In Vivo Survival and Homeostatic Proliferation of Natural Killer Cells

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Abstract

While the specificity and development of natural killer (NK) cells have been intensely studied, little is known about homeostasis of the mature NK population. Here we show that mouse NK cells undergo homeostatic proliferation when transferred into NK-deficient $Rag^{-/-}$ $\gamma C^{-/-}$ hosts. Normal NK functional activity is maintained during this process, although there are some changes in NK phenotype. Using cell sorting, we demonstrate that mature (Mac-1^{hi}) NK cells undergo homeostatic proliferation in an NK-deficient environment, yet immature (Mac-1^{lo}) NK cells also proliferate in such hosts. We find that mature NK cells survive but do not proliferate in hosts which possess an endogenous NK pool. However, we go on to show that mature NK survival is critically dependent on interleukin (IL)-15. Surprisingly, NK survival is also compromised after transfer of cells into IL-15R$\alpha^{-/-}$ mice, implying that IL-15 responsiveness by bystander cells is critical for NK maintenance. These data imply that, similar to T cells, homeostasis of the NK pool is much more dynamic than previously appreciated and this may be relevant to manipulation of NK cells for therapeutic purposes.

Key words: IL-15 • lymphopenia • cytokines • homeostasis

Introduction

NK cells are thought to play an important role in immune defense against pathogens (1–3) and tumors (4–7). The identity and specificity of both inhibitory and stimulatory receptors for class I MHC molecules on NK cells has been intensely studied, and much is known about the role of these receptors in dictating NK functional activity (8–10). Other studies have focused on the role of soluble factors in regulating NK differentiation and function, and this has lead to identification of IL-15 as a key factor in NK cell ontogeny. Thus, there is NK deficiency in mice lacking IL-15 or IL-15 receptor components (comprising of IL-15Rα, IL-2Rβ, and the common γ chain [γC]*) (11–15).

In contrast to this extensive literature on NK differentiation and effector functions, the factors which influence maintenance of the mature NK pool have received little attention. Mature NK cells are typically thought to be a terminally differentiated population, unable to be maintained by self renewal (1, 2, 16). Indeed, NK cells are typically not in cell cycle, except in response to infection with certain pathogens or stimulation with high doses of appropriate cytokines (such as type I interferons, IL-12 or IL-15; references 1 and 2). However, it is currently unclear whether NK cells have a fixed lifespan as nondividing cells or whether they can respond to homeostatic signals to maintain the size of the NK pool.

Similar questions have been addressed for the T cell pool, and it has become clear that mature T cell homeostasis is far more dynamic than previously suspected (17–20). Survival of naive mature T cells requires exposure to IL-7 and self-peptide/MHC complexes. Furthermore, naive T cells proliferate in a T lymphopenic host (a process termed homeostatic proliferation) which, like naive T cell survival, requires exposure to IL-7 and appropriate self-peptide/MHC complexes. Homeostatic proliferation does not actually replace the naive pool: naive T cells stably acquire a memory-like phenotype during homeostatic expansion and thymic output is required to replenish the naive T cell pool (21, 22). Hence, it has been speculated that T cells initiate homeostatic proliferation in an effort to repopulate lymphopenic individuals with functionally enhanced cells. Indeed, homeostatic proliferation of T cells can markedly enhance their reactivity to foreign antigens and recent work has shown this may be a therapeutic benefit in cancer immunotherapy (23–25).

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*Abbreviation used in this paper: γC, common γ chain.

Less is known about survival and homeostatic proliferation of other lymphocyte subsets. Recent data indicate that Vα14i NK-T cells, like conventional T cells undergo homeostatic proliferation (26). B cell survival requires a functional B cell receptor (27), and exposure to soluble factors (28). Recent reports suggest that B cells also undergo homeostatic proliferation in B cell-deficient hosts (29), although this may be a property of a subset of B cells (30).

Such findings raise the question of how other mature lymphoid subsets are maintained. Here we focus on homeostasis of mature NK cells and report that it is a highly dynamic process. We find that mature NK cells proliferate rapidly in an NK-deficient environment, arguing that NK cells are capable of responding to "space" by homeostatic proliferation. Furthermore, we show that IL-15 is mandatory for the survival of mature NK cells, and that this IL-15 dependence is, at least in part, mediated through responses by other IL-15Rα bearing cells. The implications of these findings for natural and therapeutic regulation of the NK pool are discussed.

Materials and Methods

Mice. Rag-2$^{-/-}$/γC$^{-/-}$ double deficient (Rag$^{-/-}$γC$^{-/-}$), CD45.1^{+} Rag-2$^{-/-}$ mice (CD45 congenic Rag$^{-/-}$), and IL-15$^{-/-}$ mice were obtained from Taconic Farms. All of these mice are on C57BL/6 (B6) or B6/B10 mixed backgrounds. IL-15Rα$^{-/-}$ mice were obtained from The Jackson Laboratory. Additional Rag-2$^{-/-}$ or Rag-1$^{-/-}$ mice were obtained from Taconic Farms or The Jackson Laboratory, respectively. B6 mice and CD45 congenic (CD45.1, also called Ly5.2) B6 mice were obtained from Jackson Laboratories or the National Cancer Institute. All mice were maintained under specific pathogen-free conditions and used at 6–12 wk of age.

Adoptive Transfer and Cell Sorting. Red blood cell–depleted splenocytes were isolated from Rag$^{-/-}$ mice or B6 mice and labeled with CFSE (Molecular Probes) as described previously (31). In some experiments, Rag$^{-/-}$ splenocytes (which were on average 25% NK1.1^{+}) were used without further purification. Splenocytes isolated from B6 mice were enriched for NK cells using SpinSep/Murine NK Cells kit (StemCell Technologies Inc.) before CFSE labeling and adoptive transfer. In this case the percentage of NK1.1^{+} (CD3^{-}) cells was on average 40%. Between 0.7×10^6 and 1.4×10^6 CFSE-labeled splenocytes were injected per recipient mouse, unless otherwise indicated. In all experiments, donor cells were injected intravenously into the tail vein.

