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Murine NK Cell Intrinsic Cytokine-Induced Memory-like Responses Are Maintained following Homeostatic Proliferation

Molly P. Keppel,* Liping Yang,† and Megan A. Cooper*

Several recent studies have demonstrated that innate immune NK cells exhibit memory-like properties with enhanced nonspecific and specific recall responses. Cytokine activation alone of murine NK cells induces the differentiation of memory-like cells that are more likely to produce IFN-γ, a key NK cell cytokine important for activation of the immune response. Using an adoptive cotransfer system, we first show that cytokine-induced memory-like responses are NK intrinsic. However, engraftment of donor NK cells in NK-competent hosts is poor because of homeostatic control mechanisms. Therefore, we used alymphoid Rag- and common γ-chain–deficient mice as recipients and observed homeostatic expansion of cotransferred cytokine-activated and control donor NK cells. Despite proliferation of all cells, NK cells derived from those cells originally activated by cytokines retained an intrinsic enhanced capacity to produce IFN-γ when restimulated in vitro with cytokines or target cells. These NK cell memory-like responses persisted for at least 4 wk in alymphoid hosts and 12 wk in NK-competent hosts. These findings indicate that memory-like NK cells can readily self-renew and maintain enhanced function in a lymphopenic host for at least a month. The Journal of Immunology, 2013, 190: 000–000.

Our immune response can be divided into two broad arms, innate and adaptive immunity. Until recently, a major distinction between these two arms was the exclusive ascription of immunologic memory to adaptive T and B lymphocytes. However, several recent reports have suggested memory-like responses by innate immune NK cells (1–4). NK cells are lymphocytes that express germline-encoded receptors and are present in patients and mice with defects in proteins necessary for T and BCR rearrangement (e.g., Rag deficient) (5, 6). NK cells are important for the early control of infection, particularly viruses (7, 8). NK cells are also capable of killing tumor cells and may play a role in tumor surveillance and are currently being evaluated for cancer immunotherapy (9). NK cells mediate their effects via two primary mechanisms, production of cytokines and target cell killing.

Several studies have suggested that NK cells can retain a cellular memory of activation to both specific and nonspecific stimuli (1–4, 10–12). Studies by von Andrian and colleagues (1, 4) demonstrated liver NK cell-mediated specific memory to multiple haplotypes of Ags using a contact hypersensitivity model. In addition, a subset of previously sensitized liver NK cells exhibited specific killing of Ag-pulsed cells and provided protection against systemic infection (4). Lanier and colleagues (3, 13) reported development of Ly49H+ memory splenic NK cells in vivo that was dependent on cytokines following infection with murine CMV (MCMV), a virus that encodes a ligand recognized by the activating Ly49H receptor. We established that cytokine activation alone induces the differentiation of memory-like NK cells that are more likely to produce IFN-γ (2). The first model suggests that some liver NK cells might exhibit features of both cellular and immune memory (i.e., the ability to retain memory of prior activation as well as specificity to protect the host against infection with the same organism), whereas cytokine-induced memory-like NK cells, including those induced by MCMV, likely represent cellular memory responses and are referred to as “memory-like” in this paper. Taken together, these studies suggest that NK cells can acquire memory-like, Ag-independent and -dependent, phenotypes. In addition to these murine studies, we recently demonstrated that human NK cells preactivated with cytokines acquire memory-like responses following prolonged in vitro culture (14). Additional studies have demonstrated possible human NK cell memory responses in vivo (15–17), although it is more challenging to accurately identify previously activated human NK cells rather than primed cells responding to persistent viral stimulation (18).

The NK cell compartment comprises ~10% of human PBLs and ~3–5% of murine splenocytes. This overall number of NK cells is tightly controlled and adoptive transfer of NK cells into NK-competent mice, including wild-type and Rag-1–/– deficient hosts, results in low engraftment and little proliferation of donor NK cells because of limited availability of the survival and growth factor IL-15 (19, 20). However, adoptive transfer into NK-deficient hosts leads to robust proliferation and long-term engraftment of mature NK cells (21–23). Similarly, Miller et al. (24) demonstrated that in patients, higher dose lymphodepleting regimens prior to adoptive immunotherapy with allogeneic NK cells led to more successful engraftment and expansion of donor NK cells. Thus, homeostatic expansion has the potential to allow for

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Abbreviations used in this article: CHS, contact hypersensitivity; γc, common γ-chain; MCMV, murine CMV; poly(I:C), polyinosine-polycytidylic acid; Rag-1–/–, Rag-1–/– deficient.

