The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2004

Turnover and Proliferation of NK Cells in Steady State and Lymphopenic Conditions[1] FREE

Amanda M. Jamieson; ... et. al

J Immunol (2004) 172 (2): 864–870.
https://doi.org/10.4049/jimmunol.172.2.864

Turnover and Proliferation of NK Cells in Steady State and Lymphopenic Conditions[1]

Amanda M. Jamieson,[2] Patricia Isnard,[2,3] Jeffrey R. Dorfman,[2,4] Mark C. Coles,[5] and David H. Raulet[6]

To gain insight into NK cell dynamics, we investigated the turnover and proliferation rates of NK cells in normal and lymphopenic conditions. In contrast to previous reports suggesting a very rapid turnover of NK cells, continuous 5-bromo-2′-deoxyuridine (BrdU)-labeling studies demonstrated that the time necessary for labeling 50% of splenic NK cells in mature mice was 17 days, similar to the rate of labeling of memory T cells. In contrast, in young mice, splenic NK cells labeled very rapidly with BrdU, although cell cycle analyses and BrdU pulse-labeling studies suggested that most of this proliferation occurred in a precursor population. A somewhat larger percentage of bone marrow NK cells was cycling, suggesting that these proliferating cells are the precursors of the mostly nondividing or slowly dividing splenic NK cells. Splenic NK cells from mature mice also did not proliferate significantly when transferred to normal mice, but did proliferate when transferred to irradiated mice. Thus, NK cells, like T cells, undergo homeostatic proliferation in a lymphopenic environment. Homeostatic proliferation of NK cells was not dependent on host cell class I molecules or host production of IL-15. Nevertheless, the number of recovered NK cells was much lower in IL-15$^{-/-}$ hosts. These results suggest that IL-15 is not essential for homeostatic proliferation of NK cells, but is necessary for survival of the NK cells. Our results provide important basic information concerning the production and replacement of NK cells. ***The Journal of Immunology*****, 2004, 172: 864–870.**

Natural killer cells play an important role in host defense in some viral and bacterial infections as well as against certain tumor cells (1, 2). NK cell specificity is determined by a balance in signaling by stimulatory and inhibitory receptors. The inhibitory receptors are generally specific for self MHC class I molecules expressed by target cells (3). Two families of class I-specific receptors have been identified in mice. The Ly-49 family of receptors recognizes class Ia molecules directly (4), while the CD94/NKG2 receptor recognizes a nonclassical class Ib molecule, Qa-1, associated with peptides derived from the signal peptides of many class Ia molecules (5). Susceptibility of target cells to NK cells is increased when the target cells are deficient in the expression of class I MHC molecules that can engage the inhibitory receptors, as shown with tumor cells (6) or nontransformed cells (7).

Cytokines have been implicated in the development and activation of NK cells. NK cell development is defective in gene-targeted mice lacking expression of common γ-chain, the γ-chain subunit common to the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (8). Although NK cells develop essentially normally in mice deficient in the expression of IL-7, IL-2, or their receptors (9, 10), mice lacking IL-15 or its unique receptor component (IL-15Rα) are highly deficient in NK cells (11, 12). In vivo treatment of normal or IL-15 knockout mice with IL-15 results in increased NK cell numbers and the induction of NK cell activity (12–15). The relative roles of IL-15 in NK cell differentiation, proliferation, and maintenance have not been clearly established, although a role in NK cell survival is suggested by in vitro and in vivo studies (16, 17).

Relatively little is known concerning NK cell homeostasis, maintenance, and turnover. Early studies suggested that mature NK cells in the spleen turn over very rapidly, which would distinguish them from most T cell subsets (18, 19). In this study, we add considerable new information concerning NK cell dynamics. We examine NK cell accumulation, cell cycle status, and turnover at various life stages in mice, the effects of inhibitory receptor expression and host MHC expression on these processes, and homeostatic proliferation of NK cells in lymphopenic mice.

Materials and Methods

Mice

C57BL/6J (hereafter designated B6) mice, which express the Ly-5.2 allele, were obtained from The Jackson Laboratory (Bar Harbor ME). B6-Ly-5.1 congenic mice were obtained from Charles River Laboratories (Frederick, MD). B6 β_2-microglobulin (β_2m)$^{-/-}$ mice (20) that had been backcrossed five times to B6. K$^{b-/-}$D$^{b-/-}$ mice on the B6 background were described previously (21). B6-IL-15$^{-/-}$ mice were generously provided by J. Pechon (Immunex, Seattle, WA) (12). All mice were bred in the animal facility at the University of California, according to institutional guidelines.

Cell labeling

For short-term labeling (3 days or less), mice were injected i.p. every 12 h with 150 μl of 9 mg/ml 5-bromo-2′-deoxyuridine (BrdU)[7] (Sigma-Aldrich, St. Louis, MO) freshly dissolved in PBS. For longer term labeling, mice

Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720

Received for publication April 1, 2003. Accepted for publication October 29, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by National Institutes of Health Grant RO1AI35021 (to D.H.R.). P.I. was a recipient of a postdoctoral fellowship from the National Cancer Center (Plainview, NY).

[2] A.M.J., P.I., and J.R.D. contributed equally to this work.

[3] Current address: Nucleis, 3 allée Pierre de Coubertin, 69007 Lyon, France.

[4] Current address: Kenya Medical Research Institute, Wellcome Trust Research Laboratories, Post Office Box 230, Kilifi, Kenya.

[5] Current address: National Institute For Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom.

[6] Address correspondence and reprint requests to Dr. David H. Raulet, Department of Molecular and Cell Biology and Cancer Research Laboratory, 485 Life Science Addition, University of California, Berkeley, CA 94720-3200. E-mail address: raulet@uclink4.berkeley.edu

[7] Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; β_2m, β_2-microglobulin.

0022-1767/04/$02.00

were administered BrdU in their drinking water (0.8 mg/ml). Water was changed every day and contained 1% glucose to overcome taste aversion.

