Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection

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Cells of the immune system undergo homeostatic proliferation during times of lymphopenia induced by certain viral infections or caused by chemotherapy and radiation treatment. Natural killer (NK) cells are no exception and can rapidly expand in number when placed into an environment devoid of these cells. We explored the lifespan and function of mouse NK cells that have undergone homeostatic proliferation in various settings of immunodeficiency. Adoptive transfer of mature NK cells into lymphopenic mice resulted in the generation of a long-lived population of NK cells. These homeostasis-driven NK cells reside in both lymphoid and nonlymphoid organs for >6 mo and, similar to memory T cells, self-renew and slowly turn over at steady state. Furthermore, homeostatically expanded NK cells retained their functionality many months after initial transfer and responded robustly to viral infection. These findings highlight the ability of mature NK cells to self-renew and possibly persist in the host for months or years and might be of clinical importance during NK cell adoptive immunotherapy for the treatment of certain cancers.

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Abbreviation used: MCMV, mouse CMV.

Immunocompetency is defined as having a functional pool of immune cells ready to respond against foreign invaders. Homeostatic, or space-driven, proliferation of lymphocytes is an important mechanism that the immune system has evolved to maintain a sufficient number of mature hematopoietic cells to mount an effective response against pathogens (Jameson, 2002; Marrack and Kappler, 2004; Ma et al., 2006; Surh and Sprent, 2008). During various viral infections, lymphopenia (i.e., the loss in number of lymphocytes below normal levels) can occur. Although HIV infection results in an extreme example of devastating T cell lymphopenia over time (Margolick and Donnenberg, 1997; Hellerstein et al., 1999), other viral infections, such as measles, which do not directly infect T cells, cause a transient lymphopenia in the host (Okada et al., 2000; Tumpey et al., 2000). In the clinical setting, lymphopenia can occur as a consequence of radiation treatment and chemotherapy, and restoration of a functional lymphoid compartment is a crucial medical objective (Bell and Sparshott, 1997; Mackall et al., 1997; Greenberg and Riddell, 1999).

Homeostatic proliferation of T cells has been thoroughly characterized, and the signals that CD4^{+} and CD8^{+} T cells require for homeostasis have been well documented. Homeostatic expansion of T cells from TCR transgenic mice transplanted into a lymphopenic recipient results in an activated effector/memory phenotype (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). A consequence of naive T cell homeostatic expansion is the generation of a pool of long-lived memory phenotype T cells dependent on IL-7 and IL-15 (members of the γ_C family of cytokines) for their survival (Ku et al., 2000; Schluns et al., 2000; Becker et al., 2002; Goldrath et al., 2002; Kieper et al., 2002; Tan et al., 2002). In contrast, there is little known about the requirements and consequences of homeostatic proliferation in the NK cell compartment (Prlic et al., 2003; Ranson et al., 2003; Jamieson et al., 2004).

Although NK cells were previously thought to have a relatively short lifespan, we have recently documented that after mouse CMV (MCMV) infection, long-lived NK cells are generated and reside for months in lymphoid tissues, as well as nonlymphoid sites, and can rapidly mount protective secondary responses when virus is reencountered (Sun et al., 2009a). Other groups have shown that NK cells contain intrinsic adaptive immune features when primed using chemical haptens and inflammatory cytokines (O'Leary et al., 2006; Cooper et al., 2009). We sought to determine whether homeostatic proliferation will drive expansion of NK cells to become long lived and able to respond months later to pathogen challenge.

RESULTS
### Long–lived NK cells are generated during homeostatic proliferation
NK cells undergo homeostatic proliferation when adoptively transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ mice or when mice are exposed to sublethal irradiation (Prlic et al., 2003; Jamieson et al., 2004), and in one study the transferred NK cells were recovered several weeks later (Ranson et al., 2003). We injected wild-type C57BL/6 NK cells (CD45.1$^+$) into $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice, and the percentage of NK cells was highest at day 5 during homeostatic expansion, followed by apparent contraction of these cells observed at later time points (days 12 and 20; Fig. 1 A). The transferred NK cells could be easily recovered 30 and 60 d later in peripheral blood (Fig. 1, A and B) and spleen (Fig. 1 C). Similar to NK cell responses measured during viral infection (Dokun et al., 2001; Robbins et al., 2004; Sun et al., 2009a), NK cell proliferation driven by lymphopenia undergoes several distinct phases: expansion, contraction, and stable cell maintenance (Fig. 1, B and C). Interestingly, NK cells were the only

lymphocyte type to undergo these distinct phases, as T and B cell subsets filled the splenic compartment without undergoing a contraction phase (Fig. S1).

Likewise, when small numbers of NK cells (CD45.1$^+$) were transferred into sublethally irradiated C57BL/6 mice (CD45.2$^+$), the donor NK cells rapidly proliferated during the first week and were readily recovered in the recipient several months later (Fig. S2). The donor cells in these experiments were sorted to high purity (>99% NK1.1$^+$ CD3$^-$ splenocytes) to ensure that hematopoietic stem cells residing in the donor splenocyte population were not being cotransferred with the donor NK cells. Furthermore, a separate group of sublethally irradiated mice were treated with a NK cell–depleting mAb (PK136) at the time of the adoptive transfer of purified CD45.1$^+$ NK cells. In this setting, both mature donor and recipient NK cells would be deleted, and if no stem cells were transferred, only host NK cells (CD45.2$^+$) would repopulate the periphery. At day 60 after adoptive transfer, no donor CD45.1$^+$ NK cells could be detected in mice treated with NK cell–depleting mAbs, and all NK1.1$^+$ cells were of host origin (Fig. S2 A), demonstrating that no stem cells were transferred. In contrast to the mAb-depleted mice, when control mice were treated with PBS, the donor CD45.1$^+$ mature NK cells underwent proliferation and contraction and became long-lived cells (Fig. S2 B). Thus, like naive T cells, mature naive NK cells transplanted into a lymphopenic environment undergo homeostatic proliferation and persist for months in the recipient.