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proliferation of mature NK cells, long-term engraftment, and more successful immunotherapy.

We investigated whether cytokine-activated NK cells retain an intrinsic memory-like phenotype following expansion and proliferation in an alymphoid host. NK cells undergo rapid proliferation in alymphoid hosts, and the long-lived pool of cells in reconstituted mice is renewed from dividing mature NK cells (19, 20).

First, we definitively demonstrated that cytokine-induced NK cells’ memory-like responses are cell intrinsic. Next, we found that despite extensive proliferation in alymphoid hosts, memory-like NK cells maintain the capacity to produce enhanced IFN-γ for at least 1 mo. Finally, we found that unlike cells in alymphoid hosts, memory-like NK cells in NK-competent mice maintain an enhanced capacity for IFN-γ production for 3 mo. These studies suggest that exogenous treatment of NK cells with cytokines for immunotherapy or activation of NK cells in the context of an infection might lead to long-lived memory-like responses that persist with proliferation and self-renewal.

Materials and Methods

Mice

All mice were on the on the C57BL/6 background and bred and maintained at our facility. Rag-1–deficient (Rag-1−/−) mice expressing the C57BL/6 CD45.2 Ag were obtained from The Jackson Laboratory (BAR Harbor, ME). Mice deficient in Rag-2 (Rag-2−/−) and common γ-chain/IL-2Rγ (γc−/−) were purchased from The Jackson Laboratory and bred to obtain Rag-2−/−γc−/− mice. Mice expressing the congenic CD45.1 receptor (CD45.1+) were obtained from The Jackson Laboratory and bred to Rag-1−/− animals to obtain CD45.1+Rag-1−/− mice. Animals were housed in specific pathogen-free conditions and studies were approved by the Washington University Animal Studies Committee.

Adoptive transfers

Splenocytes were obtained from Rag-1−/− donors (CD45.1 or CD45.2) and cultured overnight as described previously (2). For some experiments, NK cells were enriched by collection of nonadherent cells in a 10:1 ratio with Yac-1 targets for a total of 4 h. CD45.1+ or control treated (low-dose IL-15 alone or without murine IL-12 (10 ng/ml) for 4 h, with brefeldin A added after the first hour. Coculture with Yac-1 (obtained from American Type Culture Collection, Manassas, VA) targets was performed at a 10:1 ratio of splenocytes to targets with or without murine IL-12 (10 ng/ml) for 4 h, with brefeldin A added after the first hour. For CD107a (LAMP-1) assays, splenocytes were cultured at a 10:1 ratio with Yac-1 targets for a total of 4 h.

Results

Cytokine stimulation results in initial NK cell priming followed by an NK-intrinsic memory-like response

