identify NK cells, but not CD11b (or Mac-1) (2). During bone marrow (BM) maturation, NK cells acquire a number of inhibitory receptors such as the CD94-NKG2 complex and members of the Ly49 family (2), and it is thought that in the naive setting, signaling through the repertoire of inhibitory receptors specific for autologous MHC class I keeps NK cells in a quiescent state (3).

Mature NK cells, which express Mac-1+ (2), are predominantly found in peripheral organs such as the spleen and continually increase in number from birth until they reach a constant level at adulthood due to homeostatic expansion in response to the available space within the developing organ (4). This expansion is characterized by an initially high NK cell turnover rate in young mice that decreases continually until reaching a constant level at 8 wk of age (4). Homeostatic proliferation of NK cells is also observed experimentally following the transfer of NK cells into alymphoid $Rag2^{-/-}\gamma C^{-/-}$ mice; this proliferation being dependent on endogenous IL-15 and IL-15Rα expression not only to drive cell division but also to maintain cell survival through Bcl-2 (5, 6). Although IL-15 and its receptor components are required for the expansion and survival of peripheral NK cells (6–8), the mechanisms controlling the number of naive NK cells and NK cells that participate in immune responses are not clearly understood.

A number of possible mechanisms exist to control peripheral lymphoid cell numbers including failing to receive positive signals or the ligation of inhibitory receptors (9). In addition to expressing inhibitory receptors for MHC class I, a subset of NK cells also express a putative inhibitory receptor belonging to the C-lectin superfamily known as killer cell lectin-like receptor G1 (KLRG1). KLRG1 is composed of a homodimer of glycosylated 30- to 38-kDa subunits and contains a conserved YSxL motif in its cytoplasmic domain that differs from the classical ITIM motif (YxxL) (10). KLRG1 is expressed on ~30% of NK cells in the spleen of naive C57BL/6 mice and between 50 and 80% of CD56dim human NK cells (10, 11). It is has previously been shown that NK cells expressing KLRG1 accumulate following murine CMV (MCMV) infection and that these cells are mature in cell surface phenotype and undergo apoptosis preferentially during NK cell contraction (12). Virus-experienced CD8+ T cells and human effector/memory T cells can also express KLRG1 (11, 13). Recently, members of

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[4] Abbreviations used in this paper: Imm, immature; BM, bone marrow; KLRG1, killer cell lectin-like receptor G1; MCMV, murine CMV; PI, propidium iodide; M1, mature 1; M2, mature 2.

the cadherin family, which are found on Langerhans cells, keratinocytes, and epithelial cells, have been shown to bind KLRG1 and inhibit multiple KLRG1$^+$ T cell and NK cell responses, suggesting that KLRG1 is a functional inhibitory receptor (14–16).

In this study, we have investigated the expression of KLRG1 during steady-state NK cell development and following experimental homeostatic proliferation. KLRG1$^+$ NK cells accumulate during normal development in vivo to stable levels in adult mice. Expression of KLRG1 is associated with slower in vivo turnover, and following homeostatic proliferation most NK cells acquire KLRG1 expression in vivo. KLRG1 expression is restricted to the recently identified MAC-1$^+$CD27$^-$ NK cell subset in all circumstances and supports the existence of at least three peripheral NK cell subsets in vivo. Consistent with KLRG1 expression being related to NK cell turnover, $Cd45^{-/-}$ mice, which have a significantly elevated peripheral NK cell turnover rate, also have significantly elevated proportions of KLRG1$^+$ NK cells throughout development. These data identify KLRG1 as a marker of NK cell proliferation and maturation, and suggest that KLRG1 may be involved in regulating NK cell homeostasis.

Materials and Methods

Mice

C57BL/6 (B6), $Rag2^{-/-}$ (17), $\gamma C^{-/-}$ (18), and $Cd45^{-/-}$ (19) mice were bred and maintained at The Walter and Eliza Hall Institute of Medical Research. $Il18^{-/-}$ (20), $Il12^{-/-}$ (21), and $Il18^{-/-}Il12^{-/-}$ mice were bred and maintained at Peter MacCallum Cancer Centre (Melbourne, Australia). All mutants have been backcrossed at least eight times onto the C57BL/6 background before being made homozygous and maintained by brother-sister mating. Competitive BM chimeras were generated by i.v. transfer of 2×10^6 C57BL/6 (Ly5.2$^+$) and $Cd45^{-/-}$ (Ly5.2$^-$) BM cells to C57BL/6 recipients following 2 doses of 550 rads and analyzed 8 wk later.