To obtain a pure population of mature Mac-1^{hi} NK cells, splenocytes from B6 mice were enriched for NK cells by negative selection using MACS microbeads (Miltenyi Biotec). Splenocytes were labeled with FITC-coupled Abs to B220 (clone RA3–6B2), I-A^{b} (AF6–120.1), CD4 (GK1.5), and CD8 (53–6.7) (all from BD Biosciences). After staining, cells were subject to depletion using anti-FITC MACS microbeads. This protocol enriched the NK population by 5–10 fold. Cells in the flow through were then stained with anti-CD3 FITC (BD Biosciences), anti-Mac1(M1/70) PE, and NK1.1 (PK136) APC (both from eBioscience) and further purified using the FACSVantage™ DIVA system. Alternatively, splenocytes from Rag$^{-/-}$ donor mice were directly stained with anti-CD3, anti-Mac-1, and anti-NK1.1, and sorted on the FACSVantage™ DIVA. In both cases, cells used for adoptive transfer experiments were at least 93.5% pure NK1.1^{+} CD3^{-} and Mac-1^{hi}. Cells were then labeled with CFSE and transferred into recipient mice as described. 0.7×10^5 to 2×10^5 NK1.1^{+} CD3^{-} Mac1^{hi} cells were injected.

Flow Cytometry. Recipient mice were killed at the time points indicated and single cell suspensions were prepared from the spleen. Red blood cell–depleted splenocytes were treated with 2.4G2.1 ("Fc-Block") before further staining. Cells were typically stained with anti-NK1.1 APC, anti-Mac-1 PE (as above) and anti-CD69 biotin (clone H1.2F3; eBioscience), followed by Streptavidin (SA)-PerCP.

For intracellular staining, cells were prepared with the Cytofix/Cytoperm kit in the presence of brefeldin A (BD Biosciences) and stained with anti-IFN-γ PE (XMG1.2; eBioscience), anti-NK1.1 APC and Mac-1 biotin followed by SA-PerCP.

Cells were analyzed using a FACSCalibur™ (BD Biosciences) and analyzed using FLOWJO (TreeStar) software. For the experiment in Fig. 6 b, the rare donor cells were identified and enumerated in two ways. In the first approach, total live events were analyzed for CFSE^{+} donor cells. In the second method, CFSE^{+} events were acquired from a known starting number of total live cells. Results from the latter approach are shown, but both methods yielded similar data.

NK Functional Assays. Poly I:C induction of NK function was performed as described previously (32). Briefly, 150 μg poly I:C were injected intraperitoneally per mouse. Mice were killed 5.5 h after injection and single cell suspensions prepared from the spleen. Cytolytic potential of cells was tested in a ^{51}Cr release assay. Briefly, EL-4 (a syngeneic control target) and YAC-1 (an NK cell target) cells were labeled with ^{51}Cr-sodium chromate for 1 h at 37°C and washed before incubation with the effector cells. NK cells were recovered from Rag$^{-/-}$γC$^{-/-}$ hosts transferred with Rag$^{-/-}$ splenocytes 7 d before harvesting, or from untransferred Rag$^{-/-}$ mice. NK cells were enriched using the SpinSep kit (StemCell Technologies Inc.). In these experiments, this protocol yielded a population which was 40–80% NK1.1^{+}. The number of cells in the effector population was adjusted such that the effector to target (E:T) numbers listed reflect input of NK1.1^{+} cells. Cells were incubated for 4 h at 37°C and target cell lysis calculated. Spontaneous release was less than 12% in the experiment shown. A total of four ^{51}Cr release assays were performed.

Results

NK Cells Undergo Homeostatic Proliferation in NK-deficient Hosts. To study NK cell homeostasis, we used an adoptive transfer approach. NK cells were isolated from RAG$^{-/-}$ mice (which lack T and B cells, but possess NK cells) and were labeled with the dye CFSE before injection into RAG$^{-/-}$ γC$^{-/-}$ mice (which are devoid of all lymphocytes). Proliferation of the donor NK pool was observed, as monitored by dilution of the CFSE dye and increase in percentage and numbers of donor NK cells over time (Fig. 1 a). If this finding were analogous to T cell homeostatic proliferation, then donor NK proliferation would depend on the absence of an endogenous NK pool. To test this, we transferred NK cells into both Rag$^{-/-}$ and Rag$^{-/-}$ γC$^{-/-}$ hosts. Donor cells were distinguished from host NK cells in the Rag$^{-/-}$ hosts using an allelic marker. Donor NK cells proliferated extensively in the Rag$^{-/-}$ γC$^{-/-}$ hosts, as re-