On the day of analysis, spleen and bone marrow cells were harvested from labeled mice. At least one nontreated control mouse was included with each experiment, and at least three mice in each treatment group were evaluated independently in each experiment. RBC were eliminated by treating the cells with ACK (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA), and splenocytes were stained with relevant Abs. Subsequent BrdU staining was performed, as described (22), and analyzed on an EPICS XL-MCL flow cytometer (Coulter, Hialeah, FL).

Abs and immunofluorescence analysis

Anti-Ly-49C/Ly-49I mAb SW5E6 (23), anti-NKG2A mAb 16a11 (24), and anti-FcR mAb 2.4G2 (25) have been described previously. The hybridoma-producing anti-Ly-49G2 mAb 4D11 (26) was purchased from American Type Culture Collection (Manassas, VA). Abs were conjugated to FITC (Boehringer Mannheim, Indianapolis, IN) or biotin (Pierce, Rockford, IL). Anti-NK1.1 PE, DX5 PE, CD3 biotin, CD5 biotin, CD19 biotin, CD3 PE-Cy5, and Ter119 biotin conjugates were purchased from E-biosciences (San Diego, CA), except that PK136 used for the BrdU analyses was kindly conjugated to PE by P. Schow (University of Calfornia, Berkeley). Streptavidin tricolor, streptavidin PE, and streptavidin RED613 were purchased from BD PharMingen (San Diego, CA). Biotinylated anti-IL-7Rα mAb A7R34 was generously provided by C. Surh (Scripps Research Institute, La Jolla, CA). Anti-CD3 Tricolor (670) and anti-$\alpha\beta$ TCR Tricolor were purchased from Caltag (S. San Francisco, CA) and were used to exclude NK T cells from most analyses.

Cell cycle analysis

Nylon wool-nonadherent splenocytes and bone marrow cells were depleted of Gr-1$^+$ and CD19$^+$ cells using the AutoMACS system (Miltenyi Biotec, Auburn, CA) and were preincubated with 2.4G2 culture supernatant, washed, and stained with Abs specific for NK1.1 and CD3, followed by fixation with 1 ml ice-cold 70% ethanol overnight at 4°C. Cells were resuspended in 1 ml of fresh Sytox Green (Molecular Probes, Eugene, OR) staining solution (1 nM Sytox Green, 100 U/ml RNase A, 1 g/L glucose, 1× PBS), incubated at least 30 min at room temperature, and analyzed on a flow cytometer.

NK cell transfers

Single cell suspensions of spleen cells from groups of 30 donor mice (B6-Ly-5.1 mice) were prepared. Cells were incubated with biotinylated Abs for depletion (anti-CD3, anti-CD19, anti-Ter119, and anti-CD5) and magnetically sorted using the AutoMACS protocol. Enriched NK cells were recovered from the AutoMACS, counted, and washed with PBS. Analysis demonstrated that the cells were typically 60–80% NK1.1$^+$CD3$^-$ cells. The cell concentration was adjusted to 5×10^6 NK cells/ml in 3 μM CFSE solution (CFSE in PBS; Molecular Probes) and incubated 10 min at 37°C. After quenching CFSE with FCS and washing in PBS, 5×10^6 NK1.1$^+$CD3$^-$ cells were injected i.v. (subocular vein) into congenic B6 (Ly5.2) mice 2–4 h after the mice received a single sublethal dose of 6 Gy irradiation from a ^{137}Cs source. After 7 days, spleen cells were analyzed.

Quantitation of NK cell proliferation dynamics from CFSE profiles

The calculation models to define the dynamics of clonal expansion in vivo have been described previously (27). Briefly, where n is the number of division cycles achieved during clonal expansion, and E the number of events under each CFSE fluorescence peak, the size of the precursor sample pool (Ps) is given by equation 1 and the size of the precursor sample pool that underwent cell division (Psr) is given by equation 2. Equation 3 gives the proportion, or frequency, of the precursor sample pool that divided (R).

$$Ps = {}_0{}^{n}\Sigma(E_n/2^n) \quad (1)$$

$$Psr = {}_1{}^{n}\Sigma(E_n/2^n) \quad (2)$$

$$R = Psr/Ps \quad (3)$$

Results

NK cell accumulation and BrdU labeling as a function of age

The number of NK cells (defined as NK1.1$^+$CD3$^-$ cells) was determined in the spleens of B6 mice of various ages. The steady state number of NK cells rose rapidly during early life, from 1 to 3×10^5 per spleen at days 1 and 2 after birth, reaching $\sim 2 \times 10^6$ cells at 3 wk of age, and finally achieving a plateau of $2–4 \times 10^6$ per spleen in 2- to 8-mo-old mice (Fig. 1). The rise in NK cell numbers closely paralleled the increased cellularity of the spleen over the same time period (Fig. 1*B*).

Splenic NK cells incorporate BrdU at high levels early in life and at low levels later in life

As an initial assessment of NK cell turnover, mature mice were provided with the BrdU in their drinking water continuously for various periods of time. The mice were 8–10 wk old at the beginning of the labeling period. The percentage of NK cells (gated NK1.1$^+$CD3$^-$ cells; see *inset*, Fig. 2*A*) that labeled with BrdU was determined by flow cytometry at each time point. The NK cells labeled with linear kinetics, with one-half of the cells acquiring the label by ~17 days after labeling was initiated (Fig. 2*A*). This rate of labeling was faster than that of total splenic T cells (Fig. 2*B*) and similar to that of memory T cells (22) and splenic NK1.1$^+$ T cells (data not shown).