### Abs, flow cytometry, and cell sorting

Abs specific for NK1.1 (PK136), Ly5.2 (ALI 4A2), Ly5.1 (A20.1), CD11b (Mac-1; M1/70), TCR-β (H57-5921), and IFN-γ (HB170 and XMG1.2) were purified from hybridoma supernatants available from American Type Culture Collection and coupled to fluorochromes using standard techniques. Abs against CD27, KLRG1, DX5, BrdU and CD49b were purchased from (BD Pharmingen). Single-cell suspensions were prepared by passing organs through steel sieves and filtering suspensions through 100 microfilters. Suspensions were washed and red cells were lysed using a standard red cell removal buffer. Inguinal and axillary lymph nodes were pooled. Liver lymphocytes were isolated from a 40–80% Percoll gradient (Amersham Pharmacia Biotech) centrifuged for 20 min at 2,500 rpm. For flow cytometry, single-cell suspensions were stained with the appropriate mAb in PBS containing 2% FCS. Biotinylated mAbs were revealed by Cy5- or PE-streptavidin (Southern Biotechnology Associates). Cell sorting and analysis were performed using either a LSR, FacsDIVA (BD Biosciences), or MoFlo (DakoCytomation) with dead cells being excluded by propidium iodide (PI) staining. Cell sorting was performed using to >95% purity.

Homeostatic proliferation of NK cells

Sorted NK cells were washed in PBS and between 4×10^4 and 1×10^5 cells were i.v. transferred into 6- to 10-wk-old $Rag2^{-/-}\gamma C^{-/-}$ mice. Mice were sacrificed 13 days later and cell preparations counted and analyzed as described above. Alternatively, 5×10^6 in vitro-expanded NK cells were loaded with 5 μM CFSE (Molecular Probes) and transferred into 8-wk-old $Rag2^{-/-}\gamma C^{-/-}$ or B6 mice and sacrificed 2 or 6 days later.

NK cell assays in vitro

Cell proliferation was performed by culturing 3×10^4 cells/well in 96-well, flat-bottom plates in IMDM supplemented with 10% FCS and 50 ng/ml gentamicin (Sigma-Aldrich) and a titration of recombinant human IL-15 (0.5–50 ng/ml; R&D Systems) for 72 h. Plates were then pulsed with 1 μCi of [^{3}H]thymidine (NEN) and harvested after 8 h onto glass-fiber filters (Packard), and incorporation was determined by scintillation counting. Cell survival was determined by culturing 3×10^4 cells/well in 96-well, flat-bottom plates in IMDM/FCS for 72 h with dead cells being determined by PI and annexin V (BD Pharmingen) staining. KLRG1
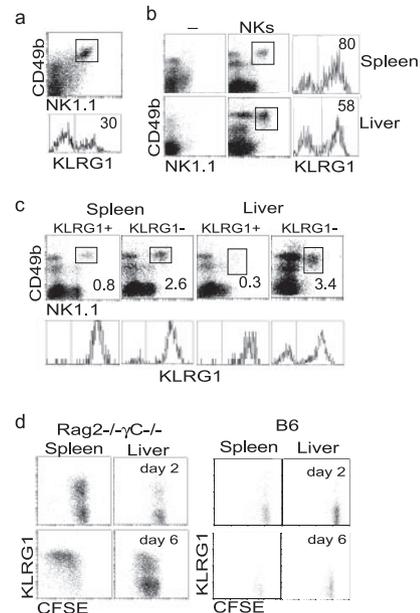
FIGURE 1. Homeostatically expanding NK cells express KLRG1. *a*, KLRG1 expression on resting adult NK cells (NK1.1$^+$CD49b$^+$). Data are representative of six mice. *b*, Splenic NK cells were sorted and i.v. transferred into lymphopenic recipients ($Rag2^{-/-}\gamma C^{-/-}$). Mice either received no NK cells (−) or 2×10^5 NK1.1$^+$CD49b$^+$ cells (NKs). Mice were sacrificed 13 days later with spleen and liver lymphocytes being stained with mAbs against NK1.1, CD49b, and KLRG1, and analyzed by flow cytometry. Data are representative of three experiments. *c*, A total of 5×10^4 splenic KLRG1$^+$ or KLRG1$^-$ NK cells was sorted and i.v. transferred into $Rag2^{-/-}\gamma C^{-/-}$ mice. Recipients were sacrificed 13 days later with spleen and liver lymphocytes being stained with NK1.1, CD49b, and KLRG1. *d*, A total of 5×10^6 IL-15-expanded KLRG1$^-$ NK cells were loaded with CFSE and transferred into $Rag2^{-/-}\gamma C^{-/-}$ or B6 mice. Recipients were sacrificed 2 and 6 days later with spleen and liver lymphocytes analyzed by flow cytometry as indicated. Data are representative of three individual experiments.

induction was performed by expanding sorted NK cells in Nunclon 6-well, flat-bottom tissue culture plates (Nunc) at 1×10^6 cells/ml in 50 ng/ml IL-15. After 5 days, IL-12 (2 ng/ml) and IL-18 (10 ng/ml; R&D Systems) were added alone and in combination, and KLRG1 expression was monitored by flow cytometry.

Measurement of NK turnover in vivo

NK cell turnover was measured as described previously (22). Briefly, 0.5 mg/ml BrdU (Sigma-Aldrich) and 2% glucose water was fed to mice for 2 wk. Single-cell suspensions were prepared, stained, and fixed in 2% paraformaldehyde. Cells were then resuspended in 50 μg/ml DNase1 (Sigma-Aldrich) in PBS/1 mM CaCl$_2$/1 mM MgSO$_4$ for 30 min at 37°C before being stained with anti-BrdU.