We previously reported cytokine-induced memory-like NK cell responses following adoptive transfer of activated and control NK cells into separate hosts. To definitively determine whether memory-like responses are NK-intrinsic, we generated congenic Rag-1–deficient donor mice (CD45.1+Rag-1−/−) and performed cotransfers of cytokine-activated and control NK cells into the same host (Fig. 1A). Cytokine-activated (IL-12 + IL-18 with low-dose IL-15) CD45.2+ or control treated (low-dose IL-15 alone) CD45.1+ enriched NK cells were labeled with CFSE and adoptively transferred into CD45.1+Rag-1−/− hosts (Fig. 1A). Cytokine activation with IL-12 plus IL-18 stimulates IFN-γ production by >90% of NK cells, whereas low-dose IL-15 maintains survival without inducing IFN-γ production (2). Following adoptive transfer, donor and host splenic NK cells were identified as NK1.1+ lymphocytes expressing (Fig. 1A): CD45.2+CFSE− (host); CD45.2+NK1.1+ (preactivated donor); and CD45.2+CFSE+ (control donor). Control-treated NK cells exhibited minimal proliferation because Rag-deficient hosts have intact NK cell compartments (Fig. 1C, 1D), allowing for reliable identification of these donor NK cells based on CFSE expression. There were slightly more control than previously activated donor cells present at day 1; however, despite proliferation of cytokine-activated cells, there was no significant difference in the numbers of control versus activated donor cells present at days 3, 7, or 21 (Supplemental Fig. 1), perhaps reflecting the limitations of reconstituting cells in mice with an intact NK cell compartment. Splenocytes from recipients were harvested and stimulated for 4 h with cytokines (IL-12 + IL-15) or media and IFN-γ production measured by intracellular flow cytometry. One day after adoptive transfer, a small percentage of previously activated donor NK cells continued to produce IFN-γ spontaneously, and they had a primed phenotype with the majority of cells producing IFN-γ after in vitro infection might lead to long-lived memory-like responses that persist with proliferation and self-renewal.
cytokine restimulation (IL-12 + IL-15) (Fig. 1B). By contrast, significantly fewer control-donor and host NK cells were positive for IFN-γ. At later time points, preactivated donor NK cells remained more likely to produce IFN-γ as compared with control or host NK cells; however, the percentage of IFN-γ–positive preactivated NK cells was less than that seen at day 1. Production of IFN-γ by control donor NK cells was similar to endogenous host cells, with the exception of day 21, when there was a small but statistically significant increased production of IFN-γ by control donor versus host NK cells (20.3 versus 17.7%). The exaggerated IFN-γ response of preactivated donor cells at day 1 suggests a residual priming effect at this early time point, whereas the later responses, particularly day 21, are consistent with a persistent memory-like response. Similar results were obtained with NK cells enriched by magnetic bead purification (94% purity) or harvest of nonadherent cells (50–90% purity) and independent of the source of cells (i.e., CD45.1 or CD45.2). These cotransfer experiments definitively demonstrate that memory-like effects are NK intrinsic and not the result of transfer of other activated cells (e.g., macrophages and/or dendritic cells), because transfer of enriched cytokine-activated NK cells did not alter the phenotype of host or control-donor NK cells.

**NK cells homeostatically proliferate in NK-deficient hosts and maintain functional capacity**

It remains possible that enhanced memory-like NK responses are due to in vivo proliferation and not related to a prior activation event. Therefore, we next examined naive and cytokine-activated NK cell responses following enforced proliferation of both subsets using an alymphoid host. NK cells were enriched from congenic (CD45.1 and CD45.2) Rag-1−/− mice, cytokine activated or control treated, CFSE labeled, and adoptively cotransferred into Rag-2−/−γc−/− hosts (Fig. 2A), which lack endogenous T, B, and NK cells. Prior reports have demonstrated that NK cells undergo homeostatic proliferation in alymphoid hosts with maintenance of effector functions (19, 21, 23). NK cells are absent in Rag-2−/−γc−/− mice because of a defect in IL-15 signaling, which is required for NK cell development (25). However, Sun et al. (26) observed IL-12–induced development of NK cells in the setting of a viral infection in mice lacking IL-15. To ensure that we were...
measuring donor NK cells and not endogenous NK cells that differentiated in response to cytokine stimulation, we injected Rag-2−/− γc−/− mice with activated CD45.1+ NK cells alone (Supplemental Fig. 2). As expected, there was no development of endogenous CD45.1-negative NK cells as late as 4 mo following transfer.

Preactivated and control NK cells both proliferated extensively in vivo, and CFSE was not detectable in either subset after 7 d, with the majority of NK cells dividing by day 3 (Fig. 2A, 2B). Relatively few NK cells were recovered from the spleens of hosts 1 d after transfer (Fig. 2C). However, total NK cell counts increased over 7 d (Fig. 2C) and were similar to those observed previously (23). The ratio of preactivated to control cells was initially low, and most splenic NK cells at day 1 were control cells with numbers of preactivated cells increasing over 7 d (Fig. 2D). It is unclear whether this is due to engraftment or homing of preactivated NK cells, although at early time points, we observed similar ratios of transferred cells in the liver and lungs, the other major organs housing NK cells (Supplemental Fig. 3).