To evaluate the BrdU-labeling rate of NK cells at different life stages, a fixed labeling period was applied to mice of various ages. In mature B6 mice of 7–15 wk of age (49–105 days of age), ~10–15% of NK cells labeled with BrdU over 3 days (Fig. 3*A*), consistent with the continuous labeling data. This rate of labeling was similar to the rate of incorporation in splenic T cells over the same age range (Fig. 3*C*). In contrast to these results, in 18-day-old mice nearly 90% of NK cells labeled with BrdU over 3 days (Fig. 3*A*). Thus, the incorporation of BrdU approximately mirrored the rate of accumulation of NK cells in the spleen: very high in young

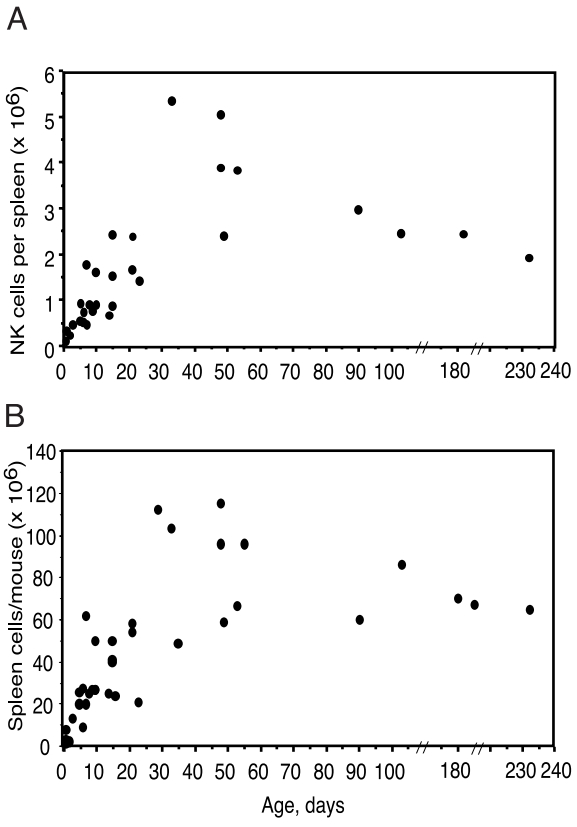

FIGURE 1. *A*, NK cellularity in the spleen in mice of different ages. NK cells were defined as NK1.1$^+$CD3$^-$ cells. Each symbol represents a separate animal. *B*, Splenic cellularity over the same time course.

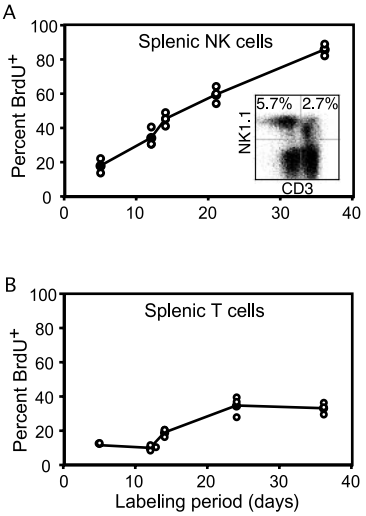

FIGURE 2. Continuous BrdU labeling of splenic NK cells ($NK1.1^+CD3^-$ cells) (*A*), and splenic T cells ($NK1.1^-CD3^+$ cells) (*B*). BrdU labeling was initiated in mice of 8–10 wk of age and continued for the time period shown. Open symbols represent individual animals, and filled symbols represent the means at each labeling period. The flow cytometry histogram shown in the *inset* depicts staining of a representative sample from this analysis with anti-NK1.1 vs anti-CD3.

mice in which NK cells were accumulating, and much lower in older mice after NK cell numbers reached a steady state.

Similar to the pattern in the spleen, nearly all $NK1.1^+CD3^-$ NK cells in the bone marrow of 18-day-old mice had incorporated BrdU after a 3-day pulse (Fig. 3*B*). As in the spleen, the percentage of $BrdU^+$ cells decreased substantially in older mice, but the plateau level of labeled cells, ~30–35% of NK cells, was substantially higher than the plateau level in the spleen (10–15%). These data suggest either that bone marrow NK cells proliferate more than splenic NK cells, and/or that a larger fraction of bone marrow NK cells corresponds to recent progeny of proliferating precursor cells.

In subsequent analyses, we pulsed 18-day-old mice with BrdU for increasingly shorter intervals, and determined the percentage of $BrdU^+$ cells among splenic cells as a function of the length of the BrdU exposure. Splenic NK cells were compared with the entire nylon wool-nonadherent spleen cell population (Fig. 4). The latter population labeled with approximately linear kinetics, but there was a lag in the labeling of NK cells, such that only 5% of NK cells were labeled at 6 or 12 h, but nearly one-half of the cells were labeled by 48 h. A lag in labeling indicates that most of the incorporation occurred in a precursor population rather than in the population under analysis (28, 29). Thus, the data suggest that most of the NK cells in the spleens of 18-day-old mice are not themselves undergoing rapid division, but most likely represent the recent progeny of proliferating precursor cells that either reside outside the spleen or do not express the NK1.1 marker.

Cell cycle analysis of NK cells in spleen and bone marrow

To assess the extent of ongoing proliferation of NK cells in the spleen and bone marrow, the cell cycle status of NK cells was determined by flow cytometric analysis of gated $NK1.1^+$ $CD3^-$ cells stained with Sytox Green. The cell cycle analysis demonstrated that in 3-wk-old mice ~3% of splenic $NK1.1^+CD3^-$ cells were in either the S or G_2/M phases of the cell cycle (Fig. 5*A*). The percentage of NK cells in cycle was nearly unchanged in mice of 6 wk of age (Fig. 5*C*), suggesting that the proliferation of splenic NK cells does not vary significantly within this age range, despite