Results

KLRG1 expression is acquired as NK cells undergo homeostatic expansion

Adult splenic NK cells undergo finite homeostatic division when transferred into alymphoid recipients ($Rag2^{-/-}\gamma C^{-/-}$) (5, 6). To determine factors that may regulate this proliferative process, KLRG1 expression was monitored on homeostatically expanding NK cells. Strikingly, the percentage of KLRG1$^+$ NK cells significantly increased from 30% on donor cells (Fig. 1*a*) to ~80 and 60% in the spleen and liver, respectively, following homeostatic proliferation in $Rag2^{-/-}\gamma C^{-/-}$ mice (Fig. 1*b*). This increase was not due to preferential expansion of KLRG1$^+$ NK cells because sorted KLRG1$^+$ NK cells remained positive and the majority of KLRG1$^-$ NK cells became KLRG1$^+$ 13 days following transfer
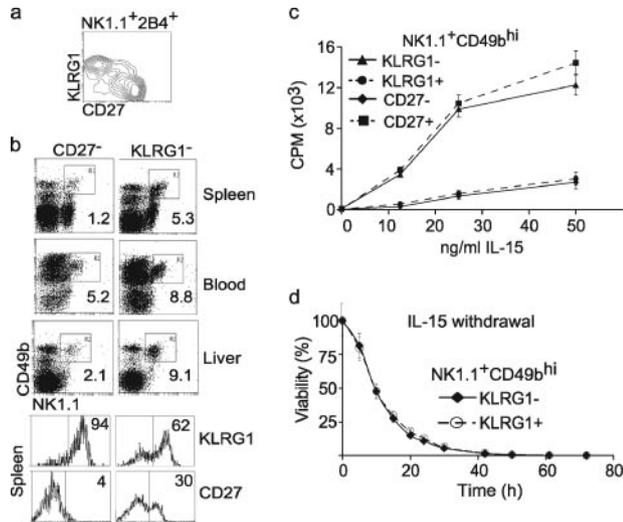
FIGURE 2. KLRG1$^-$ NK cells express CD27 and undergo greater homeostatic expansion. *a*, KLRG1 and CD27 expression were determined simultaneously on adult splenic NK cells (NK1.1$^+$2B4$^+$) by flow cytometry. Numbers in histograms represent percentages. Data are one representative of four mice. *b*, A total of 1×10^5 KLRG1$^-$ and CD27$^-$ NK cells (NK1.1$^+$CD49b$^+$) were sorted and i.v. transferred into *Rag2*$^{-/-}\gamma C^{-/-}$ mice. Recipients were sacrificed 13 days later with spleen and liver lymphocytes analyzed by flow cytometry as indicated. Numbers indicate percentages and histograms are of recipient splenic NK cells. Data are representative of two experiments. *c*, A total of 4×10^4 NK1.1$^+$CD49b$^+$ was sorted using the indicated mAbs and cultured in a range of IL-15 for 72 h with 1 μCi of [^{3}H]thymidine being added for the final 8 h and radio-labeled DNA counted. *d*, A total of 3×10^4 KLRG1$^-$ and KLRG1$^+$ NK cells (NK1.1$^+$CD49b$^+$) were sorted from the spleen and cultured medium alone for 3 days. Cell death was determined using PI and annexin V staining by flow cytometry. Data are representative of three independent experiments.

into *Rag2*$^{-/-}\gamma C^{-/-}$ mice (Fig. 1*c*). Furthermore, transferred KLRG1$^-$ NK cells generated larger NK cell numbers in the spleen and liver following homeostatic proliferation compared with KLRG1$^+$ NK cells (Fig. 1*c* and data not shown). To confirm that KLRG1 expression correlates with cell division, KLRG1$^-$ NK cells were CFSE-labeled and transferred into *Rag2*$^{-/-}\gamma C^{-/-}$ mice or B6 controls. Following transfer into *Rag2*$^{-/-}\gamma C^{-/-}$ mice, many NK cells have become KLRG1$^+$ by day 2, and this proportion increased further by day 6 with most NK cells in the spleen of *Rag2*$^{-/-}\gamma C^{-/-}$ recipients expressing KLRG1 following loss of CFSE (Fig. 1*d*). NK cells in recipient liver underwent less division, and consequently a smaller fraction of KLRG1$^+$ NK cells was observed in this organ (Fig. 1*d*). In contrast, NK cells transferred into B6 mice did not experience marked cell division, and only a very small fraction of these cells expressed KLRG1 (Fig. 1*d*). Taken together, these findings show that KLRG1$^-$ NK cells acquire KLRG1 and become less responsive to proliferative signals following homeostatic expansion in vivo.