Kinetics and activation of NK cells during homeostatic proliferation
To evaluate the kinetics of NK cell homeostatic expansion, whole C57BL/6 (CD45.1$^+$) splenocytes were labeled with CFSE and transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice. Because
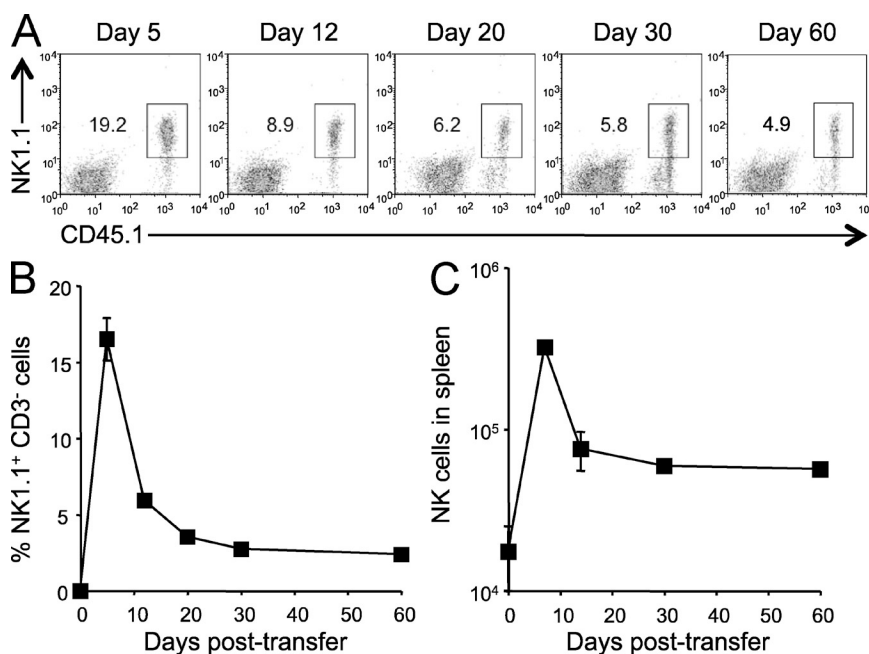

Figure 1. Homeostatic expansion of NK cells produces long–lived cells. (A) 10^6 splenic NK cells (CD3$^-$ NK1.1$^+$) from wild-type CD45.1$^+$ C57BL/6 mice were enriched and adoptively transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ CD45.2$^+$ C57BL/6 mice. Peripheral blood mononuclear cells were analyzed at the indicated days after transfer. Flow cytometric plots show percentages of transferred CD45.1$^+$ NK1.1$^+$ cells in whole blood and are representative of four mice per time point. (B) Graph shows the percentages of transferred NK cells in peripheral blood during and after homeostatic proliferation. (C) Graph shows the absolute number of transferred NK cells in the spleen at various time points after adoptive transfer into $Rag2^{-/-} \times Il2rg^{-/-}$ mice. Error bars on the graphs display SEM (n = 3–4 at each time point). All data presented are representative of three independent experiments.
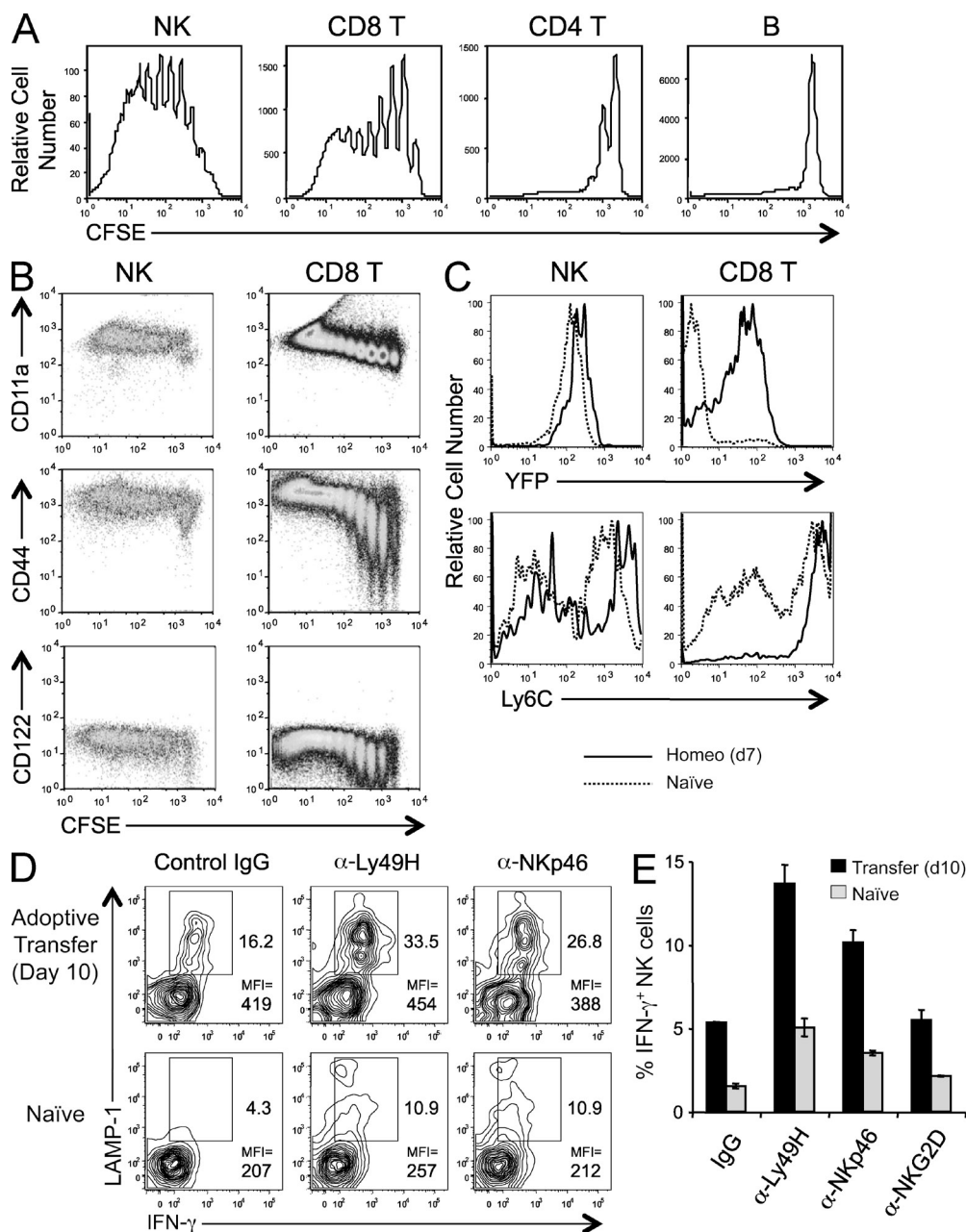
Figure 2. Kinetics and phenotype of NK cells during homeostatic proliferation. (A) 2×10^7 splenocytes from wild-type CD45.1+ C57BL/6 mice were labeled with CFSE and adoptively transferred into *Rag2$^{-/-}$* × *Il2rg$^{-/-}$* CD45.2+ C57BL/6 mice. At 7 d after transfer, various lymphocyte populations in the spleen were analyzed. (B) Flow cytometric plots are gated on transferred NK cells or CD8+ T cells and show expression of CD11a, CD44, and CD122 during CFSE dilution. (C) Splenocytes from *Yeti* C57BL/6 mice (YFP driven by the IFN-γ promoter) were transferred into *Rag2$^{-/-}$* × *Il2rg$^{-/-}$* C57BL/6 mice, and 7 d later YFP and Ly6C expression (solid lines) were analyzed on NK cells and CD8+ T cells. Naive NK cells and CD8+ T cells directly isolated from *Yeti* mice without undergoing homeostatic proliferation (dotted lines) are included as controls. (D) NK cells at 10 d after transfer or from naive C57BL/6 mice were incubated with antibodies against Ly49H and NKp46 (or a control IgG) and measured for expression of LAMP-1 and intracellular IFN-γ. Plots are gated on transferred CD45.1+ NK1.1+ cells. Mean fluorescent intensity (MFI) of intracellular IFN-γ in LAMP-1+ NK cells is indicated for each sample. (E) Graph shows the percentages of transferred NK cells producing IFN-γ after stimulation with plate-bound antibodies against Ly49H, NKp46, NKG2D, or a control IgG. NK cells from naive C57BL/6 mice were similarly stimulated (as a control). Error bars on the graph display SEM (*n* = 3–4). All data presented are representative of three independent experiments.