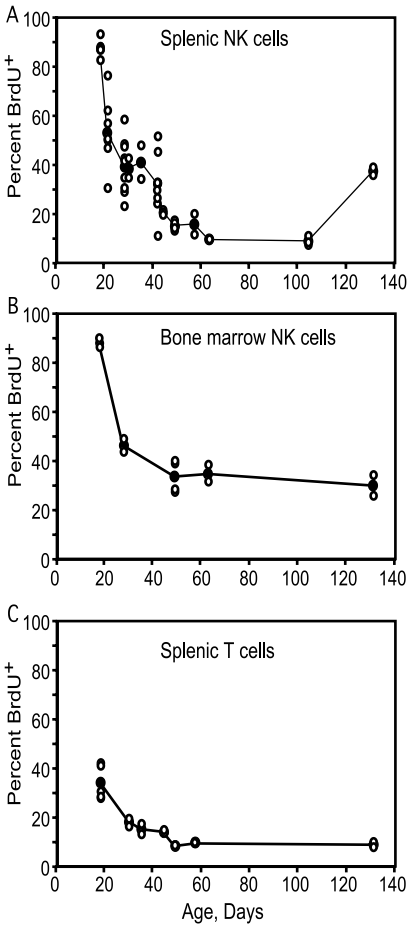

FIGURE 3. Rate of BrdU labeling of NK cells in mice of different ages. Mice of the indicated ages were exposed to BrdU for 3 days. Incorporation of BrdU in splenic $NK1.1^+CD3^-$ cells (*A*), bone marrow $NK1.1^+CD3^-$ cells (*B*), and splenic T cells (*C*) was determined by flow cytometry. Open symbols represent individual animals, and filled symbols represent the means at each labeling period. In *A*, the two mice at the latest time point showed possible signs of stress or infection (unusually small thymi), which may account for the increased BrdU incorporation in their NK cells.

the decrease in the rate of BrdU labeling over the same period (see Fig. 5 legend). These data suggest that most peripheral NK cells, even in young mice, are not proliferating at a substantial rate, consistent with the BrdU pulse-labeling data. A minor percentage of NK cells are in cycle under steady state conditions, however, suggesting either that a small subset of NK cells is dividing rapidly, or that many or all NK cells are dividing infrequently.

The percentage of cycling NK cells in the bone marrow was slightly higher than that of splenic NK cells at 6 wk of age (~5% compared with 2–4%) (Fig. 5, *C* and *D*). Although a somewhat higher percentage of bone marrow NK cells was cycling at 3 wk of age (~10%) (Fig. 5*D*), this small difference cannot account for the much higher percentage of BrdU-labeled splenic NK cells in younger mice as compared with older mice. It is likely, therefore, that the higher rate of labeling in young mice is due to the filling of the splenic compartment by recently divided $NK1.1^-$ precursor cells, which could eventually saturate splenic niches and prevent further rapid influx of additional recently divided cells. Taken together, the results suggest that most peripheral NK cells in young and old mice are nondividing or slowly dividing cells that are recently derived from proliferating precursor cells.

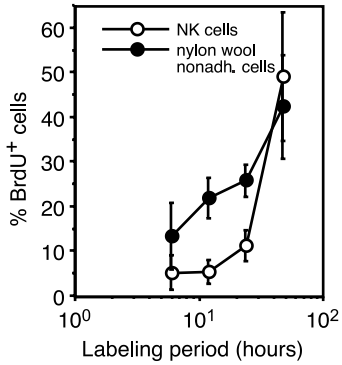

FIGURE 4. A lag in BrdU labeling of splenic NK cells. Young (18-day-old) mice were exposed to BrdU for the indicated short time periods. Spleen cells were passed through a nylon wool column before analysis. The percentages of $BrdU^+$ cells in the entire nylon wool-nonadherent population were compared with those in the $NK1.1^+CD3^-$ subset.

Proliferation of NK cells does not correlate with expression of inhibitory receptors or MHC expression

As a measure of whether expression of inhibitory MHC-specific receptors influences basal proliferation of NK cells, we examined whether BrdU incorporation correlated with inhibitory receptor expression. BrdU labeling was commenced at 8 wk of age, and lasted for 1, 2, or 3 wk before spleen cells were harvested and gated NK cells were analyzed by flow cytometry. To reveal whether more or less proliferation occurred within an NK subset defined by expression of a given inhibitory receptor, we depicted the data as a ratio of the percentage of $BrdU^+$ cells in the receptor-positive subset compared with percentage of $BrdU^+$ cells in the receptor-negative subset (Table I). The ratios were close to 1.0 when analyzing NK subsets in B6 mice defined by any of three inhibitory receptors, NKG2A/CD94 (16a11 mAb), Ly-49G2 (4D11 mAb), or Ly-49C/I (5E6 mAb), indicating that expression of these receptors did not correlate well with NK cell proliferation. Two of these receptors recognize MHC class I molecules expressed in the B6 strain: NKG2A (when paired with CD94) recognizes a D^b peptide presented by Qa-1 (30), whereas Ly-49C and Ly-49I recognize the K^b class I molecule (31, 32). In contrast, Ly-49G2 does not recognize the H-2^b-encoded class I molecules expressed in B6 mice (32, 33).

As an additional approach to investigate the possible role of MHC-receptor interactions in basal NK cell proliferation, we compared the rate of BrdU incorporation into NK cells in wild-type mice with that of mice deficient for MHC class I expression. Two types of class I-deficient mice were examined: those deficient for the K^b and D^b classical class I molecules ($K^{b-/-}D^{b-/-}$ mice), and those lacking β_2m, a molecule necessary for expression of classical and nonclassical class I molecules. Both types of mice lack ligands for both Ly-49 receptors and the CD94/NKG2A receptor. The results demonstrated that the rates of BrdU incorporation into NK cells in general, as well as into the specific subsets examined, were roughly similar in class I^+ and class I^- mice (Table I) (data not shown). Taken together, the results suggest that MHC-NK receptor interactions have little, if any, effects on basal NK cell proliferation.

Homeostatic proliferation of NK cells

We next addressed whether proliferation of mature NK cells, like that of mature T cells (34–36), is regulated by the extent of lymphoid cellularity. Splenic NK cells from mature B6-Ly-5.1 mice of ~8 wk of age were labeled with CFSE and transferred to adult B6 mice that were irradiated or not. Seven days after transfer into unirradiated mice, the vast majority of donor NK cells had not undergone any detectable proliferation, consistent with the findings above that most mature NK cells are not actively dividing (Fig. 6*A*). However, when transferred into irradiated B6 mice, a substantial percentage of the cells had undergone at least one cell division at this time point (Fig. 6*B*). These results suggest that NK cells, like T cells, undergo homeostatic proliferation in irradiated hosts.