KLRG1 is expressed on CD27$^-$ NK cells

Given the fact that KLRG1 can act as an inhibitory receptor (15, 16), sorting NK cells using an anti-KLRG1 Ab may subject KLRG1$^+$ NK cells to inhibition. To overcome this effect, we analyzed the expression of NK cell surface markers to identify proteins differentially expressed from KLRG1. In agreement with a recent finding (23), CD27, a TNF-family member capable of inducing NK cell proliferation and IFN-γ production (24), was found to be expressed almost exclusively on KLRG1$^-$ NK cells (Fig. 2*a*). Because both KLRG1 and CD27 can potentially alter NK cell
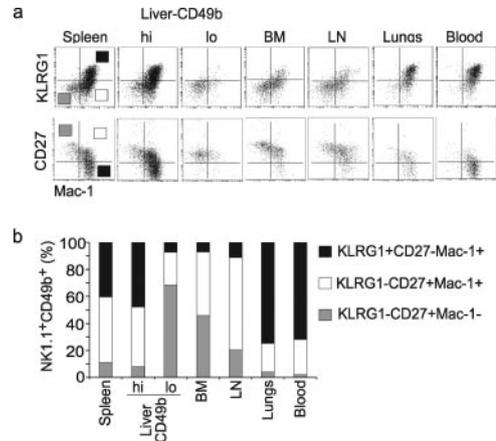

FIGURE 3. KLRG1 and Mac-1 expression define NK cell maturation. *a*, NK cells (NK1.1$^+$CD49b$^+$) from multiple adult organs were stained with Mac-1, CD27, and KLRG1. *b*, The NK cell subsets are represented as a percentage of the total NK cells in each organ. Subsets are indicated by color in *a*.

activity, CD27$^-$ NK cells were sorted to obtain a highly pure KLRG1$^+$ population without having had to ligate either receptor. Consistent with our previous findings, *Rag2*$^{-/-}\gamma C^{-/-}$ mice receiving KLRG1$^-$ NK cells had significantly more CD49$^+$NK1.1$^+$ NK cells ($p < 0.04$) in all organs tested compared with the mice that received CD27$^-$ (KLRG1$^+$) NK cells (Fig. 2*b* and data not shown). These differences in homeostatic proliferative potential correlated with IL-15 responsiveness in vitro, with KLRG1$^-$ NK cells proliferating significantly better than their CD27$^-$ counterparts across a range of IL-15 concentrations (Fig. 2*c*). Interestingly, NK cells sorted as KLRG1$^+$ proliferated identically in IL-15 to those independently sorted as CD27$^-$, likewise NK cells independently sorted as either KLRG1$^-$ or CD27$^+$ proliferated identically in IL-15, indicating that the Abs used for sorting did not alter NK responsiveness to IL-15 (Fig. 2*c*). Furthermore, no differences in survival were observed between KLRG1$^+$ and KLRG1$^-$ NK cells following ex vivo culture in medium only (Fig. 2*d*) or following transfer into naive Ly5.1$^+$ mice (data not shown), indicating that differential responsiveness to IL-15 is unlikely to be attributed to differences in NK survival.

KLRG1 and Mac-1 expression define NK subsets

Increasing expression of the adhesion protein Mac-1 (CD11b) is commonly used to track NK cell maturation in vivo (2). To determine whether KLRG1 expression differed during in vivo NK cell development, Mac-1 and KLRG1 expression were monitored simultaneously. KLRG1 expression was confined to the mature Mac-1$^+$ subset of NK cells, whereas the KLRG1$^-$ population was comprised of both Mac-1$^+$ and Mac-1$^-$ NK cells (Fig. 3*a*), these data being consistent with an earlier report of KLRG1 expression following MCMV infection (25). The distribution of KLRG1 expression on NK cells was confirmed by tracking CD27 and Mac-1 expression, because KLRG1 and CD27 appear mutually exclusively expressed on NK cells (Fig. 3*a*). The percentages of the three NK population identified by differential CD27, Mac-1, and KLRG1 staining were compared in several organs. High levels of KLRG1$^+$ NK cells were found in the lung and blood, moderate levels in spleen and among the CD49b^{high} NK cells in the liver, whereas NK cells expressing low KLRG1 levels predominate in the BM, among the CD49b^{low} NK cells in the liver and lymph node (Fig. 3*b*).
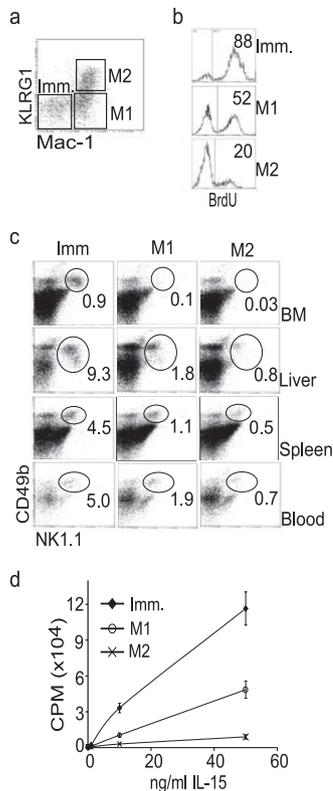
FIGURE 4. Maturational subsets have varying turnover and ability to undergo homeostatic expansion. *a*, Splenic NK cells (NK1.1$^+$CD49b$^+$) were defined as KLRG1$^-$Mac-1$^-$ (Imm), KLRG1$^-$Mac-1$^+$ (M1), and KLRG1$^+$Mac-1$^+$ (M2). Data from five independent experiments are shown. *b*, Adult mice were fed 0.5 mg/ml BrdU and sacrificed 12 days later. Splenic Imm, M1, and M2 NK cells were fixed and stained with anti-BrdU. Numbers are the average of three experiments. *c*, Splenic Imm, M1, and M2 NK cells were sorted and 5×10^5 cells were i.v. transferred into $Rag2^{-/-}\gamma C^{-/-}$ mice. Recipients were sacrificed 13 days later with spleen, BM, blood and liver lymphocytes being analyzed for NK1.1, CD49b, and KLRG1 expression by flow cytometry. Numbers indicate percentages. Data are representative of two experiments. *d*, A total of 1×10^5 splenic Imm, M1, and M2 NK cells was sorted and cultured in a range of IL-15 for 72 h with 1 μCi of [^{3}H]thymidine being added for the final 8 h and radio-labeled DNA counted. Data are representative of two independent experiments.

