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Journal
Trends in Immunology, 34(6)

ISSN
1471-4906

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Publication Date
2013-06-01

DOI
10.1016/j.it.2013.02.005

Peer reviewed

Review

Natural killer cells: walking three paths down memory lane

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Immunological memory has traditionally been regarded as a unique feature of the adaptive immune response, mediated in an antigen-specific manner by T and B lymphocytes. All other hematopoietic cells, including natural killer (NK) cells, are classified as innate immune cells, which have been considered short-lived but can respond rapidly against pathogens in a manner not thought to be driven by antigen. Interestingly, NK cells have recently been shown to survive long term after antigen exposure and subsequently mediate antigen-specific recall responses. In this review, we address the similarities between, and the controversies surrounding, three major viewpoints of NK memory that have arisen from these recent studies: (i) mouse cytomegalovirus (MCMV)-induced memory; (ii) cytokine-induced memory; and (iii) liver-restricted memory cells.

What defines an immune memory cell?

Immunological memory is the ability of certain immune cells to remember a previous encounter with a pathogen and provide an enhanced response upon secondary encounter with the same pathogen. Memory cells are long-lived, respond more robustly to secondary infection and are phenotypically and epigenetically distinct from their naïve counterparts. Memory T cell formation is well characterized, and can be divided into three stages [1]. In the first stage, the expansion phase, naïve T cells clonally expand and differentiate into effector T cells after encounter with cognate antigen. In the second stage, the contraction phase, the majority (>90%) of effector T cells undergo apoptosis and surviving T cells enter the third stage – the memory phase. These long-lived T cells reside throughout the body and maintain their numbers through self-renewal. Memory T cells undergo robust expansion and heightened effector function upon re-encounter with their cognate antigen, termed the recall response. Antigen specificity, a hallmark of adaptive immunity, is considered a prerequisite for the development of immunological memory. Innate immune cells, such as NK cells, lack the ability to undergo somatic rearrangements of their receptors and are thus thought to be incapable of forming memory. This concept is being challenged now as evidence for NK cell memory emerges. Once considered purely short-lived, rapid responders, NK cells now straddle the line between innate and adaptive immunity, possessing limited antigen specificity, an extended lifespan, and mediating enhanced recall responses. Notably, not all of the classic features of adaptive immune memory have been demonstrated for memory NK cells. Recent NK cell research is challenging the concept of immunological memory, and is providing evidence for: (i) antigen-specific NK memory cells induced by MCMV infection; (ii) NK memory cells induced by exposure to cytokines alone; and (iii) liver-restricted NK memory cells with highly antigen-specific recall responses. Here, we discuss how these emerging three perspectives differ in their definition of NK memory, and how memory NK cells diverge from classic adaptive memory. These features will be critical for understanding how, and to what extent, NK cells might enhance vaccination strategies.

Memory NK cells after MCMV infection: mimicking their T cell cousins?

Although NK cells are classically referred to as innate cells, they share many properties with their adaptive lymphocyte cousins; in particular, $CD8^+$ T cells [2]. NK and T cells share a common lymphoid progenitor [3], express many common cell surface markers [4], and most importantly, function in a similar way in response to stimuli, producing cytokines and mediating cytotoxicity through the release of perforin and granzymes [1,4,5]. Could these shared attributes with T cells extend to the recently described memory-like properties of NK cells? Work by our group and others has established a view of NK cell memory that is based on viral antigen-driven proliferation through specific ligand/receptor interaction; this in turn generates a self-renewing, long-lived memory population with enhanced ability to respond to a secondary challenge [6].

Infection of mice with MCMV is a well-established model of NK cell–virus interaction and highlights the critical nature of NK cells in resistance to viral infection. C57BL/6 mice carry the activating Ly49H receptor on ~50% of NK cells, which specifically recognizes the m157 MCMV protein on infected cells; this ligand–receptor interaction drives Ly49H-dependent expansion of NK cells during the acute phase of infection [7–9]. Using a system in which $Ly49H^+$ NK cells were adoptively transferred into mice lacking this receptor, we have shown prolific antigen-driven expansion of these $Ly49H^+$ cells after MCMV infection. Expansion is

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Keywords: natural killer cells; adaptive immunity; immunological memory.