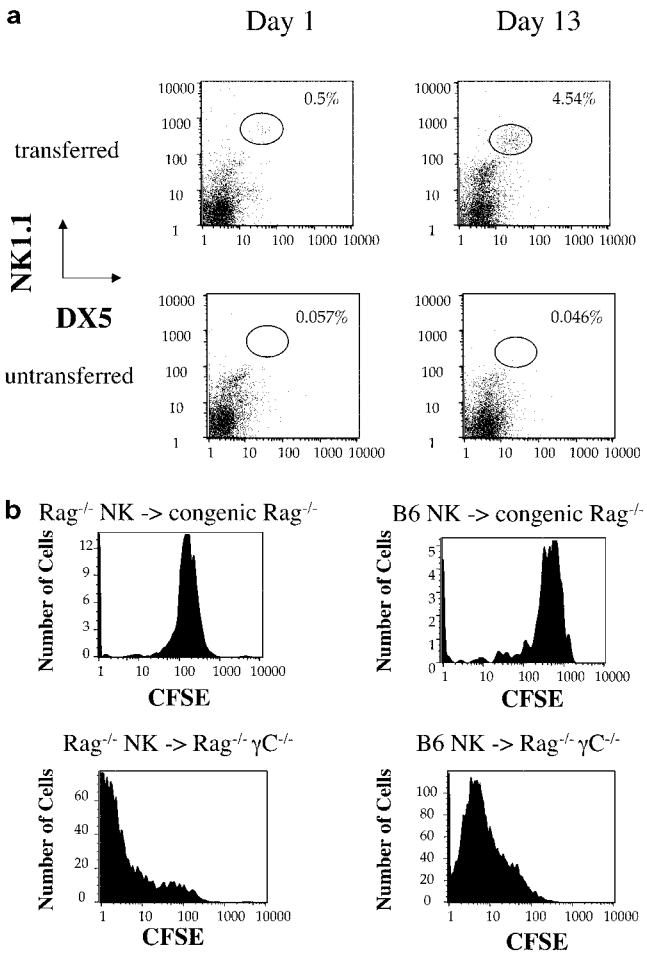


Figure 1. Expansion of NK1.1$^+$ cells in an NK-deficient host. (a) Splenocytes were isolated from Rag$^{-/-}$ mice, CFSE labeled and transferred into Rag$^{-/-}\gamma C^{-/-}$ hosts. Hosts and untransferred controls were killed at days 1 and 13 post transfer and stained for NK1.1 and DX5. (b) Splenocytes were isolated from Rag$^{-/-}$ mice (left hand panels) or enriched from B6 mice (right hand panels). The cells were labeled with CFSE and ~1 × 10^6 cells transferred into congenic (CD45.1$^+$) Rag$^{-/-}$ or Rag$^{-/-}\gamma C^{-/-}$ hosts. After 7 d, the hosts were killed and splenocytes analyzed. The CFSE profile is shown for cells in the Rag$^{-/-}$ hosts (cells gated for CD45.1$^-$NK1.1$^+$ events) in the top panels, and cells in the Rag$^{-/-}\gamma C^{-/-}$ host (gated on NK1.1$^+$ events) in the bottom panels.

vealed by extensive CFSE dye dilution, but there was minimal proliferation of donor NK cells in the Rag$^{-/-}$ hosts (Fig. 1 b). Some CFSE negative cells (~20% on average) were detected in the congenic donor NK gate after transfer into Rag$^{-/-}$ hosts. These events could be caused by host (CFSE negative) NK cell contamination in the gate or this population may represent a small population of rapidly dividing donor NK cells (see below). In either case, the bulk of donor NK cells do not appear to divide in Rag$^{-/-}$ recipients. In these experiments we tested the response of NK cells derived from Rag$^{-/-}$ donors and also NK cells enriched by negative selection from normal B6 donors. As shown in Fig. 1 b, both NK populations behaved similarly, and further experiments were performed using either B6 or Rag$^{-/-}$ animals as a source for NK cells.

In addition to genetic mutation, lymphocyte space can be induced acutely by irradiation. Indeed, we also found that NK cells proliferate in sublethally irradiated B6 or Rag$^{-/-}$ hosts, although to a lesser extent than in Rag$^{-/-}\gamma C^{-/-}$ hosts (unpublished data). This less vigorous proliferative response may relate to the fact that NK cells are more radioresistant than most lymphocytes (33, 34) and thus the extent of NK-lymphopenia induced by sublethal irradiation may be inadequate.

Thus, our data suggest that NK cells undergo extensive proliferation in NK cell–deficient recipients, but are maintained without cell division in an NK-full host. These data suggest that NK cells are capable of undergoing homeostatic expansion in response to NK 'space'.

Mature and Immature NK Cells Homeostatically Proliferate. Although the NK development typically occurs in the bone marrow, some NK precursors are found in the spleen (32), the source of our NK donor population. This raised the possibility that we might be studying NK development rather than mature NK homeostatic proliferation in the adoptive transfer model. Recent work from Kim et al. has elegantly defined the maturation stages of murine NK cells (32): fully mature NK cells (Stage V cells) express high levels of the Mac-1 ($\alpha M\beta 2$) integrin, while their immediate precursors (Stage IV NK cells) are Mac-1^{lo}. This maturation step is accompanied by acquisition of full NK functionality. About 90% of the NK cells in the spleen are Mac-1^{hi} and the remaining 10% are Mac-1^{lo} (32), and this ratio was typically maintained after homeostatic proliferation in Rag$^{-/-}\gamma C^{-/-}$ hosts (Fig. 2 a). The proliferating cells included both Mac-1^{hi} and Mac-1^{lo} subsets, although the cells with the Mac-1^{lo} phenotype (immature NK cells) had undergone more rounds of proliferation, as judged by the extent of CFSE dilution (Fig. 2, b and c).

As Mac-1^{lo} NK cells can give rise to fully mature Mac-1^{hi} NK cells, it was possible that Mac-1^{lo} cells in our donor innoculum were solely responsible for the proliferation observed in Rag$^{-/-}\gamma C^{-/-}$ hosts. To test this possibility, we sorted Mac-1^{hi} NK-1.1$^+$ donor NK cells before transfer into either Rag$^{-/-}$ and Rag$^{-/-}\gamma C^{-/-}$ hosts (Fig. 3 a). This approach revealed that the fully mature Mac-1^{hi} NK subset was capable of homeostatic proliferation, demonstrated by extensive proliferation in the NK-deficient Rag$^{-/-}\gamma C^{-/-}$ hosts but not in the NK-replete Rag$^{-/-}$ hosts (Fig. 3 b). Similar findings were obtained whether the Mac-1^{hi} NK population was prepared from Rag$^{-/-}$ or B6 donor mice (unpublished data). In addition, we performed preliminary experiments involving transfer of sorted Mac-1^{hi} donor NK cells into congenic Rag$^{-/-}$ hosts. Gating on the donor-derived cells in this case demonstrated that at least 95% of the donor NK cells were in a single CFSE^{hi} peak at day 5 after transfer, implying no cell division (unpublished data). This suggests that mature (Mac-1^{hi} /Stage V) NK cells do not proliferate after transfer into the NK "full" Rag$^{-/-}$ host.