Turnover and proliferative potential of NK cell subsets

From the differential expression pattern described above, we propose that NK cells can be divided into Mac-1$^-$KLRG1$^-$CD27$^+$ (Imm), Mac-1$^+$KLRG1$^-$CD27$^+$ (mature 1; M1), and Mac-1$^+$ KLRG1$^+$CD27$^-$ (mature 2; M2) (Fig. 4*a*). Because a proportion of splenic KLRG1$^-$CD27$^+$ NK cells are Mac-1$^-$, and given the fact that Mac-1$^-$ NK cells display faster proliferation and turnover than Mac-1$^+$ NK cells (2, 5), we next quantified the proliferative capacity of mature NK cell subsets based on KLRG1 expression, and in comparison to Imm NK cells. As expected, the turnover of M1 and M2 NK cells was less than that of Imm NK cells following 12 days of BrdU feeding (Fig. 4*b*). However, consistently more M1 NK cells (52%) had divided in vivo during this period compared with M2 NK cells (20%) (Fig. 4*b*). Next, we investigated the homeostatic proliferation potential of the three NK cell subsets. Transfer of Imm NK cells resulted in 2.5- to 9-fold more NK cells in $Rag2^{-/-}\gamma C^{-/-}$ recipient organs compared with organs of mice that received the mature subsets (Fig. 4*c*). Consistent with the difference in turnover rate of mature NK cell subsets, $Rag2^{-/-}\gamma C^{-/-}$ recipients transferred with M1 NK cells possessed 2- to 3-fold
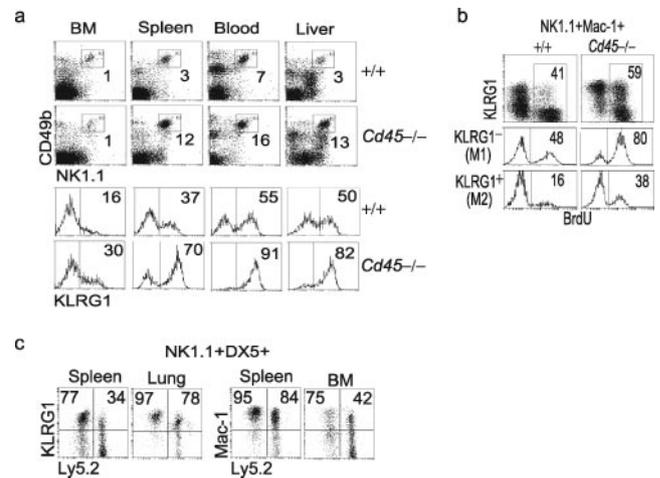

FIGURE 5. Enhanced turnover leads to an increased pool of KLRG1$^+$ NK cells. *a*, NK cells (NK1.1$^+$CD49b$^+$TCR-β^-) from BM, liver, spleen, and blood of adult $Cd45^{-/-}$ and control mice were enumerated, and KLRG1 expression was determined by flow cytometry. Data are representative of six mice. *b*, Adult $Cd45^{-/-}$ and control mice were fed 0.5 mg/ml BrdU and sacrificed 12 days later. Mature splenic NK cells (NK1.1$^+$Mac-1$^+$) were analyzed for KLRG1 expression and BrdU incorporation by flow cytometry. Numbers are percentages and are representative of three mice. *c*, BM chimeras were generated from equal amounts of control (Ly5.2$^+$) and $Cd45^{-/-}$ BM (Ly5.2$^-$). NK cells were analyzed by flow cytometry 8 wk later. Number represent percentage positive of Mac-1 or KLRG1 for Ly5.2$^-$ or Ly5.2$^+$ NK cells.

more NK cells in all organs analyzed compared with $Rag2^{-/-}$ $\gamma C^{-/-}$ recipients transferred with M2 NK cells (Fig. 4*c*). The percentage of donor KLRG1$^+$ NK cells in recipient spleens was ~40, 80, and 99% in recipients that received Imm, M1, and M2, respectively, whereas almost all the NK cells in the BM of recipients that received Imm NK cell recipients were KLRG1$^-$ (data not shown). Consistent with our earlier finding, differences in homeostatic expansion among the NK cell subsets correlated with IL-15 responsiveness, with Imm cells proliferating better than M1, which in turn proliferated more than M2 NK cells (Fig. 4*d*). Taken together, these findings confirm the existence of three peripheral NK cell subsets with varying proliferative capacity.