To address the role of MHC expression in homeostatic proliferation of NK cells, CFSE-labeled NK cells were transferred in parallel to irradiated $\beta_2m^{-/-}$ recipients. The extent of NK cell proliferation in irradiated $\beta_2m^{-/-}$ hosts was only slightly lower than that in irradiated wild-type mice (Fig. 6*C*). Therefore, homeostatic NK cell proliferation does not depend to a significant extent on host expression of MHC class I molecules, a finding that was consistent with our expectations because most of the MHC-specific receptors are inhibitory in function. Conversely, however, host MHC class I molecules appear to play no discernible role in limiting the extent of donor cell-NK cell proliferation.

Because IL-15 has been implicated in the development and proliferation of NK cells, we addressed whether homeostatic proliferation of NK cells depends on IL-15. This was accomplished by transferring NK cells to irradiated $IL\text{-}15^{-/-}$ mice. Because IL-15 is produced by stromal cells and not lymphoid cells, no IL-15 should be available to transferred NK cells in this experiment. Surprisingly, the percentage of donor NK cells that had undergone cell division (see *Materials and Methods* for calculations) was reduced only modestly in $IL\text{-}15^{-/-}$ hosts, suggesting that commitment to cell division was largely independent of IL-15 (Fig. 6*D*). In contrast, the number of donor NK cells recovered from $IL\text{-}15^{-/-}$ hosts was reduced by >15-fold compared with the number recovered from wild-type hosts (percent recovery of transferred cells is indicated to the *right* of each panel in Fig. 6). These results suggest that under conditions favoring homeostatic proliferation of NK cells, IL-15 is not required for cell division per se, but is essential for survival of NK cells.

Discussion

Our study adds substantially to previous studies of NK cell dynamics. The earlier studies showed that most NK cells in the secondary lymphoid organs of normal nonimmune adult mice are relatively small cells (37) and that spontaneous NK cell cytotoxic activity is mediated by relatively small cells (37) and is resistant to short-term exposure to hydroxyurea, which kills proliferating cells (18, 38). Most of these and other early studies (19, 39) examined spontaneous NK cell activity rather than NK cells themselves. Our results provide important new information by examining the dynamics of phenotypically defined NK cell populations with respect to cell cycle status and BrdU-labeling kinetics. The data confirm that most splenic NK cells in adults are not dividing rapidly. However, we did detect a small cycling splenic NK population in steady state conditions, suggesting either that a small NK subset is rapidly dividing or that many NK cells divide infrequently.

Contrary to our findings, it is widely believed that NK cells in mature mice turn over very rapidly. This notion is based on early kinetic studies, in which it was concluded that splenic NK cells in mature mice turn over rapidly, with a $t_{1/2}$ of ~1 day (18). We obtained strikingly contradictory data, showing that splenic NK cells label with BrdU relatively slowly, with a $t_{1/2}$ of ~17 days (Fig. 2*A*). This rate is similar to that of $NK1.1^+$ T cells in the spleen (data not shown) and that reported for T cells with a memory phenotype (22), although slower than that of conventional splenic T cells. Resident splenic T cells with a memory phenotype

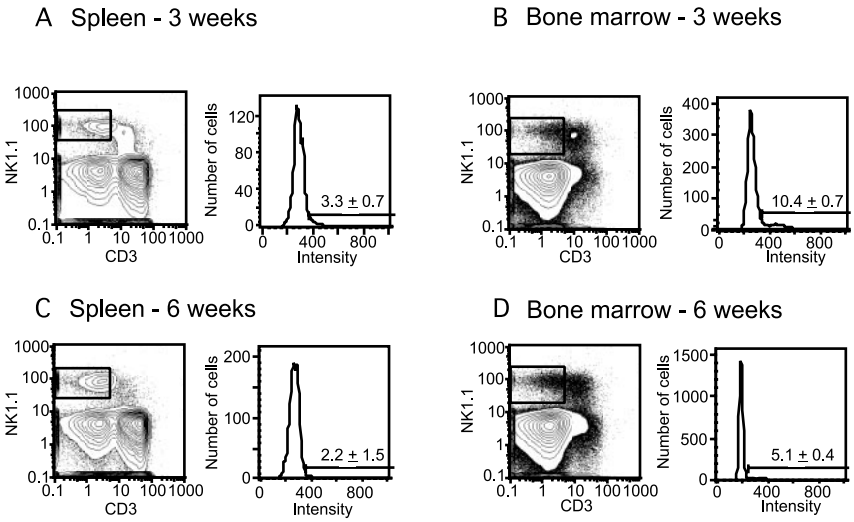

FIGURE 5. Cell cycle analysis of NK cells demonstrates a small percentage of cycling cells. Gated NK1.1$^+$CD3$^-$ cells (as shown) were analyzed. NK cells from spleen (*A* and *C*) or bone marrow (*B* and *D*) of B6 mice were compared. The donor mice were either 3 wk old (*A* and *B*) or 6 wk old (*C* and *D*). The percentages of cells in the S or G_2/M phase of the cell cycle were determined by staining with Sytox Green and measuring the fluorescence of individual nuclei by flow cytometry. The bracketed numbers within the panels correspond to the percentages ± SD of positive cells ($n = 4$).

are similar to NK cells in several other respects, such as cell surface markers and responsiveness to poly(I:C).

The earlier kinetic study examined the disappearance of spontaneous NK cell activity (lysis of YAC-1 cells) at various times after treatment of mice with hydroxyurea (18). Although activity was not reduced immediately after hydroxyurea treatment, it waned very rapidly after a short delay. It was concluded that the short delay represented the time it took for dividing precursor cells to reach the spleen, and the rapid decline thereafter was interpreted to indicate that active NK cells are very short-lived. A problem with this interpretation is that it examined only spontaneous NK cell cytolytic activity. Recent studies have shown that the NK cell population in mature mice is nearly devoid of cytolytic activity until activated by stimuli (priming) such as poly(I:C), viral infection, or target cells that express ligands such as Rae1 that stimulate NK cells (40, 41). NK cells with spontaneous cytolytic activity are therefore likely to represent only a small subset of the total NK1.1$^+$CD3$^-$ population in normal untreated mice. These cells may exhibit a rapid replacement rate because they have been recently stimulated by environmental cues, such as infectious agents. Alternatively, it is possible that such cells are generally more frequent among recently differentiated NK cells. Finally, hydroxyurea may be toxic to NK cells for reasons unrelated to cell division. In any case, our data clearly establish that the bulk of NK cells in mature mice is replaced at a much slower rate than previously thought.