Thus, our data with bulk and Mac-1^{hi} purified NK donor populations indicate that both mature and immature

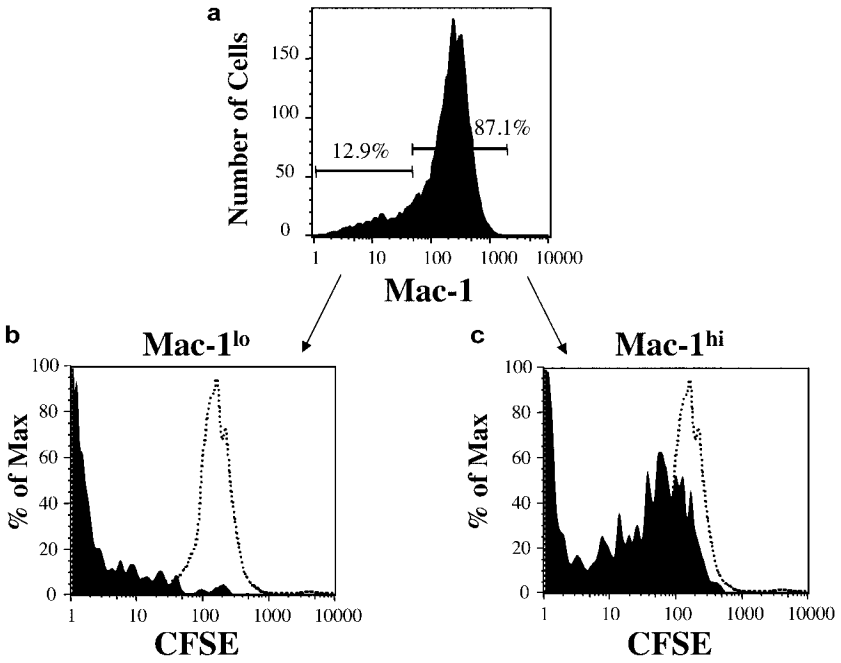


Figure 2. Both Mac-1^{lo} and Mac-1^{hi} NK cells proliferate in NK-deficient hosts. Splenocytes from $Rag^{-/-}$ animals were harvested, CFSE labeled, and adoptively transferred into $Rag^{-/-}\gamma C^{-/-}$ hosts. Host animals were harvested 7 d post transfer and stained for NK1.1 and Mac-1. (a) Mac-1 staining in the $NK1.1^{+}$ population. The CFSE profile for (b) Mac-1^{lo} (immature phenotype) and (c) Mac-1^{hi} (mature phenotype) NK cells is shown. The CFSE level in donor NK cells 7 d after transfer into $Rag^{-/-}$ hosts is also shown (open histogram with dotted line), to indicate the extent of CFSE dilution in the $Rag^{-/-}\gamma C^{-/-}$ hosts.

NK populations are capable of homeostatic proliferation in an NK-deficient host.

NK Cells Maintain Phenotype and Function during Homeostatic Proliferation. Homeostatic proliferation of naive T cells results in dramatic changes in phenotype and activity. In most cases, naive T cells acquire memory markers and functional properties without transition through a typical activated stage. On the other hand, some reports indicate homeostatic proliferation can induce T cell anergy or result in overt T cell activation (for a review, see reference 20). For these reasons it was of interest to test whether and how NK phenotype and function were affected by homeostatic proliferation.

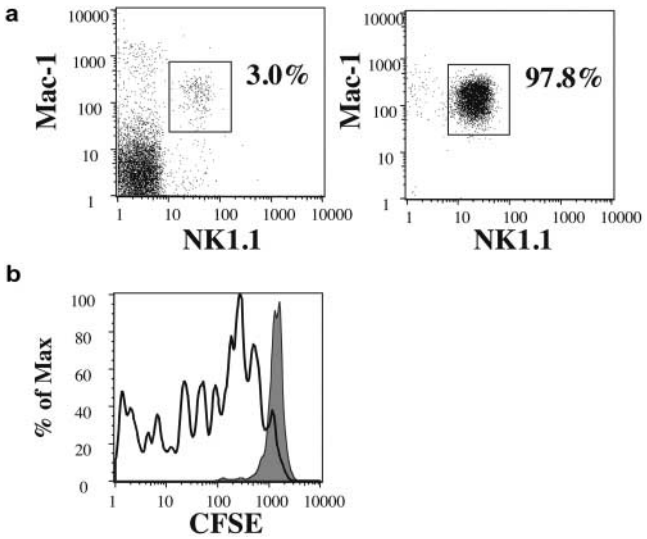


Figure 3. Homeostatic proliferation of mature (Mac-1^{hi}) NK cells. (a) B6 splenocytes were sorted for $NK1.1^{+}$ $CD3^{-}$ Mac-1^{hi} cells. Purity of the population before and after sorting is shown. (b) $NK1.1^{+}$ $CD3^{-}$ Mac-1^{hi} cells were CFSE labeled and transferred into $Rag^{-/-}$ and $Rag^{-/-}\gamma C^{-/-}$ recipients. Hosts were harvested 5 d after transfer. CFSE profile from representative $Rag^{-/-}$ (gray filled histogram) and $Rag^{-/-}\gamma C^{-/-}$ hosts (unfilled histogram) are shown.

We examined cell surface expression of NK1.1, Mac-1, and CD69 on donor NK cells after transfer into $Rag^{-/-}$ versus $Rag^{-/-}\gamma C^{-/-}$ hosts. In addition, we monitored the size of these cells (forward scatter, FSC). For the most part the phenotype of the cells after homeostatic proliferation was similar to that of normal mature NK cells. However, we did observe a consistent rise in FSC (commensurate with the cells becoming blasts) and elevated expression of Mac-1 (Fig. 4, a and b). CD69, an early activation marker, was also elevated on a subset of NK cells undergoing homeostatic proliferation (Fig. 4 c), although this finding was more variable than the changes in Mac-1 expression. Lastly, NK1.1 expression was typically unchanged after NK homeostatic proliferation (Fig. 4 d).