KLRG1$^+$ NK cells accumulate in mice with enhanced NK cell cellularity

Because our data suggested that KLRG1$^+$ NK cells accumulate following periods of in vivo proliferation, we examined KLRG1 expression on NK cells with enhanced turnover rates. We recently reported that mice lacking the type 1 transmembrane phosphatase CD45 (26) have elevated peripheral NK cell numbers due to enhanced turnover in vivo (22). $Cd45^{-/-}$ mice had significantly more KLRG1$^+$ NK cells in spleen, blood, and liver, with both NK cells numbers (data not shown) and percentage being significantly elevated ($p < 0.01$; Fig. 5*a*). We previously reported that enhanced NK cell turnover in $Cd45^{-/-}$ mice occurred in the spleen, with the number of NK cells and their turnover rate in the BM being comparable to control mice (22). We therefore examined the turnover rate of mature Mac-1$^+$ NK cell subsets in $Cd45^{-/-}$ mice. M1 NK cells (KLRG1$^-$) had a greater turnover rate than M2 (KLRG1$^+$) in both control and $Cd45^{-/-}$ mice (Fig. 5*b*), with both M1 and M2 subsets in $Cd45^{-/-}$ mice having significantly ($p < 0.044$) greater turnover compared with M1 and M2 in control mice (Fig. 5*b*). Next, we determined whether the increased KLRG1$^+$ NK population in $Cd45^{-/-}$ mice was cell intrinsic. Irradiated host
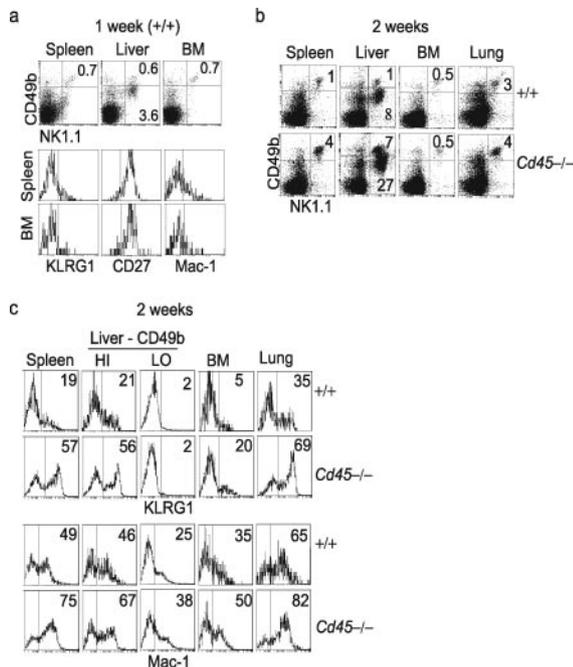
FIGURE 6. KLRG1 is acquired on NK cells during development. *a*, NK cells (NK1.1$^+$CD49b$^+$) from BM, liver, and spleen of 1-wk-old *Cd45$^{-/-}$* and control mice were enumerated by flow cytometry. Numbers indicate percentages in quadrant. *b*, NK cells (NK1.1$^+$CD49b$^+$) from BM, liver, spleen, and lung of 2-wk-old *Cd45$^{-/-}$* and control mice were enumerated by flow cytometry. Numbers indicate percentages in quadrant. *c*, NK cells (NK1.1$^+$CD49b$^+$) from BM, liver, spleen, and lung of 2-wk-old *Cd45$^{-/-}$* and control mice were analyzed for KLRG1 and Mac-1 expression by flow cytometry. Numbers indicate percentages. Data are representative of three mice.

mice were reconstituted with equal amounts of control (Ly5.2$^+$) and *Cd45$^{-/-}$* BM (Ly5.2$^-$), and NK cells were analyzed. *Cd45$^{-/-}$*-derived NK cells were more mature in phenotype than NK cells derived from control mice, with higher fractions of Mac-1$^+$ and KLRG1$^+$ NK cells observed in all organs investigated (Fig. 5*c*), indicating that the enhanced maturation of *Cd45$^{-/-}$* NK cells was cell autonomous.