Despite proliferation of all control and preactivated NK cells by day 7, progeny of preactivated NK cells, never before exposed to cytokines, maintained an enhanced capacity to produce IFN-γ, whereas control NK cell progeny maintained a naive phenotype (Fig. 3). Donor-derived NK cells were easily identified based on cell surface expression of NK1.1 and CD45.1 or CD45.2 (Fig. 3A). Upon stimulation with IL-12 plus IL-15, significantly more NK cells derived from preactivated cells produced IFN-γ than control NK cells (Fig. 3). Similar to cotransfer studies in Rag-deficient hosts, 1 d after transfer, a very high percentage of preactivated NK cells produced IFN-γ, likely due to priming of those cells. At 3 and 7 d after transfer, overall IFN-γ production was lower, and NK cells that originated from preactivated cells continued to produce significantly more IFN-γ upon cytokine stimulation than control cells from the same host. These enhanced responses could represent a residual priming effect, rather than a memory-like response, although it is interesting that this enhanced capacity for IFN-γ production was passed on to daughter cells. It was reported that MCMV-induced memory NK cells express high levels of KLRG1, and we measured expression of this Ag as well as CD11b and the activation marker CD69 (Supplemental Fig. 4). We saw high expression of KLRG1 on cytokine-activated and control NK cells 1 and 4 wk after cotransfer into Rag-2−/− γc−/− hosts. Expression of KLRG1 on both donor subsets was much higher than that seen on unmanipulated Rag-1−/− NK cells, suggesting that expression of this Ag is related to in vivo proliferation (Supplemental Fig. 4). Cytokine-activated and control cells also had similar expression of CD69 and CD11b, both of which were higher than seen on unmanipulated Rag-1−/− NK cells. These results demonstrate that cytokine-activated NK cells maintain an enhanced capacity for IFN-γ production early following homeostatic expansion, and proliferation upregulates multiple markers of NK cell activation/mature.

**Expanded memory-like and control NK cells respond more robustly to activation with target cells, whereas responses to in vivo TLR activation are dependent on early priming**

We next determined NK cell IFN-γ production in response to tumor targets in vitro and TLR3 activation in vivo. Cytokine-activated and control-treated NK cells were adoptively transferred into separate Rag-2−/− γc−/− hosts. After 7 d (Fig. 4A) or 4 wk (Fig. 4B) of expansion in vivo, NK cells were assayed for production of IFN-γ by culturing in vitro for 4 h with Yac-1 tumor cells alone or with IL-12, which enhances IFN-γ production via costimulation. Significantly more donor NK cells derived from cytokine-activated cells produced IFN-γ as compared with control cells at both time points. Although responses at 1 wk could represent NK cell priming, enhanced NK cell responses at 4 wk are consistent with a memory-like phenotype in response to tumor cell stimulation.

We next evaluated in vivo memory-like responses using the TLR3 ligand poly(I:C). One or 4 wk after adoptive cotransfer of cytokine-activated and control NK cells into Rag-2−/− γc−/−, mice were injected i.p. with poly(I:C) and NK cell IFN-γ production measured directly ex vivo. We observed that the progeny of pre-
vously activated NK cells had increased IFN-γ production at 1 wk (Fig. 4C). However, this effect was not observed at 4 wk, suggesting that the earlier response could be related to residual NK cell priming and not memory.

Cytotoxic potential of memory-like and control NK cells
To determine whether homeostatic proliferation has any effect on memory-like NK cell killing, we assessed cell surface expression of CD107a following target cell engagement as a surrogate for degranulation and the ability to kill targets. One or 4 wk after adoptive transfer into allogeneic hosts, cytokine-activated and control NK cells exhibited similar levels of cell surface CD107a in response to Yac-1 cells (Fig. 5A, 5B), suggesting that there is no difference in their ability to kill target cells, similar to our previous studies. Interestingly, both cytokine-activated and control NK cells expressed very high levels of granzyme B protein 1 and 4 wk after adoptive transfer (Fig. 4C, 4D). As previously reported, naïve NK cells from Rag-1−/− mice did not express appreciable granzyme B protein (Fig. 4C, shaded) (27).