To examine NK cell function, we initially tested whether homeostatic proliferation influenced the response of NK cells to poly I:C, a potent inducer of NK activity (probably via IL-15). Both normal NK cells and NK cells which were allowed to expand in a $Rag^{-/-}\gamma C^{-/-}$ host were induced with poly I:C and assayed for IFN-γ production (Fig. 5 a) and up-regulation of CD69 (Fig. 5 b). In addition, we noted that Mac-1 levels are elevated by poly I:C stimulation on both populations of NK cells (Fig. 5 c). We also tested the impact of homeostatic proliferation on the ability of NK cells to lyse target cells ex vivo. NK cells which had undergone homeostatic proliferation in $Rag^{-/-}\gamma C^{-/-}$ hosts were able to lyse YAC-1 target cells at least as well as NK cells derived from $Rag^{-/-}$ mice (Fig. 5 d). Thus, these functional assays suggest that homeostatic proliferation did not significantly enhance or diminish NK reactivity.

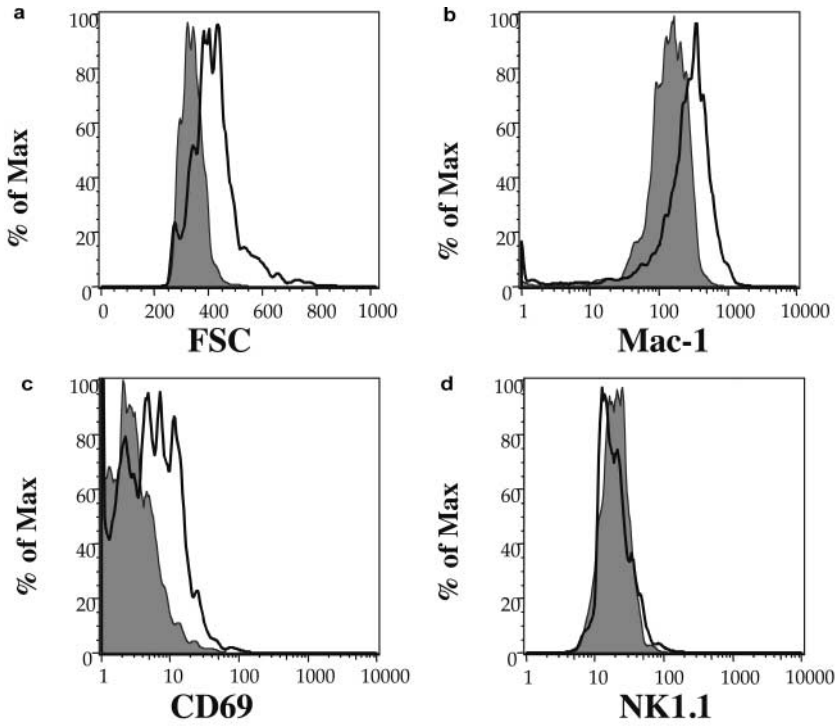
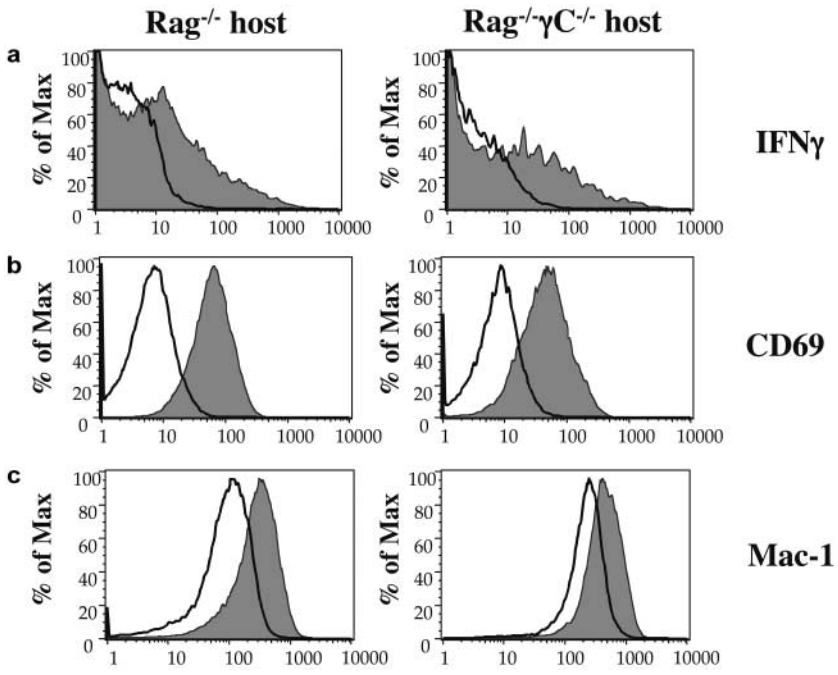


Figure 4. NK phenotype after homeostatic expansion. Splenocytes were harvested from $Rag^{-/-}$ mice, CFSE labeled, and transferred into $Rag^{-/-}$ (gray histograms) and $Rag^{-/-}\gamma C^{-/-}$ (open histograms) hosts. Recipients were killed at day 7 after transfer. Cells were analyzed for (a) size (forward scatter), (b) Mac-1, (c) CD69, and (d) NK1.1.

Overall these findings suggest that homeostatic proliferation had no discernable effect on NK function and a moderate effect on NK phenotype. Importantly, these findings also suggested that the proliferation observed in $Rag^{-/-}\gamma C^{-/-}$ hosts did not arise from activation by endogenous pathogens in these mice: were that to be the case, we might have expected to see strong expression of activation markers on the donor NK population from these hosts (1).