Rag2$^{-/-}$ × *Il2rg$^{-/-}$* mice lack B and T cells in addition to NK cells, we could compare rates of homeostatic proliferation between different lymphocyte subsets. NK cells and CD8+ T cells rapidly divided more than seven to eight times

over the course of 1 wk, in contrast to CD4+ T cells and B cells which divided only one to two times (Fig. 2 A). The kinetics of expansion of NK cells measured in the liver of *Rag2$^{-/-}$* × *Il2rg$^{-/-}$* mice was similar to the spleen (Fig. S3).
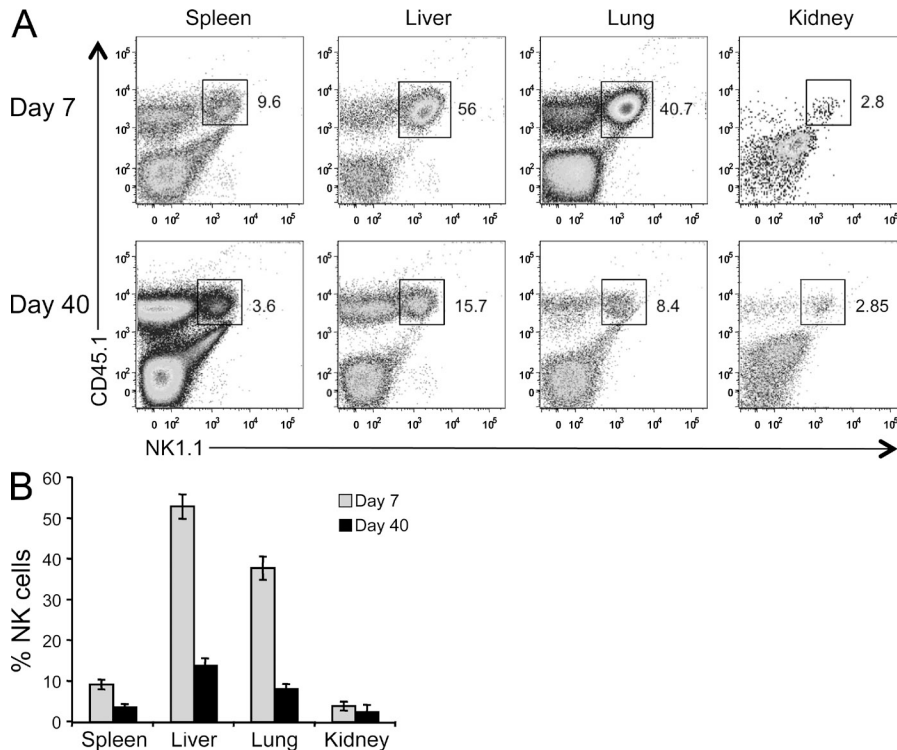
Figure 3. Long–lived NK cells reside in lymphoid and nonlymphoid tissues after homeostatic proliferation. CD45.1+ spleen cells were adoptively transferred into *Rag2*−/− × *Il2rg*−/− CD45.2+ C57BL/6 mice, and different tissues were harvested 7 and 40 d later. (A) Percentages of donor NK cells (CD45.1+ NK1.1+) within the total cell population are shown for spleen, liver, lung, and kidneys. Flow cytometric plots shown are representative of three to four mice at each time point. (B) Graph shows the percentages of transferred NK cells in different tissues at days 7 and 40. Error bars on the graph display SEM (*n* = 3–4). All data presented are representative of three independent experiments.

When examining activation markers that have previously been described to be up-regulated on CD8+ T cells during homeostatic proliferation (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000), we found NK cells to similarly up-regulate CD11a (LFA-1), CD44, and CD122 (IL-2Rβ chain) with an increased number of divisions (Fig. 2 B). Furthermore, 7 d after transfer of NK cells and CD8+ T cells from *Yeti* mice (expressing YFP under the IFN-γ promoter; Stetson et al., 2003) into *Rag2*−/− × *Il2rg*−/− C57/BL6 mice, both NK cells and CD8+ T cells increased YFP expression (Fig. 2 C). The detection of amplified IFN-γ transcripts in *Yeti* CD8+ T cells is consistent with prior studies demonstrating the increased ability of CD8+ T cells to secrete IFN-γ in response to peptide stimulation with each successive cell division during homeostatic proliferation (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). Because NK cells from *Yeti* mice have been described to maintain basal expression of IFN-γ transcripts and YFP (Stetson et al., 2003), the increase in YFP expression in NK cells during homeostatic proliferation is not as pronounced as in CD8+ T cells; however, overall levels of YFP were greater in NK cells undergoing homeostatic proliferation (Fig. 2 C). Interestingly, whereas all CD8+ T cells strongly up-regulated Ly6C during 7 d of homeostatic proliferation, NK cells showed only slightly increased expression of Ly6C and maintained both low- and high-expressing populations (Fig. 2 C). Together, these findings demonstrate that both NK cells and CD8+ T cells become activated during rapid cell division in a lymphopenic environment.

Using TCR transgenic models, CD8+ T cells undergoing homeostatic proliferation become activated and are able respond to TCR ligation by rapidly producing IFN-γ (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). We tested if NK cells that have undergone homeostatic proliferation also acquired the ability to mount rapid effector function, in addition to up-regulating activation markers. When stimulated using plate-bound antibodies against activating NK cell receptors, a greater percentage of NK cells undergoing homeostatic expansion expressed IFN-γ and LAMP-1 compared with resting NK cells from naive C57BL/6 mice (Fig. 2 D). These findings are consistent with the increased amount of IFN-γ transcripts in NK cells undergoing homeostatic proliferation (Fig. 2 C). 10 d after residing in lymphopenic hosts, the transferred NK cells were specifically activated via the Ly49H, NKp46, or NKG2D receptors, and this resulted in a two- to threefold increase in the percentage of cells making IFN-γ (measured intracellularly) compared with NK cells from naive mice (Fig. 2 E). In addition, homeostatically dividing NK cells produced nearly twice as much IFN-γ on a per-cell basis than naive NK cells (as measured by mean fluorescent intensity; Fig. 2 D). Interestingly, a percentage of homeostatically proliferating NK cells expressed LAMP-1 and produced IFN-γ even without receptor triggering (when incubated with control IgG; Fig. 2, D–E). However, the difference between the basal activity and receptor-induced stimulation of the transferred NK cells was still greater than in naive NK cells (Fig. 2, D–E). This high background of basal cytokine production and degranulation in homeostatically expanding NK cells (compared with naive resting NK cells) suggests that proliferative forces alone can activate NK cells to secrete effector cytokines.