1471-4906/$ – see front matter
 http://dx.doi.org/10.1016/j.it.2013.02.005

followed by a contraction phase and the establishment of a long-lived pool of memory Ly49H$^+$ cells, detectable as late as 70 days post-infection [10]. This memory NK cell pool undergoes secondary expansion, displays enhanced effector function *ex vivo*, and offers increased protection against MCMV challenge, compared to naïve NK cells [10]. In a manner similar to a CD8$^+$ T cell response, the initial expansion is dependent on interaction with antigen (m157); MCMV lacking this protein does not induce Ly49H$^+$ proliferation or the establishment of a memory pool [10].

This antigen-driven expansion is critically dependent on interleukin (IL)-12 signaling [11]. NK cells lacking the IL-12 receptor do not proliferate in response to MCMV and fail to generate NK memory cells that can protect mice against challenge [11]. In addition, signal transducer and activator of transcription (STAT)4, but surprisingly not interferon (IFN)-γ, signaling is important for the NK cell response to MCMV and the generation of NK memory [11]. These findings highlight the importance of cytokine signaling together with the m157–Ly49H interaction in driving NK expansion. It is unclear if the deficiencies in cytokine signaling directly impede these NK cells from becoming memory cells, or if there is simply a requirement for NK cell proliferation prior to the establishment of memory. Moreover, the contraction phase may also be a critical determinant in the development of NK memory [1,8,10]. Effector T cell apoptosis and memory T cell formation after viral infection are dependent on B cell lymphoma (Bcl)-2 and Bcl-2-regulating proteins such as BCL-2 like 11 (BCL2L11 or BIM) [12–14]. Similarly, NK effector cell contraction and transition into memory relies on appropriate apoptotic signaling. Notably, the kinetics of the NK cell contraction phase after MCMV infection resemble the gradual and continuous contraction of CD4$^+$ T cells, rather than the rapid decline and plateau of CD8$^+$ T cells [15]. Which cues inhibit apoptosis and allow a subset of Ly49H$^+$ NK cells to become long-lived memory NK cells? This question remains to be fully addressed, but it is likely that prosurvival cytokines, including IL-15, are involved [2].

The identification of memory NK cells using specific cell surface markers, as is the standard for memory T cells, is an important unmet need in the field. NK cells in different phases of the viral response show remarkably distinct transcriptional profiles [4]. Using data generated by the ImmGen Consortium (www.immgen.org), we recently interrogated the differences between NK cells isolated at various times following MCMV infection, including Ly49H$^+$ memory NK cells [16]. Owing to the nature of the systematically generated ImmGen dataset, memory NK transcriptional profiles can be readily compared to naïve NK cells and effector NK cells isolated early (36 h) and late (7 days) after MCMV infection, as well as with memory CD8$^+$ T cells isolated following infection by *Listeria monocytogenes* or vesicular stomatitis virus [16]. This analysis yielded a subset of genes that are specifically upregulated in the memory NK and CD8$^+$ T cell compartments, compared to naïve or effector cells, including Ly6c and CD49a [16]. Whether these genes are representative of long-lived NK cells in general, or whether antigen-driven proliferation is required to generate this memory profile, remains to be addressed. Additionally, it is of interest to compare memory NK to memory T cells isolated from the same mouse following MCMV infection. Interestingly, expansion of NKG2C$^+$ NK cells has also been observed during some types of virus infection in humans (Box 1). Despite progress in identifying NK memory signature genes using transcriptional profiling, there is still a need for specific cell surface markers that would allow for the study of NK memory without the dependence on an adoptive transfer system that relies on known ligand–receptor interactions.