NK Cells Depend on Host IL-15 and IL-15Rα Expression for Survival. As IL-15 plays an important role in NK cell development and activation (35, 36), we were interested in



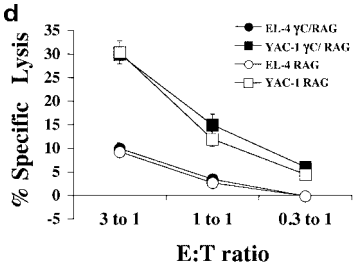


Figure 5. NK cells remain functional after homeostatic expansion Splenocytes were isolated from $Rag^{-/-}$ mice, CFSE labeled and injected into $Rag^{-/-}\gamma C^{-/-}$ hosts. 7 d after transfer, these animals and control $Rag^{-/-}$ animals were challenged with poly (I:C) or left as controls. Spleens of challenged and unchallenged mice were harvested 5.5 h later. Splenocytes were stained for (a) IFN-γ, (b) CD69, and (c) Mac-1. Poly (I:C) treated animals are represented in the gray filled profiles, with overlapping unfilled profiles from untreated controls. Data representative from two independent experiments, with $n = 3$ per condition in each experiment. (d) Splenocytes were harvested from $Rag^{-/-}$ animals (open symbols) and from $Rag^{-/-}\gamma C^{-/-}$ animals which were transferred with $Rag^{-/-}$ NK cells 7 d previously (filled symbols). NK cells were enriched from the pooled cells of each group and these cells were tested in a 4-h ^{51}Cr-release assay against YAC-1 (squares) versus control EL-4 targets (circles). The percentage of $NK1.1^+$ cells in each effector pool was calculated and cell numbers adjusted so that the E:T ratio reflects input of NK cells into the assay.

the requirement for IL-15 in NK homeostasis. We enriched NK cells from normal B6 donors, labeled them with CFSE, and transferred the cells into IL-15$^{-/-}$ versus B6 hosts. These host animals are similar in terms of overall lymphoid cellularity but the IL-15$^{-/-}$ mice are profoundly NK deficient (15). While donor NK cells persisted well in the B6 recipients, we saw a striking loss of NK cells in the IL-15$^{-/-}$ hosts by day 4 after transfer (Fig. 6 a). These findings were unlikely to reflect rejection of the donor cells nor reduced efficiency of transfer into the IL-15$^{-/-}$ host, since donor T cells (NK1.1^{-} cells which were contaminants in the donor input) were present in both hosts (Fig. 6 a). In the same experiment, both donor NK and T cell populations proliferated vigorously in Rag$^{-/-}\gamma$C$^{-/-}$ hosts (Fig. 6 a), demonstrating that these cells were not compromised by the purification procedures. A small population (<0.4% of total splenocytes) of CFSE negative NK1.1^{+} cells was observed in the IL-15$^{-/-}$ hosts: these cells were present at a similar frequency to the residual NK population observed previously in IL-15$^{-/-}$ mice (15), and we concluded that these were host-derived cells.

In preliminary experiments, we assayed the time course for donor NK cell survival after transfer into congenic B6 and IL-15$^{-/-}$ hosts. These data suggested that at 6 h donor NK cells were present in both types of host, but that the NK population had already started to decline at 24 h post adoptive transfer into IL-15$^{-/-}$ hosts (unpublished data). These data are consistent with a recent report from Cooper and colleagues, which also suggested a critical role for IL-15 in mature NK survival (37).

Finally, we studied NK persistence in IL-15R$\alpha^{-/-}$ hosts. One might predict that these hosts would support NK survival at least as well as B6 controls, as IL-15R$\alpha^{-/-}$ mice express IL-15 but lack endogenous NK cells (14, 35, 38). Surprisingly, however, donor NK cells decline precipitously in IL-15R$\alpha^{-/-}$ recipients, behaving similarly to NK cells transferred into IL-15$^{-/-}$ hosts (Fig. 6 b). Again, poor engraftment or rejection of the donor cells is unlikely, as non-NK cells (identified as being NK1.1^{-}) in the donor innoculum were detected at similar numbers in all the host animals. These data suggest that host expression of both IL-15 and IL-15Rα is necessary for donor NK survival. The implications of these findings on NK homeostasis are discussed below

Discussion

This report focuses on homeostasis of the mature NK population. NK cells (in contrast to T and NK-T cells) develop extrathymically, in the absence of CD3 chains and RAG proteins (9). NK cells have several characteristics that partially overlap with those of T cells, including the ability to mediate target specific lysis and produce IFN-γ upon stimulation.

While the requirements for NK development are being determined (9, 32), little is known about how the size and