KLRG1$^+$ NK cells increase in frequency during development

NK cells in newborn mice undergo pronounced homeostatic proliferation, resulting in a continuous increase in NK cell numbers in the spleen with a maximum being reached at 8 wk of age (4). Because KLRG1$^+$ NK cells accumulated following division, we next monitored the appearance of KLRG1$^+$ NK cells during normal development in control and *Cd45$^{-/-}$* mice. At 1 wk of age, KLRG1$^+$ NK cells were absent from the spleen and BM, with the majority having the phenotype of Imm NK cells (Mac-1$^-$KLRG1$^-$CD27$^+$) (Fig. 6*a*). By 2 wk of age, NK cells increased in frequency (Fig. 6*b*) with a small proportion of KLRG1$^+$ NK cells observed in the periphery (Fig. 6*c*). A comparison between control and *Cd45$^{-/-}$* mice revealed that by 2 wk of age, *Cd45$^{-/-}$* mice already display enhanced NK cell development characterized by increased NK cell numbers and a greater proportion of NK cells expressing KLRG1 and Mac-1 compared with control mice (Fig. 6, *b* and *c*, and data not shown). These data indicate that mature NK cells acquired KLRG1 expression during the normal postnatal development of the NK cell compartment and that the enhanced development observed in *Cd45$^{-/-}$* mice resulted in more KLRG1$^+$ NK cells at an earlier stage.
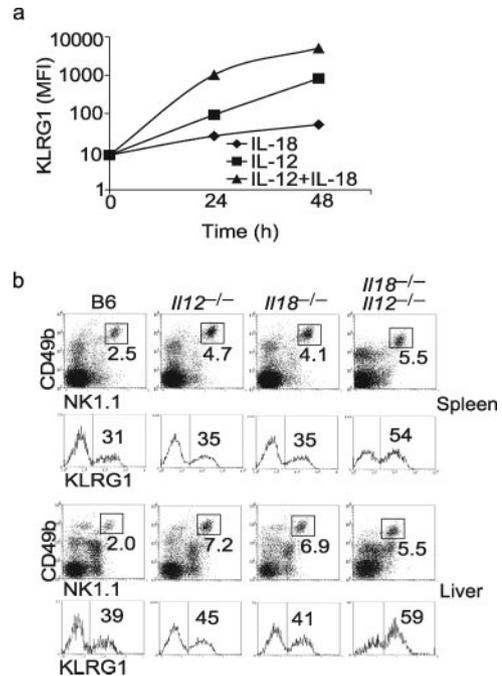
FIGURE 7. KLRG1 can be induced by IL-12 and IL-18. *a*, NK cells (NK1.1$^+$CD49b$^+$) were expanded in IL-15 for 7 days then cultured in 20 ng/ml IL-18 and 2 ng/ml IL-12. Cells were harvested 24 or 48 h later and KLRG1 expression was determined by flow cytometry. MFI, Mean fluorescence index. *b*, Spleen- and liver-derived NK cells from *Il12$^{-/-}$*, *Il18$^{-/-}$*, *Il12$^{-/-}$ Il18$^{-/-}$*, and control mice were analyzed for KLRG1 expression by flow cytometry.

KLRG1 expression is induced by cytokines

Previous reports suggest that KLRG1 expression may be regulated by cytokines such as IFNα (12). IL-12 and IL-18 are both potent proinflammatory cytokines capable of activating NK cells (27). Culturing IL-15 expanded KLRG1$^-$ NK cells in IL-12 or IL-18 in vitro induced KLRG1 expression in a time-dependent manner, with IL-12 being more efficient at this process at the concentration of cytokines used (Fig. 7*a*). Furthermore, these two cytokines functioned synergistically to induce KLRG1, with the NK cells appearing large and granular and down-regulating NK1.1 expression, consistent with earlier reports (27) (Fig. 7*a* and data not shown). However, IL-12 and IL-18 were not required in vivo for KLRG1 expression because *Il18$^{-/-}$* and *Il12$^{-/-}$* mice possessed KLRG1$^+$ NK cells at normal proportions. Surprisingly, *Il18$^{-/-}$ Il12$^{-/-}$* mice possessed more KLRG1$^+$ NK cells compared with control mice, suggesting that, by analogy with phenotype observed in *CD45$^{-/-}$* mice, they to have a defect in NK cell homeostasis (Fig. 7*b*).

Discussion

Lymphopoiesis is a multistage process involving proliferation, maturation, and survival. The importance of NK cells in controlling infectious pathogens and in the immunotherapy of cancers suggests that understanding mechanisms that govern NK cell lymphopoiesis could greatly improve therapeutic approaches for cancer and infectious disease. In this study, we describe the phenotypic maturation of NK cells during development and homeostatic expansion and highlight functional differences between mature NK cells based on the expression of an inhibitory receptor KLRG1.

All current data suggest that KLRG1 is acquired on NK cells during development. First, KLRG1 is only expressed on NK cells of mature phenotype (Mac-1$^+$CD49b^{high}CD27$^-$) (23), with few

KLRG1$^+$ NK cells present in the BM, the predominate location of Imm NK cells (2), and greater numbers are found in the spleen, lungs, and peripheral blood. Second, the proportion of KLRG1$^+$ NK cells in the mature pool increase during development from being almost undetectable at 7 days of age to >30% at >8 wk of age in unchallenged mice. Third, transferred KLRG1$^-$ NK cells can become KLRG1$^+$, but not vice versa. Although these data are compatible with a model whereby KLRG1$^+$ NK cells derive from KLRG1$^-$ precursors in peripheral organs such as spleen, the possibility that some KLRG1$^+$ cells are produced directly from BM progenitors has not been formally excluded.