To date, the evidence supporting this particular CD8$^+$ T cell type view of NK memory has been limited to MCMV. Are similar NK memory cells generated in response to other pathogens? Such studies have been hampered by a

Box 1. The existence of human virus-induced NK memory

Does infection by viral pathogens lead to the development of memory NK cells in humans? NK cells are critical in controlling viral infections in humans; this has been revealed through individuals with rare NK cell-specific deficiencies that present clinically with uncontrolled viral infections, particularly those of the herpes virus family [cytomegalovirus (CMV), Epstein–Barr virus, HSV, and varicella zoster virus], and human papillomavirus [54]. The human CMV (HCMV) ligand–NK receptor pair has not been identified, but the activating CD94–NKG2C receptor appears to be important in CMV recognition. Human NK cells expressing NKG2C exist at a high frequency in HCMV-seropositive healthy subjects, as compared with HCMV-seronegative individuals [55]. Similar to the response of Ly49H$^+$ NK cells during MCMV infection in mice, human NKG2C$^+$ NK cells expand greatly in allogeneic transplantation patients during acute HCMV viremia [55]. A unique subset of NK cells that expresses NKG2C at high levels and the maturation marker CD57 persists at an elevated frequency in HCMV-seropositive individuals and increases after HCMV reactivation [55–57].

Interestingly, expansion of NKG2C$^+$ NK cells has also been observed in HCMV-seropositive patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [58]. Furthermore, NKG2C$^+$ NK cells rapidly proliferate and persist for more than 2 months following hantavirus infection [59]. The kinetics of these NK cell responses strikingly resemble the expansion of Ly49H$^+$ NK cells after MCMV infection. In patients with chikungunya virus (CHIKV) infection, there is a transient expansion and persistent survival of NK cells coexpressing NKG2C and CD57 [60]. Of note, NKG2C$^+$ NK cells only expand during other viral infections if the individual had previously been infected with HCMV. In contrast to infection with HCMV, infection with HSV-2 does not significantly expand a specific subset of NK cells [61]. There have been a limited number of studies in humans investigating pathogen-induced expansion of specific NK cell subsets. For example, the frequencies of 2B4^{bright} and NKp46^{dull} human NK cells increase following both inoculation of influenza virus vaccine and exposure of NK cells to influenza virus *in vitro* [62]. CD57^{bright} NK cells, but neither CD57 $^-$ nor CD57^{dim} NK cells, are enriched in patients with chronic HIV-1 infection [63]. Finally, Wu and colleagues have described a population of NK cells from individuals infected with *Mycobacterium tuberculosis* that express the memory-associated marker CD45RO [64]. These CD45RO$^+$ NK cells are found in the pleural fluid, but not the peripheral blood, of infected patients. When stimulated with IL-12 *ex vivo*, these cells respond more robustly than their CD45RO$^-$ counterparts: a higher frequency expresses IFN-γ; they display greater cytotoxicity against K562 cells, and they express higher levels of both subunits of the IL-12R (β1 and β2). In theory, if a vaccine could be designed to induce preferential expansion of effector NK cells and subsequent differentiation into memory NK cells specific to pathogens, this could increase vaccine efficacy and provide novel therapeutic approaches to clinically relevant viral infections such as CMV, herpes, hepatitis, and HIV.

lack of known pathogen ligand–NK receptor pairs in other infection models. Although it has been reported that the NKp46 receptor specifically recognizes influenza hemagglutinin (HA) [17,18], this interaction does not lead to NK cell proliferation and may only induce bystander NK cell activation due to cytokines induced during viral infection [19]. Local proliferation of NK cells in the lungs of mice infected intranasally with influenza has not been observed [19]. Using a mouse model of genital herpes simplex virus (HSV)-2 infection, Abdul-Kareem and colleagues have shown that previously exposed NK cells are able to respond more robustly to challenge, as measured by IFN-γ production and protection against lethal challenge. This recall response is HSV-2 specific and is independent of B and T cells [20]. Although no specific NK receptor was identified as mediating this protection, this study supports an antigen-specific recall response of memory NK cells in a distinct model of herpes virus infection.