Long-term maintenance of memory-like NK cell phenotype
Having determined that NK cell memory-like responses are intrinsic and maintained in allogeneic hosts, we next sought to determine how long memory-like responses are preserved. First, we examined the longevity of memory-like responses in NK-competent hosts (Rag-1−/−). Cytokine-activated NK cells adoptively transferred into separate NK-sufficient hosts (Rag-1−/−) do not undergo homeostatic proliferation and engraft in low numbers but maintained a memory-like phenotype for 12 wk with enhanced production of IFN-γ following cytokine restimulation (Fig. 6A). To determine whether expanded and proliferating NK cells also maintain this phenotype, cytokine-activated and control NK cells were cotransferred into Rag-2−/−γc−/− hosts and analyzed 4 and 12 wk later. NK cell counts remained relatively stable 1–12 wk after transfer (Fig. 6B); however, the ratio of previously activated to control cells decreased by 12 wk, when the majority of NK cells were derived from control donors. Following activation with IL-12 plus IL-15 in vitro, previously activated NK cells maintained a memory-like phenotype at 4 wk (Fig. 6C), similar to results seen with tumor cell stimulation (Fig. 4). At this time point, memory-like NK cells constitute the majority of the NK compartment in the host (Fig. 6B). However, by 12 wk, when control cells outnumber previously activated cells (Fig. 6B), control-treated NK cells actually produced slightly more IFN-γ (Fig. 6C). Interestingly, the levels of IFN-γ were higher at 12 wk in both Rag-1−/− and control NK cells from Rag-2−/−γc−/− mice (Fig. 6A, 6C). This could be experimental variability or a gain of function in NK cells because of aging or proliferation (in Rag-2−/−γc−/− hosts).

Discussion
Although immunologic memory has traditionally been the hallmark of adaptive immunity, several recent studies have demonstrated that innate immune NK cells have the capacity for memory-like responses (1–4). In this study, we found that cytokine-induced memory-like NK cells expand in allogeneic hosts via homeostatic proliferation and maintain their phenotype for at least a month despite extensive proliferation of all NK cells. As expected, activated NK cells made very high amounts of IFN-γ shortly after adoptive transfer to in allogeneic hosts, likely because of a primed phenotype. However, both cytokine-activated and control NK cells quickly proliferated, and by day 7, nearly all cells present in the hosts had never before been primed in vitro. Memory-like NK cells had enhanced IFN-γ responses to restimulation with cyto-
kines and tumor cells in vitro up to 4 wk after adoptive transfer. In vivo TLR activation showed enhanced responses by preactivated NK cells at early (7 d) but not later (4 wk) time points. No difference in expression of granzyme B or the ability to degranulate with tumor targets (Yac-1 cells) alone or with IL-12. Significantly more memory-like (preact) than control cells produced IFN-γ at both time points. Data represent mean ± SEM of seven and four independent experiments. *p < 0.05, **p < 0.005. (C and D) Cytokine-activated and control-treated NK cells were adoptively cotransferred into Rag-2−/−γc−/− hosts and injected 7 d (C) or 4 wk (D) later with poly(I:C) and NK cell IFN-γ production measured (mean ± SEM of four independent experiments). ****p < 0.0001; no significant difference at 4 wk.

Our data demonstrate that only a portion of daughter cells from previously activated NK cells retain a capacity for enhanced IFN-γ production, because the majority of cells did not produce IFN-γ and identification of a cell surface marker to identify memory-like NK cells will be important for future studies. As shown in this study and based on expression profiling (M.A. Cooper, unpublished data), we have not found a cell surface marker that distinguishes memory-like from naive splenic NK cells. KLRG1, an inhibitory cell surface receptor (28), has been proposed as a marker of splenic NK cell memory and was elevated on the cell surface of MCMV-induced memory NK cells (3). In this study, we found that KLRG1 was highly expressed by both cytokine-induced memory-like and naive expanded NK cells (Supplemental Fig. 4), suggesting that KLRG1 may be a marker of mature NK cell proliferation, as was previously suggested (28, 29), rather than memory.