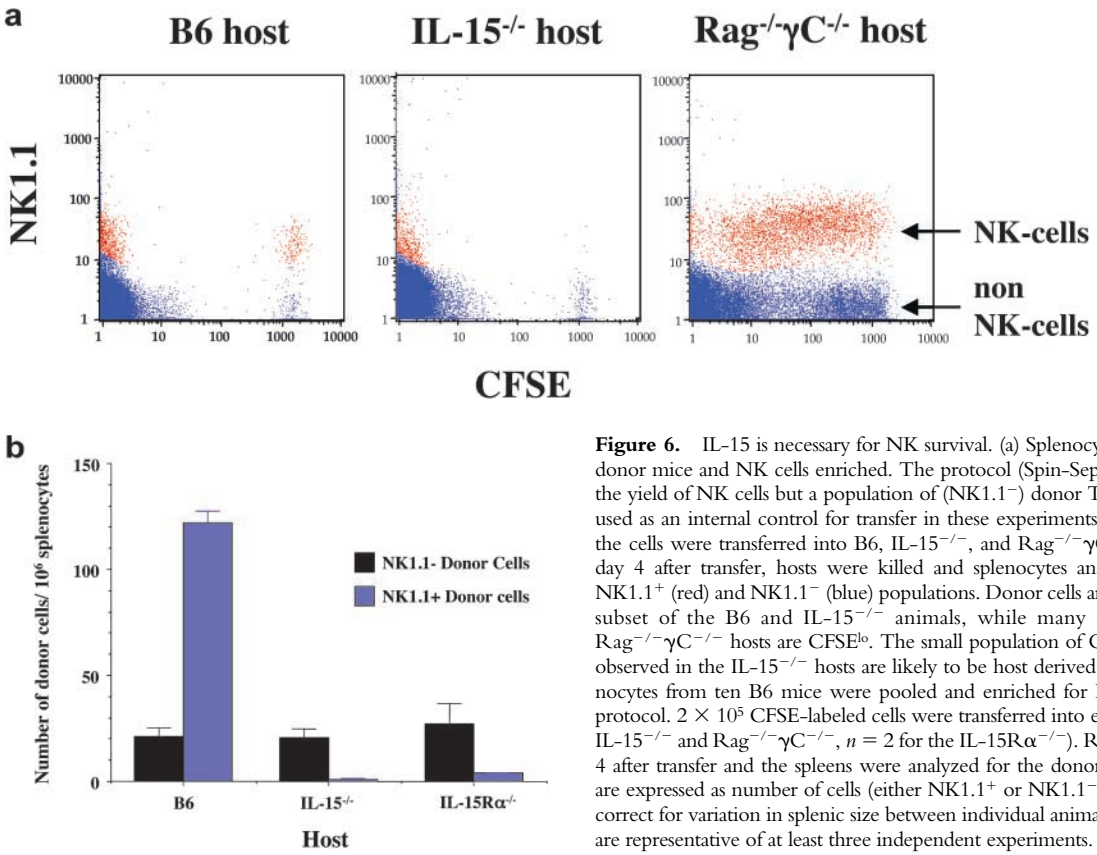


Figure 6. IL-15 is necessary for NK survival. (a) Splenocytes were harvested from B6 donor mice and NK cells enriched. The protocol (Spin-Sep) used significantly increases the yield of NK cells but a population of (NK1.1^{-}) donor T cells remains which can be used as an internal control for transfer in these experiments. After labeling with CFSE, the cells were transferred into B6, IL-15$^{-/-}$, and Rag$^{-/-}\gamma$C$^{-/-}$ hosts, as indicated. At day 4 after transfer, hosts were killed and splenocytes analyzed. The data show the NK1.1^{+} (red) and NK1.1^{-} (blue) populations. Donor cells are represented in the CFSE^{+} subset of the B6 and IL-15$^{-/-}$ animals, while many of the donor cells in the Rag$^{-/-}\gamma$C$^{-/-}$ hosts are CFSE^{lo}. The small population of CFSE negative NK1.1^{+} cells observed in the IL-15$^{-/-}$ hosts are likely to be host derived cells (see Results). (b) Splenocytes from ten B6 mice were pooled and enriched for NK cells using the SpinSep protocol. 2 × 10^{5} CFSE-labeled cells were transferred into each recipient (n = 3 for B6, IL-15$^{-/-}$ and Rag$^{-/-}\gamma$C$^{-/-}$, n = 2 for the IL-15R$\alpha^{-/-}$). Recipients were killed on day 4 after transfer and the spleens were analyzed for the donor (CFSE^{+}) population. Data are expressed as number of cells (either NK1.1^{+} or NK1.1^{-}) per million splenocytes, to correct for variation in splenic size between individual animals in each group. These data are representative of at least three independent experiments.

composition of the mature NK population is maintained. Here we demonstrate that mature NK cells are not end stage cells with a fixed lifespan, but that they respond to endogenous cues which can lead to their proliferation (in hosts lacking endogenous NK cells), survival (in animals with a normal NK pool) or loss (in animals lacking IL-15 or IL-15Rα).

An important element in interpreting our NK cell transfers was to distinguish between homeostasis versus development of NK cells. NK cells develop in the bone marrow but other tissues, including spleen and liver, also contain NK precursors (32). A recent report from Yokoyama's group has delineated the stages for mouse NK cell development, and identified a Mac-1^{hi} population as the final stage of NK cell development (termed Stage V; reference 32). By sorting for Stage V (Mac-1^{hi}) NK cells we show that they are able to survive in NK-replete (Rag$^{-/-}$) hosts and expand in NK-deficient (Rag$^{-/-}\gamma C^{-/-}$) hosts, arguing that this population can enter cell division when exposed to NK 'space'. In addition, however, experiments in which bulk NK cells were transferred indicated that a Mac-1^{lo} NK cell population proliferate extensively in NK-deficient hosts. Based on previous work, these cells are unlikely to arise from Stage V mature NK cells and are more likely to be proliferating Mac-1^{lo} Stage IV NK cells or earlier precursors from the spleen (32). In keeping with this, we observed that the extensively-proliferating, Mac-1^{lo} subset showed reduced IFN-γ production (relative to the Mac-1^{hi} subset) after poly-I:C induction (unpublished data), a hallmark of Stage IV NK cells (32). When transferring bulk NK cells into Rag$^{-/-}$ hosts, we observed a small population of CFSE negative cells, which might represent a small but rapidly dividing population of donor cells (Fig. 1b). As we did not observe a similar population after transfer of Mac-1^{hi} NK cells into Rag$^{-/-}$ hosts, it seems likely that such proliferating cells would be derived from Mac-1^{lo} Stage IV precursors, although we did not directly address that hypothesis here.

These findings suggest that mature NK cells, like T cells, are able to undergo homeostatic proliferation in appropriately lymphopenic hosts. In the case of T cells, homeostatic proliferation is accompanied by conversion of naive T cells to "memory-like" cells which show the functional and phenotypic features of bona fide memory T cells. In our studies we observe moderate phenotypic changes on NK cells after homeostatic proliferation, including up-regulation of Mac-1 and slightly elevated expression of CD69, but our data also indicate that NK function is neither compromised nor enhanced by homeostatic proliferation (Fig. 5). This diverges from the T cell literature where homeostatic proliferation leads to memory-like functions not observed in the naive pool (e.g., cytolytic activity by CD8 T cells; references 39 and 40). On the other hand, NK cells are unusual in that they are constitutively primed for reactivity, as measured by ex vivo cytolytic activity and rapid IFN-γ production upon induction. In this regard, NK cells perhaps most closely resemble preformed memory CD8 T cells, a similarity strengthened by their shared reliance on IL-15 (41).