We have shown that NK cells can mimic the virus-specific $CD8^+$ T cell response and undergo activation, expansion, and contraction and develop into long-lived memory cells (Figure 1, left panel). These memory NK cells can be recovered from a variety of tissues, including spleen, liver, and peripheral blood [10]. Furthermore, in this model, memory NK cells are generated only through viral antigen-driven expansion, and although they clearly have an enhanced recall response to challenge with the same pathogen [10], these MCMV studies have not addressed whether these cells can offer protection against heterologous challenge.

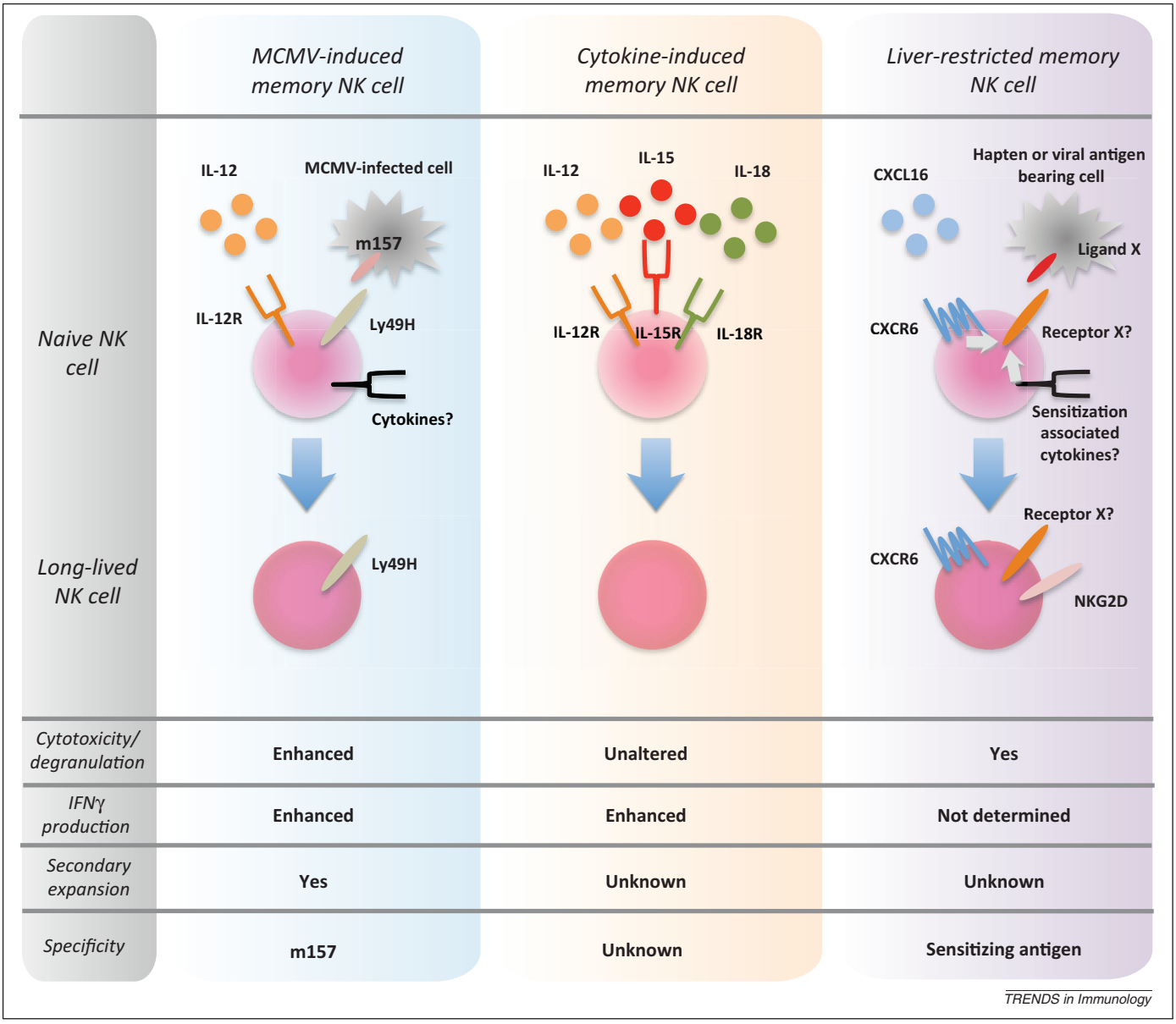

Figure 1. Natural killer (NK) cells can take one of three paths towards becoming a memory cell. Left panel: mouse cytomegalovirus (MCMV)-induced NK memory cells are generated after the cognate recognition of the m157 MCMV protein on infected cells by the activating Ly49H receptor. Memory generation requires interleukin (IL)-12 and NK cell signaling through the IL-12 receptor. Additional co-stimulatory signals by other cytokines and/or adhesion molecules might be required. Center panel: cytokine-induced memory NK cells are generated after exposure to IL-12, IL-15, and IL-18. Sensitization with antigen is not required in this model. Right panel: liver-restricted memory NK cells. Sensitization with haptens or specific antigens, in conjunction with chemokine CXC ligand (CXCL)16, is required to generate liver-restricted memory NK cells. The precursors might be selected by the cognate recognition of hapten-modified self-proteins or foreign antigens, and develop into memory NK cells. Receptors responsible for the antigen specificity have not been identified.

Cytokine-induced memory NK cells

Although some NK cell memory is antigen driven, it is unlikely that NK cells can generate antigen-specific memory against a broad range of pathogens, given that they possess only germline-encoded receptors. NK cells do, however, express receptors for and are exquisitely responsive to many cytokines, including IL-12, IL-15, and IL-18. Indeed, Yokoyama and colleagues have found that activation by cytokines alone leads to the generation of NK cells with memory-like properties [21]. NK cells from Rag1-deficient mice were activated overnight *in vitro* with IL-12, IL-18, and IL-15 and transferred into recombination activating gene (Rag) 1-deficient