## Long-lived NK cells persist in peripheral organs after homeostatic expansion

Long-lived memory CD4+ and CD8+ T cells that have encountered cognate peptide/MHC via their T cell receptors

(in the context of bacterial or viral infection) traffic to lymphoid and nonlymphoid tissues in the body and reside in those peripheral sites for months (Masopust et al., 2001; Reinhardt et al., 2001; Lefrançois and Masopust, 2002). Similarly, memory NK cells generated after MCMV infection reside in lymphoid as well as peripheral organs (Sun et al., 2010). We examined whether NK cells that have undergone homeostatic expansion traffic to different tissues and reside as long-lived cells. We transferred whole splenocytes (CD45.1$^+$) into $Rag2^{-/-} \times Il2rg^{-/-}$ (CD45.2$^+$) C57BL/6 mice and measured the proliferation of transferred NK cells in the spleen, liver, lung, and kidneys of recipient mice 7 d later. NK cell percentages were strikingly high in the liver and lung (56 and 40% of total cells recovered, respectively), compared with the spleen and kidneys (Fig. 3 A). At 40 d after adoptive transfer, homeostatically expanded NK cells were recovered in all peripheral tissues (Fig. 3, A and B). Not surprisingly, the highest percentages of the transferred NK cells were still found in the liver and lung, although the percentages were much lower than at day 7 after transfer. Whether the long-lived NK cells in these peripheral tissues represent cells that have preferentially homed to the organ or are merely trapped after initial entry remains to be determined. The apparent contraction of cell numbers between day 7 and day 40 in liver and lung were comparable to the contraction in the spleen, suggesting similar regulatory mechanisms of NK cell maintenance in the those tissues. Thus, like antigen-driven memory T and NK cells that can reside in peripheral tissues, homeostasis-driven NK cells are maintained in lymphoid and nonlymphoid organs for long durations of time.

Long-lived NK cells are self-renewing after homeostatic proliferation

CD8$^+$ T cells retain activation markers months after adoptive transfer into RAG-deficient mice and basally turn over as long-lived cells (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). Although NK cells are initially activated after homeostatic proliferation, they do not maintain high expression of all activation markers once they have become long-lived cells (Fig. S4), in sharp contrast to memory NK cells generated after viral infection which retain high expression of markers such as KLRG1, Ly6C, and CD43, demonstrating a mature phenotype (Sun et al., 2009a). We addressed whether the more quiescent state of homeostasis-driven NK cells influenced their ability to turn over at steady state (i.e., in the absence of infection or inflammation). We performed a BrdU pulse for various durations at different time points after transfer and compared long-lived NK cells generated by homeostatic proliferation to resting NK cells adoptively transferred into immunocompetent wild-type mice. CD45-congenic NK cells transferred to normal unirradiated C57BL/6 mice are better controls than endogenous NK cell populations in naive mice because endogenous NK cell progenitors from the bone marrow can also incorporate BrdU and would contribute to the overall
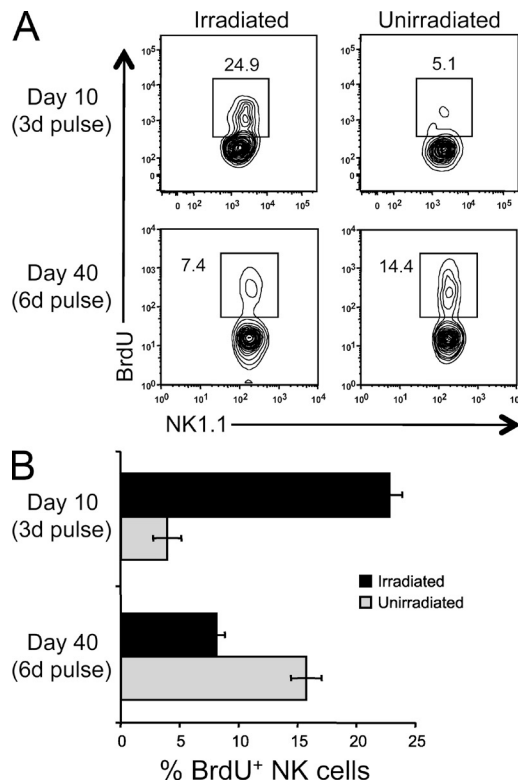
Figure 4. Long-lived NK cells generated during homeostatic proliferation slowly turn over in steady state. 5×10^5 sorted NK cells were adoptively transferred into sublethally irradiated mice and BrdU incorporation was assessed at days 8–10 (3-d pulse) and days 35–40 (6-d pulse). NK cells were also separately transferred into unirradiated wild-type mice and treated with BrdU for 3 or 6 d in parallel with the experimental group. (A) Flow cytometric plots show percentages of NK1.1$^+$ cells that have taken up BrdU and are representative of three to four mice per time point. (B) Graph shows the percentages of transferred NK cells in irradiated or unirradiated mice that were BrdU$^+$ at days 10 and 40. Error bars on the graph display SEM (n = 3–4). All data presented are representative of three independent experiments.