Unlike the results in mature mice, NK cells in young mice label very rapidly with BrdU, similar to the rate of labeling of newly produced mature T cells within the thymus (29). Our study presents several lines of evidence that indicate that the rapid BrdU labeling of NK cells early in life is due to production of new NK cells during this period, and that much of the proliferation does not occur in mature splenic NK cells. First, rapid BrdU incorporation occurred coincident with the rapid accumulation of splenic NK cells during ontogeny, and waned after NK cell numbers reached a steady state after 3 wk of age (Fig. 3). Second, there was a short lag in BrdU labeling of splenic NK cells, suggesting that most of the proliferation was not occurring in splenic NK cells themselves (Fig. 4). Third, direct analysis revealed that only a small fraction of splenic NK cells was in the S or G_2/M phases of the cell cycle, even at 3 wk of age, a time of rapid BrdU incorporation into NK cells, and that the percentage of cycling NK cells in the spleen did not change significantly at later time points (Fig. 5). We propose that splenic NK cells are derived from recently divided precursor cells, most of which cease dividing coincident with or shortly before they either arrive in the spleen or up-regulate the NK1.1 Ag. To account for the difference in labeling of splenic NK cells in young vs older mice, we propose that the rate of export of these precursor populations to the spleen or their differentiation into NK1.1$^+$ cells is much higher in younger mice than in older mice, presumably reflecting a saturation of the spleen with mature NK cells in the older mice. Our findings suggest that cell proliferation during NK cell differentiation may be similar in some respects to T cell differentiation, which is also characterized by extensive cell division in the immature stages, but little or no cell division in the terminal stages (at least until Ag is introduced).

Interestingly, neither BrdU incorporation nor cell cycling by splenic NK cells correlated well with the presence or absence of particular inhibitory receptors specific for the host's MHC molecules, as might have been expected if the inhibitory receptors inhibit basal NK cell proliferation. Consistent with these findings, the rate of BrdU incorporation into NK cells was similar regardless of whether the mice expressed MHC class I molecules normally, or were deficient for class Ia molecules ($K^{b-/-}D^{b-/-}$) or all class I molecules ($\beta_2m^{-/-}$) (data not shown). Therefore, neither MHC class I molecules nor the inhibitory receptors that recognize them play a significant role in regulating NK cell proliferation, at least under steady state conditions. Previously published data demonstrating that the NK cell repertoire is influenced by MHC class I expression (42) are not in conflict with our new findings, because MHC class I expression may influence which receptor genes are expressed in developing NK cells without affecting cell proliferation, as proposed elsewhere (43).

One of our most interesting findings was that splenic NK cells undergo homeostatic proliferation, as shown by the much greater proliferation after transfer to irradiated mice vs unirradiated mice. As has been previously observed for T cells, the lymphopenic environment in the irradiated mice apparently promotes the proliferation of NK cells. The NK cells fail to proliferate when transferred to normal mice with their abundant supply of endogenous lymphocytes, much as has also been reported for T cells. The results suggest that the lymphocyte count in the animal is somehow used as a sensor, such that NK cell proliferation is induced when lymphocyte counts are low. This mechanism may serve to rapidly expand NK cell counts back to normal in animals suffering from lymphoid cell depletion.

Homeostatic proliferation and survival of naive T cells are dependent on host MHC expression (34–36). In the case of NK cells,

Table I. *Proliferation of NK subsets in B6, $\beta_2m^{-/-}$, and $K^bD^{b-/-}$ mice*[a]

Weeks of Treatment	NK Receptors		
	16A11	4D11	5E6
B6 mice			
1	0.8 ± 0.3	1.3 ± 0.1	0.7 ± 0.0
2	0.8 ± 0.4	1.3 ± 0.3	1.0 ± 0.2
3	0.8 ± 0.3	1.2 ± 0.1	1.1 ± 0.2
$\beta_2m^{-/-}$ mice			
1	1.1 ± 0.2	1.3 ± 0.3	0.6 ± 0.1
2	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
3	1.2 ± 0.3	1.2 ± 0.03	0.9 ± 0.2
$K^{b-/-}D^{b-/-}$ mice			
1	0.8 ± 0.3	1.2 ± 0.1	0.6 ± 0.1
2	1.0 ± 0.1	1.6 ± 0.3	0.7 ± 0.1
3	1.0 ± 0.3	1.1 ± 0.2	0.8 ± 0.5

[a] Splenic NK cells were isolated from animals and stained, as described in *Materials and Methods*. Data represent the ratio of the percentage of receptor-positive cells in the $BrdU^+$ subset to the percentage in the $BrdU^-$ subset among $NK1.1^+CD3^-$ cells.

however, we found that neither homeostatic proliferation nor cell survival (judged by cell yield) was much reduced in MHC class I-deficient hosts. In the case of NK cells, this is perhaps not a surprising finding, because most of the MHC-specific receptors expressed by these cells are inhibitory in function. Furthermore, even in the case of T cells, MHC expression is required only for homeostatic proliferation of naive T cells and not for that of memory T cells (44). Most NK cells have a surface phenotype more similar to that of memory T cells than naive T cells. It remains unclear whether homeostatic proliferation of NK cells requires stimulation of some unidentified NK receptor(s) or is induced solely by other types of mediators such as cytokines.