Analysis of KLRG1 expression on NK cells has predominantly been restricted to the analysis of NK cells responding to viral infection. These studies identified the fact that NK cells responding to MCMV acquired KLRG1, and these cells contained less intracellular IFN-γ compared with the KLRG1$^-$ population (12, 25). This finding was extended by our recent report on NK subsets where Mac-1$^+$CD27$^-$KLRG1$^+$ cells were found to produce poor levels of IFN-γ following stimulation with IL-12 and IL-18, cytokines required for the NK cell expansion during MCMV infection (28) or when cocultured with dendritic cells (23). In addition, KLRG1$^+$ NK cells appear to preferentially down-regulate bcl-2 during the course of MCMV infection (25). These findings, together with our report that KLRG1$^+$ NK cells have impaired turnover and responsiveness to IL-15, suggest a model where expression of KLRG1 may dampen or terminate NK cell responses, although the mechanism for this function is yet to be elucidated. Indeed, the reduced responsiveness of KLRG1$^+$ NK cells to IL-15 could explain the down-regulation of bcl-2 following expression of KLRG1, because IL-15 has been demonstrated to maintain bcl-2 levels (6).

The phenotypic maturation of NK cells during MCMV-mediated NK cell division could be a result of exposure of enhanced levels of IL-18 and IL-12 because these cytokines activate NK cells, induce KLRG1 expression, and are known to be important for NK cell response to MCMV (28). Our study monitoring NK cell expansion during development and following transfer to lymphopenic hosts, without an overt inflammatory stimulus to induce these cytokines, provides strong evidence for NK cell maturation being induced by cell division. Furthermore, the striking finding that most peripheral $Cd45^{-/-}$ NK cells express KLRG1 indicates that this maturation can be accelerated by increased NK cell turnover in vivo. Although the turnover rate of both M1 and M2 NK cells in $Cd45^{-/-}$ mice are increased compared with control mice, the M2 NK cells turnover slower than M1 NK cells, indicating that KLRG1 expression also correlates with impaired turnover in $Cd45^{-/-}$ mice.

Induction of KLRG1 is also observed on virus-specific CD8$^+$ T cells following repetitive and persistent Ag stimulation in mice and chronic viral infections in humans (11, 13, 29). In addition, our finding of slower turnover of KLRG1$^+$ NK cells is consistent with the slow turnover of KLRG1$^+$ effector/memory T cells (11). Given the fact that KLRG1 expression correlates with impaired IL-15-mediated proliferation of naive NK cells, it is likely that this function represents a normal stage of NK cell maturation. Furthermore, this finding suggests the hyporesponsiveness of KLRG1 NK cells is unlikely to result from replicative senescence, although this possibility cannot be totally excluded at present.

Another role for NK cells is that of priming Th1 cells in draining LN by secreting IFN-γ in response to activated dendritic cells (30). KLRG1$^+$ NK cells are largely excluded from the LN, likely due to the low expression of CXCR3 and subsequent poor responsiveness to CXCL10 and CXCL11 (23). This discrimination would be beneficial for the host because KLRG1$^+$ NK cells are poor producers of IFN-γ in response to proinflammatory cytokines such as IL-12 and IL-18 (23) and thus would be less potent inducers of Th1 responses.

Taken together, our results demonstrate that NK cells undergo a phenotypic and functional maturation following periods of nonviral-induced proliferation that correlates with the acquisition of the inhibitory receptor KLRG1. NK cells that lack KLRG1 expression are more capable of eliciting NK cell effector functions; therefore, the expression of KLRG1 potentially serves as a mechanism to inhibit NK cell expansion and function. Recently, E-, R-, and N-cadherins, found on Langerhans cells, keratinocytes, and epithelial cells, were identified as ligands for KLRG1 (14–16). Indeed, E-cadherin was shown to inhibit both Ag-specific proliferation of KLRG1$^+$ T cells and cytotoxic potential (15) while also inhibiting NK cell cytotoxicity (16), suggesting that this interaction is physiologically important. If this is true, induction of KLRG1 expression whether during viral responses or normal development could serve as a mechanism to inhibit NK cell growth, function, and potential immunopathology in the absence of Ag exhaustion. This model also proposes that NK cells in blood and lung would require a greater level of stimulation or costimulation to overcome KLRG1-mediated inhibition because the majority these NK cells express KLRG1. The lung is constantly exposed to foreign aerosols, thus KLRG1 expression may prevent premature or unnecessary activation whereas other less inflammatory immune cells removed foreign matter.

A potential caveat to this model is that peripheral NK cells develop in KLRG1 transgenic (KLRG1-Tg) mice, in which KLRG1 is expressed on all NK and T cells (31). Although neither the phenotype nor function of KLRG1-Tg NK cells were examined in this study (31), their presence suggests that constant KLRG1 expression on NK cells does not completely block their development in vivo. However, it is also possible that these NK cells become desensitized to constant KLRG1 ligation during development or use other compensatory mechanisms to develop and persist in the presence of KLRG1 expression.

The use of multiple inhibitory mechanisms throughout development and activation has direct parallels to the T cell lineages. T cell specificity and homeostasis are controlled by central and peripheral tolerance, regulatory T cells, and the induction of inhibitory molecules. The data presented here suggest that peripheral NK cells are potentially controlled in a similar fashion. Although it is yet to be shown that binding of KLRG1 can suppress NK cell effector function in vivo, we provide evidence that KLRG1 expression is acquired during NK cell development and correlates with reduced proliferative capacity and differential tissue residency.

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Disclosures

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