BrdU$^+$ percentage during the pulse phase. Early after transfer into lymphopenic mice, when NK cells are undergoing extensive proliferation, homeostasis-driven NK cells incorporated far more BrdU than NK cells transferred into a lymphoreplete mouse (Fig. 4 A). Not surprisingly, fivefold more NK cells in a lymphopenic environment (24.9%) incorporated BrdU than in a lymphoreplete environment (5.1%) during a 3-d pulse (Fig. 4, A and B). However, at day 40 after transfer, when NK cells have undergone homeostatic proliferation, contraction, and are residing in a filled compartment, these cells incorporated half as much BrdU in a 6-d pulse than NK cells newly transferred to unirradiated mice (Fig. 4, A and B). We have previously observed that memory NK cells generated during viral infection turn over slower than naive NK cells (Sun et al., 2010). Whether the reduced ability to turn over (as measured by BrdU) results in increased survival of these long-lived NK cells compared with naive NK cells remains to be examined.
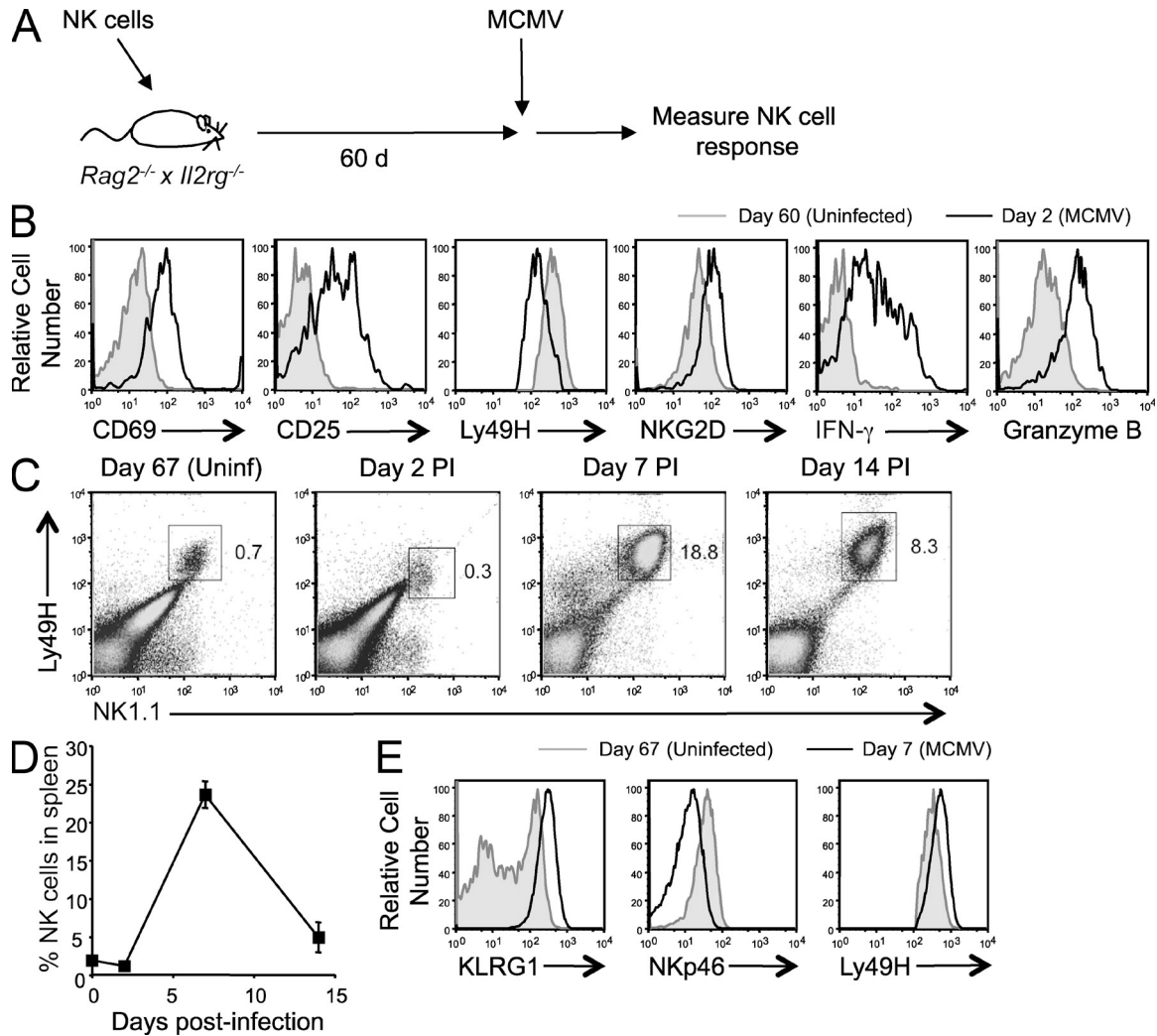
Figure 5. Homeostatic-expanded NK cells are rapidly activated and undergo antigen-specific proliferation during MCMV infection. (A) Purified CD45.1⁺ NK cells were adoptively transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ CD45.2⁺ C57BL/6 mice and, 60 d later, mice were infected with MCMV. After infection, the NK cell response was analyzed. (B) NK cells from spleen were analyzed 2 d after infection (compared with uninfected $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice containing transferred NK cells) for surface expression of CD69, CD25, Ly49H, and NKG2D and intracellular staining for IFN-γ and granzyme B. Cells from infected mice are shown as solid black lines and uninfected mice as gray lines (filled). (C) The percentages of Ly49H⁺ NK1.1⁺ cells in the spleen were determined at 2, 7, and 14 d after infection (compared with uninfected mice). (D) Graph shows the percentages of Ly49H⁺ NK cells in the spleens of uninfected and infected mice at various time points. Error bars on the graph display SEM (n = 3–4 at each time point). (E) Splenic Ly49H⁺ NK cells from uninfected (gray line, filled) and day-7-after-infection (solid black line) mice were analyzed for surface expression of KLRG1, NKp46, and Ly49H. Flow cytometric plots shown are representative of three to four mice at each time point. All data presented are representative of three independent experiments.

Long-lived NK cells display effector function and proliferate during infection

Lastly, we examined the ability of long-lived NK cells generated during homeostatic proliferation to become activated and secrete effector cytokines during virus infection (Fig. 5 A). Indeed, the long-lived NK cells present 60 d after transfer into $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice became rapidly activated after MCMV infection, expressing increased levels of CD69, CD25, and NKG2D at day 2 after infection (Fig. 5 B). Activating Ly49H receptor expression was slightly diminished at day 2 after infection, suggesting productive interactions with the MCMV–encoded ligand m157 expressed on infected cells

(Arase et al., 2002; Smith et al., 2002). Furthermore, intracellular IFN-γ and granzyme B were readily detected in the activated NK cells after infection (Fig. 5 B), together demonstrating that long-lived NK cells generated by homeostatic proliferation are poised to immediately respond against viral infection.