NK cell numbers are normally tightly regulated in healthy hosts. Homeostatic expansion occurs in the setting of lymphopenia, and mature NK cells can proliferate and expand to “fill” the empty NK compartment (21–23). In patients, this can occur as the result of infection or chemotherapy-induced lymphopenia. Following adoptive transfer into lymphoid hosts, both cytokine-activated and control NK cells proliferated extensively within a week of adoptive transfer. Cytokine-activated NK cells maintained a memory-like phenotype for 12 wk in NK-competent Rag-1−/− hosts, whereas memory-like cells lost this phenotype by 3 mo in lymphoid hosts. The observed differences may be related to NK cell homeostasis in the two models. In lymphoid, Rag-2−/−γc−/− hosts, mature NK cells undergo homeostatic proliferation driven by a lack of endogenous lymphocytes. Whereas in NK-competent, Rag-1−/− hosts, only a subset of memory-like cells proliferate 3–7 d after adoptive transfer. Thus, one explanation for the loss of memory in Rag-2−/−γc−/− hosts at 12 wk is that multiple rounds of proliferation ultimately alters the memory-like phenotype of NK cells, whereas NK cells with low turnover in NK-competent hosts maintain a memory-like phenotype. An alternative hypothesis is that there is a gain of function in all (or only control) NK cells related to homeostatic proliferation, analogous to the memory-phenotype observed in T cells following homeostatic proliferation in a lymphopenic host (30). It will be interesting to determine whether NK cells exhibit a similar type of proliferation-driven memory–phenotype differentiation at late time points.

Regardless, the maintenance of clear memory-like effects after 4 wk of proliferation demonstrates that cytokine-induced memory
likely to produce IFN-γ experiments; black bars, IL-12 + IL-15 stimulation; gray bars, media; **p (mean

Twelve weeks after cotransfer into alymphoid hosts, slightly more control donor NK cells produce IFN-γ, as shown in Figure 2C, 2D). The ratio of preactivated to control cells decreased between 1 and 12 wk after adoptive transfer (mean ± SEM of three to eight mice per time point; data shown for 1 wk is also shown in Fig. 2C, 2D). (C) Four weeks after adoptive transfer, significantly more preactivated than control-treated NK cells were measured 4 and 12 wk later. (A) Following transfer into separate NK-competent hosts, preactivated NK cells were more likely to produce IFN-γ than naive host NK cells (*p = 0.03 at 4 wk and **p = 0.007 at 12 wk; mean ± SEM of three to five independent experiments). There was no significant difference between the percentage of control-treated donor and host IFN-γ NK cells. (B) Following cotransfer of cells into alymphoid Rag−2−/−γ−/− hosts (as schematized in Fig. 2, without CFSE labeling), the absolute number of splenic NK cells remained stable, whereas the ratio of preactivated to control cells decreased between 1 and 12 wk after adoptive transfer (mean ± SEM of three to eight mice per time point; data shown for 1 wk is also shown in Fig. 2C, 2D). (C) Four weeks after adoptive transfer, significantly more preactivated than control NK cells produced IFN-γ. Twelve weeks after cotransfer into alymphoid hosts, slightly more control donor NK cells produce IFN-γ (mean ± SEM of four to five independent experiments; black bars, IL-12 + IL-15 stimulation; gray bars, media; **p < 0.004).

is stable and hereditary. From a therapeutic standpoint, clinically relevant lymphopenia in patients (i.e., infection or chemotherapy induced) and inhibition of NK cell development is likely temporary, lasting from a few days to months (31). Thus, transient lymphopenia in patients might allow for the expansion of adoptively transferred or infection-induced memory-like NK cells that, with resumption of normal NK cell differentiation and resolution of lymphopenia, are subsequently maintained in a quiescent state. On the basis of the model in this study, such non-dividing memory-like NK cells would be predicted to maintain their phenotype long-term.

There are currently three experimental models of murine NK cell memory: 1) cytokine-induced splenic NK cell memory-like function (2), as discussed in this study; 2) splenic NK memory conferred via stimulation with cytokines and a germline-encoded NK cell receptor following recognition of a pathogen-encoded ligand (3, 13); and 3) Ag-specific liver NK cell memory not mediated by a known germline-encoded receptor, which was the first type to be described previously (1, 4). Taken together, these models are complementary, rather than mutually exclusive, and definitively demonstrate that NK cells have the capacity for recall responses. Our model suggests that, after an initial cytokine activation event and production of IFN-γ, NK cells retain the capacity for enhanced IFN-γ responses to multiple stimuli. Thus, cytokine-induced NK cell memory responses are predicted to provide nonspecific protection. This is physiologically relevant, because NK cells express only a limited number of activation receptors and often rely upon cytokine signals to mediate effector function (6).