T cell homeostatic proliferation has been shown to be influenced by the TCR, and there is considerable data suggesting that homeostatic proliferation can bias the TCR repertoire (42). Indeed, it has been suggested that homeostatic proliferation is a key element in generation of autoaggressive T cells in some models of autoimmunity (43). NK cells also express a variety of MHC class I–specific receptors, some of which inhibit and others activate NK responses. While we have not yet comprehensively tested changes in NK receptor repertoire, preliminary data suggest that there are not wholesale alterations in the expression of several Ly-49 receptors (unpublished data). However this is clearly an important issue to pursue.

It is interesting to compare these data on NK cells to the well established requirements for T cell homeostasis (20). Our data suggest that, like T cells, homeostasis of mature NK cells depends on cytokines. In the case of T cells, several cytokines are able to support naive T cell survival and homeostatic proliferation in vitro, but compelling data argue that IL-7 is the obligatory factor in vivo (44–46). Likewise, we show here that IL-15 is critical for mature NK cell survival (Fig. 6). Interestingly, both cytokines also play a role in development of the respective lymphoid subsets, IL-7 being required for development of immature T cells (47, 48) and IL-15 being essential for NK cell development (14, 15).

In addition to supporting NK development, IL-15 can induce mature NK proliferation and activation (1, 49). Previous reports suggested IL-15 could support human NK survival in vitro (50), but our data extend these findings to suggest that IL-15 is an obligatory survival cytokine for murine NK cells in vivo. These findings confirm and extend a recent report from Caligiuri's group (37). It has been shown that IL-15 can act directly on mature NK cells, and so one might expect that IL-15 also acts directly on mature NK cells to mediate their survival. However, our data (Fig. 6 b) and those of Ma and colleagues (50a), show that NK survival is also compromised in IL-15Rα–deficient hosts. This is analogous to a previous report showing that IL-15 driven proliferation of normal memory CD8 T cells is compromised in IL-15Rα$^{-/-}$ hosts (38). These data argue that the capacity of IL-15 to mediate survival of both NK and memory CD8 T cells is critically dependent on IL-15Rα expression by a bystander cell. Interestingly, our data suggest that this (unknown) cell utilizes an unusual form of the IL-15 receptor. The standard lymphoid IL-15 receptor comprises of the IL-15Rα, IL-2Rβ and γC chains. Yet in our studies NK cells transferred into Rag$^{-/-}\gamma C^{-/-}$ hosts survive (and proliferate) while NK cells transferred into IL-15Rα$^{-/-}$ hosts expire (Fig. 6 b; reference 50a). An implication from these data is that the IL-15 receptor responsible for NK maintenance contains the IL-15Rα chain but not γC. This might occur if the function of the host cells is to bind IL-15 (via the high affinity IL-15Rα chain) without a need for cell signaling (via the γC chain). Interestingly, a very recent report from Dubois et al. support just such a scheme, with clear demonstration that one cell, expressing only the α-chain of the IL-15R can efficiently "present"

IL-15 to a second cell expressing only the β- and γ-chains, inducing the latter's proliferation (51). Alternatively, the discrepancy between support of NK homeostasis in the $Rag^{-/-}\gamma C^{-/-}$ hosts but not the IL-15R$\alpha^{-/-}$ hosts could concern other cytokines which are impacted by the γC deficiency. In this regard, it is interesting to note that IL-21 has been reported to support NK activation but not NK viability (52). In IL-15R$\alpha^{-/-}$ animals, IL-21 but not IL-15 is able to act through its receptor, while in $Rag^{-/-}\gamma C^{-/-}$ animals the response to both cytokines is blocked (53). In any case, a bystander cell seems to be involved in regulating NK survival. Clearly, further analysis will be needed to define the mechanism by which IL-15 and its receptor chains are involved in maintenance of the NK cell pool.

A potential caveat in interpreting these experiments is that $Rag^{-/-}\gamma C^{-/-}$ and IL-15R$\alpha^{-/-}$ hosts differ widely in lymphoid cellularity, raising the possibility that NK cells simply fail to find a niche in IL-$15^{-/-}$ and IL-15R$\alpha^{-/-}$ hosts. This seems unlikely when we consider that transferred NK cells are maintained in B6 hosts, which are similar to IL-$15^{-/-}$ and IL-15R$\alpha^{-/-}$ hosts in terms of overall cellularity and which also contain an endogenous NK population (Fig. 6). Furthermore, preliminary data indicated that NK cells proliferated (albeit moderately) in Flt-$3L^{-/-}$ host mice (unpublished data). These mice have a partial defect in NK development but are mostly normal for other splenic lymphoid subsets (54). Thus, NK homeostatic proliferation depends less on the size of the overall lymphoid compartment than on the size of the NK pool, analogous to the criteria for T cell homeostatic proliferation (20).

Although the physiological relevance of homeostatic proliferation in T cells is unclear, this process appears to occur in clinical settings of lymphopenia (for example as a result of radio-/chemotherapy or after recovery from pathogen-induced T cell deficiencies; references 55–58). Indeed, recent data suggest T cell homeostatic proliferation can be used to boost tumor immunotherapy (23–25). Likewise, our data suggest that NK homeostatic proliferation can be used to reconstitute a defective NK pool. There is growing evidence that NK cells can be a critical line of defense against pathogens (2, 3) and cancers (4–7), but that NK cells may also compromise allogeneic transplants (59). For these reasons, regulation of NK homeostasis has therapeutic potential, and our data suggest strategies of how it may be manipulated.

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