The long-lived NK cells generated by homeostatic proliferation were able to proliferate robustly, as measured 7 and 14 d after MCMV infection (Fig. 5 C). Strikingly, Ly49H-bearing NK cells represented ~20% of the lymphocytes at the peak of the response (day 7 after infection), a 30-fold increase in percentage compared with homeostatically expanded
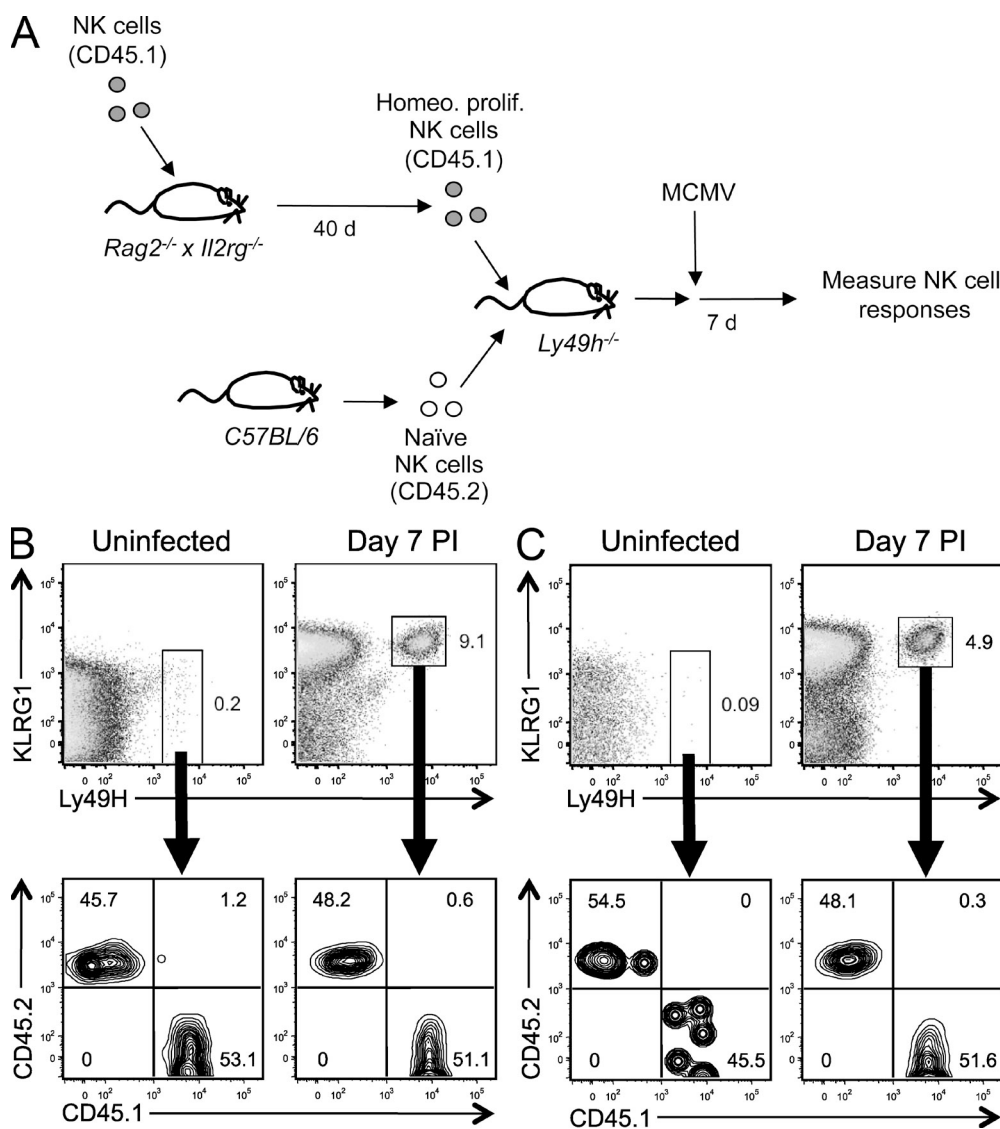
Figure 6.   Kinetics of naive and long-lived NK cells during MCMV infection. (A) Equal numbers (10⁵) of naive NK cells (CD45.2⁺) and long-lived NK cells generated by homeostatic proliferation (CD45.1⁺) were adoptively transferred into Ly49H-deficient recipient mice. 7 d after MCMV infection, the NK cell response was analyzed. (B and C) Adoptively transferred Ly49H⁺ NK cells (NK1.1⁺ CD3⁻) were analyzed for expression of KLRG1, CD45.1, and CD45.2 in spleen (B) and liver (C). All data presented are representative of three independent experiments, with three mice per time point.

NK cells in uninfected mice (Fig. 5 D). NK cells at day 7 after infection highly expressed the maturation marker KLRG1 (Fig. 5 E) and, interestingly, showed decreased expression of the activating NKp46 receptor. The cell surface density of Ly49H was increased on NK cells at day 7 after infection (Fig. 5 E), which is consistent with prior observations (Sun et al., 2009a).

To directly compare naive NK cells to long-lived NK cells generated by homeostatic proliferation in the response against viral infection, equal numbers of enriched naive NK cells (CD45.2⁺) and long-lived NK cells (CD45.1⁺) were co-adoptively transferred into Ly49H-deficient recipient mice followed by infection with MCMV (Fig. 6 A). Although few but equal numbers of cells in both populations were present

in uninfected mice, large numbers of adoptively transferred Ly49H⁺ NK cells in both spleen (Fig. 6 B) and liver populations (Fig. 6 C) were observed at 7 d after MCMV infection, showing similar proliferation kinetics and up-regulation of KLRG1 expression. Recently, virus-specific memory Ly49H⁺ NK cells were also shown to have surprisingly similar expansion compared with an equal number of naive Ly49H⁺ NK cells during MCMV infection, even though the memory NK cells demonstrated enhanced effector function (Sun et al., 2009a). Thus, although longevity and effector function may differ dramatically between naive and previously activated NK cells (by viral infection or homeostatic proliferation), the cell division program initiated in all NK cell populations studied appears similar.
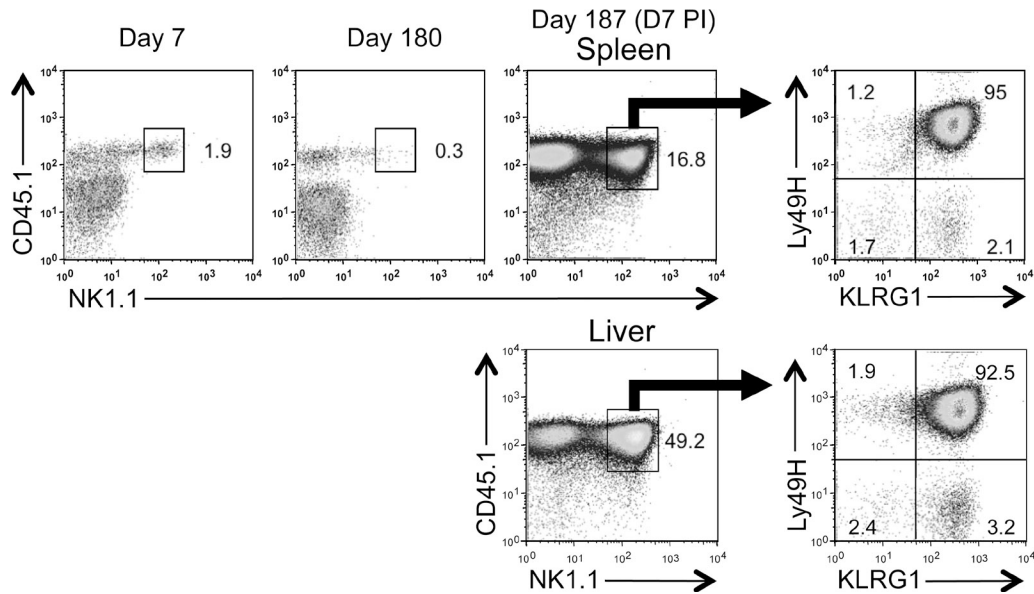
Figure 7.   Long-lived NK cells are capable of robust expansion after MCMV infection. $Rag2^{-/-} \times Il2rg^{-/-}$ CD45.2+ C57BL/6 mice containing CD45.1+ NK cells transferred 180 d prior were infected with MCMV, and NK cells in spleen and liver were analyzed at 7 d after infection (compared with uninfected $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice containing NK cells at days 7 and 180 after adoptive transfer). NK cells at day 7 after infection were analyzed for surface expression of Ly49H and KLRG1, and represent three to four mice per time point. All data presented are representative of three independent experiments.