Studies from the Lanier group (3) using an adoptive transfer system demonstrated that MCMV-induced expansion of long-lived Ly49H+ NK cells with enhanced cytokine production and the ability to provide superior protection against MCMV infection in neonatal mice. Similar to our system, this model was also dependent on cytokines, in particular IL-12 (13). Additional studies by Schlub et al. (32) in an intact host found that the kinetics of Ly49H+ NK cell expansion and contraction are different from T cells and there may not be a physiologically significant pool of memory-like NK cells that can expand in a secondary response, perhaps more consistent with the idea of enhanced cellular function rather than long-lived protective immunologic memory. It is not known whether Ly49H+ MCMV-induced memory NK cells are virus specific; however, because they respond more robustly to nonviral stimuli (i.e., anti-NK1.1) (3), it is possible that they might also provide enhanced protection against other infections. Indeed, it may be the inherent nonspecific nature of cellular memory-like responses in NK cells that could allow them to better protect the host, rather than specific immunologic memory to infections.

Studies from von Andrian’s group (1) were the first to demonstrate the capacity for liver NK cell immunologic memory with the finding that NK cells can mediate hapten-specific contact hypersensitivity (CHS) responses in the absence of T cells. Subsequent studies demonstrated a small subset of liver lymphocytes from Rag-deficient mice expressing NK1.1, CXCR6, Thy1, and other markers had the capacity for Ag-specific intrinsic memory but not splenic cells expressing the same markers (4). This type of memory appears more distinct from the two previous models based on its Ag specificity and limitation to lymphocytes from the
liver. Memory NK1.1⁺ liver cells were present in genetically diverse strains of mice (BALB/c and C57BL/6); capable of recognizing haptons and viral-like particles from HIV, influenza, and vesicular stomatitis virus; and conferred specific protection against viral infection in vivo (4). The recognition of such a diverse pool of Ags, and the fact that no Ag failed to induce NK cell memory responses (4), is reminiscent of T and B lymphocyte diversity and suggests that these NK1.1⁺ cells have the capacity for receptor editing, either via posttranscriptional modification or non-Rag-mediated recombination events. A study from another group demonstrated that the character and quality of CHS responses mediated by NK cells may be distinct from T cell CHS, suggesting a unique mechanism for liver NK1.1⁺ cell memory (33). It will be interesting to determine the mechanism of specific Ag recognition in these NK cells.

Collectively, these studies demonstrate that NK cells “learn” from prior experiences. In all of the experimental systems, the memory phenotype appears to be heritable and persists longer than the estimated 7- to 17-d half-life of NK cells (34, 35). In the case of cytokine and MCMV-induced memory, NK cells were more responsive not only to the original stimuli but also other activation signals (e.g., cytokines, receptor ligation, and tumor target cells), suggesting a generalized enhanced state of activation that could be exploited for immunotherapy (2, 3). Indeed, a recent study by the Cerwenka laboratory (36) demonstrated that IL-12, -15, and -18 preactivation of NK cells prior to adoptive transfer into sublethally irradiated tumor-bearing hosts led to significant reduction in established tumors. In this study, we discovered that cytokine-induced memory-like NK cells proliferate and expand in allogeneic hosts. There are currently multiple studies investigating NK cell adoptive therapy for cancer (9), and homeostatic expansion may be critical for treatment success as evidenced by the finding that patients treated with more intense conditioning regimens have enhanced donor NK cell engraftment (24). A recent study from Gill et al. (37) found that murine NK cells adoptively transferred into Rag-2⁻/⁻ α/β⁻ mice hosts completely lost the ability to produce IFN-γ within 5 d. They concluded that these NK cells become exhausted because of homeostatic proliferation and that caution should be used with NK cell adoptive therapy (37). However, we and others (19, 21, 23) clearly demonstrate that adoptively transferred NK cells retain the capacity to produce IFN-γ following proliferation in allogeneic hosts. The cause for discrepancy between these studies is unclear; however, the murine NK cells used by Gill et al. (37) were expanded with IL-2 prior to adoptive transfer, which may result in NK exhaustion, whereas we demonstrate that control (low-dose IL-15) or IL-12 plus IL-18-activated NK cells retain the capacity to produce IFN-γ following proliferation in allogeneic hosts. In vivo in response to recipient CMV antigen. J. Immunol. 189: 5082–5088.