We were surprised to observe that the fraction of NK cells that had undergone cell division after transfer into irradiated IL-$15^{-/-}$ mice was only slightly lower than the fraction of dividing cells observed in irradiated IL-$15^{+/+}$ hosts. Because lymphocytes do not make IL-15, these data suggest that NK cell division that occurs during homeostatic proliferation is not dependent on IL-15. Homeostatic proliferation of naive T cells is IL-7 dependent (45), but we found that IL-7Rs were only barely detectable on NK cells compared with T cells, where they were easily detected (data not shown). The latter finding raises the possibility that NK cell proliferation is independent of IL-7. In contrast to our findings concerning proliferation, the survival of NK cells was dramatically reduced in the irradiated IL-$15^{-/-}$ mice compared with irradiated IL-$15^{+/+}$ hosts, indicating that NK cell survival under these conditions is highly dependent on IL-15. These results are consistent with a recent report demonstrating that survival of nondividing NK cells after transfer to unirradiated mice is also dependent on host-derived IL-15, and suggest that IL-15 is required for survival of both proliferating and quiescent NK cells (17). It is well known that IL-15 is required for the development of NK cells as well, although it has not been clearly established whether the cytokine is primarily responsible for proliferation, differentiation, or survival of differentiating NK cells (11, 12).

In results reported after submission of this study, Prlic et al. (46) elegantly documented homeostatic proliferation of NK cells in a different system using unirradiated hosts that were lymphopenic as the result of mutations in the Rag-2 and γC genes. Their study and an accompanying paper (47) also demonstrated poor survival of NK cells in IL-$15^{-/-}$ hosts.

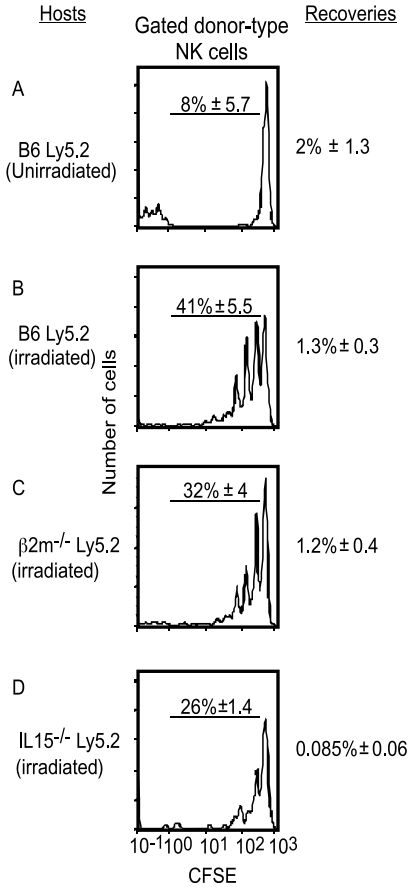

FIGURE 6. Homeostatic proliferation of NK cells in vivo. Splenic NK cells were highly enriched from B6-Ly-5.1 mice by negative selection ($CD3^-CD19^-Ter119^-CD5^-$), labeled with CFSE, and injected into unirradiated or irradiated (with a sublethal dose of 6 Gy, 2–4 h before injection) B6-Ly-5.2 hosts, as indicated. The hosts included wild-type, class I-deficient ($\beta_2m^{-/-}$), and IL-$15^{-/-}$ hosts, as also indicated. Spleens from host mice were harvested 7 days after donor cell injection. Gated donor NK cells ($NK1.1^+CD3^-Ly\text{-}5.2^-$) were analyzed for CFSE fluorescence intensity. The numbers within the panels are the means ± SDs of the percentages of transferred cells that had undergone proliferation in the host (see *Materials and Methods*). At least five individual recipient mice were analyzed. The means (± SD) of the recoveries of donor cells (expressed as 100% × recovered donor cells/injected donor cells) are depicted to the *right* of the panels. The small CFSE-negative population in *A* was not evident in all such samples and may correspond to a small subset of NK cells that proliferates extensively.

Our results help to clarify the dynamics of NK cell production in early and midlife, the rates of cell division and turnover, and the role of MHC molecules and cytokines in NK cell proliferation and survival. Furthermore, we provide the first evidence that NK cells, like T cells, undergo homeostatic proliferation under conditions of lymphocyte depletion. These findings are essential for a comprehensive understanding of NK cell biology in health and disease.

Acknowledgments

We thank Nadine Fernandez for help with i.v. injections, Eric Jensen for advice, Jennifer Hsia for technical assistance, Hector Nolla for expert assistance with flow cytometry and cell sorting, and Peter Schow for help with PE-Ab conjugations. We are grateful for the gift of IL-15-deficient mice from Jacques Pechon, and anti-IL-7Rα Ab from Charles Surh.