In long-term studies, whole splenocytes were transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice, and homeostatically driven NK cells were examined 180 d later for their ability to mount effector responses. Although observed at low frequency before infection, donor Ly49H+ NK cells underwent prolific expansion in the spleen and liver after MCMV infection (Fig. 7). Over 7 d, the antigen-specific Ly49H-bearing NK cells represented nearly 15% of all cells in the spleen and, astoundingly, 50% of all cells in the liver (Fig. 7). Altogether, these findings demonstrate the ability of mature NK cells to persist for many months as long-lived memory cells at both lymphoid and nonlymphoid sites, patrolling for and robustly responding against pathogen invaders.

DISCUSSION

The studies in this paper focus on the long-term consequences of homeostatic proliferation in NK cells. NK cells undergo homeostatic proliferation when adoptively transferred into a lymphopenic environment (Prlic et al., 2003; Ranson et al., 2003; Jamieson et al., 2004). These prior studies evaluated NK cell proliferation during the first few days and weeks after adoptive transfer but did not examine the long-term consequences of such an expansion or the lifespan or functional competence of the transferred mature NK cells. In this study, we discovered that homeostatic proliferation of naive NK cells resulted in an unexpected longevity of this lymphocyte subset. Furthermore, viral infection of these mice demonstrated that these long-lived NK cells could respond robustly >6 mo after initial transfer.

Previously, mature NK cells in the periphery were considered a terminally differentiated effector cell population incapable of self-renewal. NK cells were thought to exit the bone marrow and survey the peripheral tissues for virally infected, transformed, or stressed cells (Raulet, 2003; Yokoyama et al., 2004; Lanier, 2005). If a mature NK cell became activated (by encounter with foreign ligand or missing/altered self) and performed its effector duties, it was thought to die soon thereafter; if the same NK cell did not become activated, it was also thought to die with a half-life of ∼2 wk (Jamieson et al., 2004). Thus, like many cells of the innate immune system, NK cells were considered short-lived effector cells. This dogma has recently been challenged by several studies that demonstrate the ability of NK cells to become long-lived cells after ligation of their activating receptor or when stimulated by inflammatory cytokines (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009a). During MCMV infection, naive antigen-specific NK cells were shown to undergo many rounds of division leading to the generation of memory NK cells detectable months later (Sun et al., 2009a). Our current study demonstrates yet another setting in which NK cells can become long lived and be maintained for months in both lymphoid and nonlymphoid tissues.

The longevity of memory CD8+ T cells is dependent on cytokines, including IL-7 and IL-15 (Jameson, 2002; Marrack and Kappler, 2004; Ma et al., 2006; Surh and Sprent, 2008). Mature NK cells similarly require IL-15 for homeostasis (Lodolce et al., 1998; Kennedy et al., 2000; Cooper et al., 2002; Burkett et al., 2004; Huntington et al., 2007a), with trans-presentation of IL-15 on IL-15 receptor α-bearing cells, in part mediated by dendritic cells (Lucas et al., 2007; Mortier et al., 2009; Guimond et al., 2010). IL-15 signaling in NK cells results in the increased expression and activity of Bcl-2 (and its family members), which may shield peripheral NK cells from apoptosis (Cooper et al., 2002; Ranson et al., 2003;

Huntington et al., 2007a). Conversely, depriving NK cells of IL-15 leads to the rapid accumulation of the proapoptotic factors Bim and Noxa (Huntington et al., 2007a). Furthermore, adoptive transfer of mature NK cells into settings of IL-15 deficiency leads to an accelerated and dramatic loss in survival of these cells (Koka et al., 2003; Prlic et al., 2003; Ranson et al., 2003; Jamieson et al., 2004). Thus, both NK cell homeostatic proliferation and subsequent cell longevity are likely to be dependent on IL-15 in the environment. Other cytokines that may influence survival of long-lived NK cells need to be determined experimentally as well. Interestingly, NK cell proliferation and survival are independent of IL-15 (and other members of the γ_C family of cytokines) during viral infection but are dependent on IL-12 (Sun et al., 2009b).

What are the additional signals that NK cells acquire as they undergo homeostatic proliferation? TCR signaling is believed to mold the peripheral TCR repertoire of the naive T pool during homeostatic proliferation (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Correia-Neves et al., 2001; Ge et al., 2004). Memory CD8+ T cells become independent of TCR–MHC class I interactions for their survival and homeostatic maintenance (Murali-Krishna et al., 1999). Similarly, although MHC class II molecules are required for the long-term maintenance of the naive CD4+ T cell compartment (Clarke and Rudensky, 2000), they are not required for the homeostatic proliferation of CD4+ T cells or the maintenance of memory CD4+ T cell numbers (Swain et al., 1999; Clarke and Rudensky, 2000). Analogous to T cell–MHC interactions in the thymus, the "education" of NK cells in the bone marrow is influenced by interactions between NK cell receptors and their cognate ligands (Fernandez et al., 2005; Kim et al., 2005; Sun and Lanier, 2008b; Tripathy et al., 2008). NK cells possessing inhibitory receptors that interact with self-MHC class I ligands during development are programmed to become functional effectors in the periphery (a process designated "licensing"), whereas NK cells lacking inhibitory receptors that recognize host MHC class I are hyporesponsive or "unlicensed" (Fernandez et al., 2005; Kim et al., 2005). However, this unlicensed status is not detrimental in all circumstances; in fact, NK cells lacking inhibitory receptors that interact with host self-MHC class I can be rescued during viral infection and can surprisingly respond more robustly than their counterparts that do bind self-MHC (Sun and Lanier, 2008a; Orr et al., 2010). In the periphery, do interactions between inhibitory NK receptors with their cognate MHC class I ligands influence the ability of certain NK cell subsets to undergo homeostatic proliferation or be maintained as long-lived cells? Although there are studies that suggest NK cells proliferate with similar kinetics in sublethally irradiated wild-type and β2-microblobulin–deficient hosts (lacking MHC class I; Ranson et al., 2003; Jamieson et al., 2004), we have evidence that NK cell subsets possessing inhibitory receptors that do not engage self-MHC class I in fact proliferate more robustly in a lymphopenic environment (unpublished data), similar to the virus-driven expansion of

unlicensed NK cells (Sun and Lanier, 2008a; Orr et al., 2010). Although these questions require further investigation, NK cell receptor–MHC class I interactions might play some role in the homeostatic maintenance of certain NK cell subsets.

Several surprising differences were found to exist between activated and long-lived NK cells generated during lymphopenia compared with viral infection. Long-lived "memory" NK cells resulting from activation and proliferation in response to MCMV infection stably express high levels of KLRG1 and other activation or maturation markers (Huntington et al., 2007b; Sun et al., 2009a). On the contrary, even though they have undergone extensive proliferation, homeostatic-driven NK cells retained a phenotype more similar to the naive resting NK cells found in uninfected mice. Interestingly, unlike with CD8+ T cells, homeostatic proliferation of CD4+ T cells also leads to a different activation profile than the same cells activated via antigen/MHC, with the homeostasis-driven memory CD4+ T cells maintaining a naive phenotype (Clarke and Rudensky, 2000). Even though they reverted to a more quiescent phenotype, these homeostasis-expanded NK cells did not die quickly like naive NK cells but became long lived, similar to NK cells expanded in response to MCMV infection, and slowly turned over at steady state, similar to memory T cells (Tough and Sprent, 1994; Murali-Krishna et al., 1999; Goldrath et al., 2002; Wherry et al., 2003; Williams et al., 2006). What constitutes the epigenetic programming required for NK cell longevity? Does rapid proliferation alone induce a stable molecular program that imprints the NK cell with the ability to survive for longer periods of time? What are the factors that shield these long-lived cells from apoptotic cell death, allowing them to survive for many months? These questions will be the subject of future studies that endeavor to define the molecular mechanisms that stabilize and prolong the lifespan of NK cells.

The reconstitution of the immune system after radiation treatment or chemotherapy of cancer patients or in humans with natural immunodeficiencies (such as SCID patients) given hematopoietic stem cell transplants represent situations in humans where there is homeostatic proliferation of NK cells and other hematopoietic cell types. In addition, the adoptive transfer of activated human NK cells has been used in the treatment of certain cancers (Ruggeri et al., 2002; Hsu et al., 2005; Miller et al., 2005, 2007; Passweg et al., 2005; Shi et al., 2008; Cooley et al., 2009; Gill et al., 2009; Moretta et al., 2010; Rubnitz et al., 2010). Therefore, the use of mouse models to better understand the nature of NK cells undergoing homeostatic proliferation in lymphopenic recipients may provide insights that are important in the clinic. For example, are there detrimental side effects (i.e., graft-versus-host disease) or unforeseen dangers to healthy host tissues associated with having an enduring population of self-renewing activated NK cells poised to mediate destructive responses? Conversely, there may be benefits to immunotherapy involving the adoptive transfer of NK cells beyond the destruction of leukemias or other tumors. Generally thought to be a transient therapy, these adoptively transferred NK cells might

persist for months, years, or possibly the lifetime of the patient, patrolling against transformed cells and providing protection against relapse of cancer. Additionally, in patients receiving hematopoietic stem cell transplants, adoptively transferred mature NK cells might provide protection against infectious agents, whereas B cells and T cells are reconstituted over a period of several months. The potential pros, and cons, of an NK cell adoptive transfer strategy must be taken into consideration in light of discoveries that unveil new attributes of a cell type previously thought to be short lived.

MATERIALS AND METHODS

Mice. C57BL/6 (CD45.2$^+$) mice and CD45.1$^+$ congenic C57BL/6 mice were purchased from the National Cancer Institute. $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice were purchased from Taconic. $Klra8^{-/-}$ (Ly49H-deficient) C57BL/6 mice were provided by S. Vidal (McGill University, Montreal, Canada). R. Locksley (University of California, San Francisco [UCSF], San Francisco, CA) provided C57BL/6 Yeti mice (Stetson et al., 2003). Experiments were done according to the UCSF Institutional Animal Care and Use Committee guidelines.

Adoptive transfer of cells and viral infections. $Rag2^{-/-} \times Il2rg^{-/-}$ C57BL/6 mice or sublethally irradiated (650 rads given 2 d before adoptive transfer) wild-type C57BL/6 mice received enriched NK cells populations (50–80% NK1.1$^+$ CD3$^-$), highly purified sorted NK cells (99.5–99.9% NK1.1$^+$ CD3$^-$), or whole splenocytes (2–5% NK1.1$^+$ CD3$^-$) intravenously. In some experiments, unirradiated wild-type or $Klra8^{-/-}$ C57BL/6 mice received NK cells before BrdU treatment or viral infection. BrdU treatment of mice was performed as previously described (Sun et al., 2009a). CFSE labeling of cells was performed according to the manufacturer's instructions (Invitrogen). In the indicated experiments, 200 µg of a depleting antibody against NK1.1 (clone PK136) was injected intravenously at the time of adoptive transfer. 5×10^4 PFU of a salivary gland stock of MCMV (Smith strain) was injected intraperitoneally, as described previously (Sun and Lanier, 2008b).

Flow cytometry. Fc receptors on cells isolated from peripheral blood, spleen, lymph nodes, liver, lung, and kidneys were blocked using anti-CD16 + CD32 mAb 2.4G2 before surface staining. Cells were stained with antibodies against NK1.1, CD3, CD45.1, CD45.2, Ly49H, KLRG1, NKp46, NKG2D, CD25, CD69, CD11a, CD44, CD122, and Ly6C (eBioscience or BD). W. Yokoyama (Washington University, St. Louis, MO) provided the anti-Ly49H mAb (clone 3D10). Intracellular IFN-γ and granzyme B stainings were performed according to the manufacturer's instructions (BD). BrdU staining was performed using a BrdU Flow kit (BD). Flow cytometry was performed on an LSRII, and data were analyzed with CellQuest software (BD).

Ex vivo stimulation assay. Splenocytes were enriched for NK cells using an NK cell isolation kit (Miltenyi Biotec), followed by AutoMACS magnetic bead separation. Tissue culture plates treated with N-(1-(2,3-dioleoyloxyloxyl)propyl)-N,N,N-trimethylammonium methylsulphate (Sigma-Aldrich) were coated with antibodies against Ly49H, NKp46, and NKG2D (or IgG as a negative control; Sun et al., 2009b). NK cells were blocked with anti-CD16 + CD32 mAb (hybridoma 2.4G2) and incubated on antibody-coated plates for 5 h at 37°C in the presence of brefeldin A (BD), followed by staining for LAMP-1 (CD107a) and intracellular IFN-γ (BD), as previously described (Sun et al., 2009b).

Online supplemental material. Fig. S1 shows that the kinetics of NK cell homeostatic proliferation differs from B and T cells. Fig. S2 demonstrates that mature NK cells adoptively transferred into irradiated recipients are long lived. Fig. S3 shows that NK and CD8$^+$ T cell homeostatic proliferation is more rapid than that for CD4$^+$ T and B cells. Fig. S4 reveals that mature NK cells transferred into $Rag2^{-/-} \times Il2rg^{-/-}$ mice are initially activated but become phenotypically quiescent. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100479/DC1.

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