References

1. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol. 17:189.*

2. Diefenbach, A., and D. H. Raulet. 2002. The innate immune response to tumors and its role in the induction of T cell immunity. *Immunol. Rev. 188:9*.
3. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359*.
4. Yokoyama, W. M. 1998. Natural killer cell receptors. *Curr. Opin. Immunol. 10:298*.
5. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical MHC class I molecule Qa-1^b. *J. Exp. Med. 188:1841*.
6. Storkus, W. J., D. N. Howell, R. D. Salter, J. R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J. Immunol. 138:1657*.
7. Liao, N., M. Bix, M. Zijlstra, R. Jaenisch, and D. Raulet. 1991. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science 253:199*.
8. DiSanto, J. P., W. Müller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA 92:377*.
9. Kundig, T. M., H. Schorle, M. F. Bachmann, H. Hengartner, R. M. Zinkernagel, and I. Horak. 1993. Immune responses in interleukin-2 deficient mice. *Science 262:1059*.
10. Moore, T. A., U. von Freeden-Jeffry, R. Murray, and A. Zlotnik. 1996. Inhibition of $\gamma\delta$ T cell development and early thymocyte maturation in IL-7$^{-/-}$ mice. *J. Immunol. 157:2366*.
11. Lodolce, J. P., D. L. Boone, S. Chai, R. E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity 9:669*.
12. Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med. 191:771*.
13. Evans, R., J. A. Fuller, G. Christianson, D. M. Krupke, and A. B. Troutt. 1997. IL-15 mediates anti-tumor effects after cyclophosphamide injection of tumor-bearing mice and enhances adoptive immunotherapy: the potential role of NK cell subpopulations. *Cell. Immunol. 179:66*.
14. Munger, W., S. Q. DeJoy, R. Jeyaseelan, Sr., L. W. Torley, K. H. Grabstein, J. Eisenmann, R. Paxton, T. Cox, M. M. Wick, and S. S. Kerwar. 1995. Studies evaluating the antitumor activity and toxicity of interleukin-15, a new T cell growth factor: comparison with interleukin-2. *Cell. Immunol. 165:289*.
15. Fehniger, T. A., K. Suzuki, A. Ponnappan, J. B. VanDeusen, M. A. Cooper, S. M. Florea, A. G. Freud, M. L. Robinson, J. Durbin, and M. A. Caligiuri. 2001. Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8$^+$ T cells. *J. Exp. Med. 193:219*.
16. Carson, W. E., T. A. Fehniger, S. Haldar, K. Eckhert, M. J. Lindemann, C. F. Lai, C. M. Croce, H. Baumann, and M. A. Caligiuri. 1997. A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J. Clin. Invest. 99:937*.
17. Cooper, M. A., J. E. Bush, T. A. Fehniger, J. B. VanDeusen, R. E. Waite, Y. Liu, H. L. Aguila, and M. A. Caligiuri. 2002. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood 100:3633*.
18. Miller, S. B. 1982. Production and renewal of murine natural killer cells in the spleen and bone marrow. *J. Immunol. 129:2282*.
19. Hochman, P. S., G. Cudkowicz, and J. Dausset. 1978. Decline of natural killer cell activity in sublethally irradiated mice. *J. Natl. Cancer Inst. 61:265*.
20. Zijlstra, M., M. Bix, N. E. Simister, J. M. Loring, D. H. Raulet, and R. Jaenisch. 1990. β_2-Microglobulin deficient mice lack CD4$^-$8$^+$ cytolytic T cells. *Nature 344:742*.
21. Perarnau, B., M.-F. Saron, B. R. S. M. Martin, N. Bervas, H. Ong, M. Soloski, A. G. Smith, J. M. Ure, J. E. Gairin, and F. A. Lemonnier. 1999. Single *H2K^b*. *H2D^b* and double *H2K^{b}D^b* knockout mice: peripheral CD8$^+$ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. *Eur. J. Immunol. 29:1243*.
22. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med. 179:1127*.
23. Sentman, C. L., J. J. Hackett, T. A. Moore, M. M. Tutt, M. Bennett, and V. Kumar. 1989. Pan natural killer cell monoclonal antibodies and their relationship to the NK1.1 antigen. *Hybridoma 8:605*.
24. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med. 190:1801*.
25. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med. 150:580*.
26. Mason, L., S. Giardina, T. Hecht, J. Ortaldo, and B. Mathieson. 1988. LGL-1: a nonpolymorphic antigen expressed on a major population of mouse natural killer cells. *J. Immunol. 140:4403*.
27. Gudmundsdottir, H., A. D. Wells, and L. A. Turka. 1999. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J. Immunol. 162:5212*.
28. Egerton, M., R. Scollay, and K. Shortman. 1990. Kinetics of mature T-cell development in the thymus. *Proc. Natl. Acad. Sci. USA 87:2579*.
29. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell 66:533*.
30. Kraft, J. R., R. E. Vance, J. Pohl, A. M. Martin, D. H. Raulet, and P. E. Jensen. 2000. Analysis of Qa-1(b) peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J. Exp. Med. 192:613*.
31. Yu, Y. Y., T. George, J. Dorfman, J. Roland, V. Kumar, and M. Bennett. 1996. The role of Ly49A and 5E6 (Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. *Immunity 4:67*.
32. Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, E. G. Pamer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemonnier, et al. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity 11:67*.
33. Mason, L. H., J. R. Ortaldo, H. A. Young, V. Kumar, M. Bennett, and S. K. Anderson. 1995. Cloning and functional characteristics of murine LGL-1: a member of the Ly-49 gene family (Ly-49G2). *J. Exp. Med. 182:293*.
34. Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity 11:173*.
35. Kieper, W. C., and S. C. Jameson. 1999. Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proc. Natl. Acad. Sci. USA 96:13306*.
36. Goldrath, A. W., and M. J. Bevan. 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8$^+$ T cells in lymphopenic hosts. *Immunity 11:183*.
37. Biron, C. A., and R. M. Welsh. 1982. Blastogenesis of natural killer cells during viral infection in vivo. *J. Immunol. 129:2788*.
38. Rahal, M. D., G. C. Koo, and D. G. Osmond. 1991. Population dynamics of "null" and Thy1lo lymphocytes in mouse bone marrow: genesis of cells with natural killer cell lineage characteristics. *Cell. Immunol. 134:111*.
39. Pollack, S. B., and C. Rosse. 1987. The primary role of murine bone marrow in the production of natural killer cells. *J. Immunol. 139:2149*.
40. Glas, R., L. Franksson, C. Une, M. L. Eloranta, C. Ohlen, A. Orn, and K. Karre. 2000. Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype: an adaptive component of NK cell-mediated responses. *J. Exp. Med. 191:129*.
41. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumor immunity. *Nature 413:165*.
42. Held, W., J. R. Dorfman, M.-F. Wu, and D. H. Raulet. 1996. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor repertoire. *Eur. J. Immunol. 26:2286*.
43. Raulet, D. H., R. E. Vance, and C. W. McMahon. 2001. Regulation of the natural killer cell receptor repertoire. *Annu. Rev. Immunol. 19:291*.
44. Surh, C. D., and J. Sprent. 2002. Regulation of naive and memory T-cell homeostasis. *Microbes Infect. 4:51*.
45. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat. Immun. 1:426*.
46. Prlic, M., B. R. Blazar, M. A. Farrar, and S. C. Jameson. 2003. In vivo survival and homeostatic proliferation of natural killer cells. *J. Exp. Med. 197:967*.
47. Koka, R., P. R. Burkett, M. Chien, S. Chai, F. Chan, J. P. Lodolce, D. L. Boone, and A. Ma. 2003. Interleukin (IL)-15Rα-deficient natural killer cells survive in normal but not IL-15Rα-deficient mice. *J. Exp. Med. 197:977*.