recipient mice. Up to 3 weeks after transfer, a higher frequency of these preactivated NK cells produced IFN-γ upon restimulation, compared to those NK cells that were not preactivated. This robust response was elicited by restimulation with either cytokines (IL-12 and IL-15) or by engagement of the activating Ly49H or NK1.1 receptors. Interestingly, this enhanced response occurs both in cells that have not undergone division and those that are a product of division. Unlike viral antigen-driven memory NK cells [10], these memory-like NK cells do not possess a distinct cell surface phenotype nor do they respond with enhanced cytotoxicity upon restimulation [21].

Although cytokine signaling is required for the generation of MCMV-induced memory NK cells [11], Yokoyama and colleagues showed that cytokine activation alone can elicit long-lived NK cells that provide an enhanced response upon restimulation [21]. The mechanism by which cytokine signaling generates memory-like function in NK cells is still unknown. No similar properties are found in B and T cell adaptive immune memory, because activation itself requires antigen-receptor signaling and co-stimulation [22]. However, it is possible that upon cytokine-induced activation, epigenetic changes occur at certain loci in NK cells, imprinting a memory-like phenotype. Indeed, a similar situation occurs during T helper 1 and T helper 2 differentiation at the *Ifng* and *Il4* loci, and during memory T cell generation [23–25]. An epigenetic mechanism could account for why the progeny of the transferred cells – which have not been exposed to the activating cytokines *in vitro* – display this memory-like enhanced function [21]. However, it is important to note that, on a per cell basis, memory-like NK cells are not producing more IFN-γ. Rather, the frequency of these memory-like NK cells producing this cytokine is higher than it is in the control NK cell population. NK cells constitutively express IFN-γ mRNA transcripts [26], so it is likely that epigenetic modifications are not being made at the *Ifng* locus, but rather at other loci responsible for post-transcriptional or post-translational regulation of this cytokine.

Although MCMV-induced memory NK cells are found in both lymphoid and nonlymphoid tissues [10], and hapten-induced memory NK cells are found in the liver [27], there is no defined reservoir for cytokine-induced memory-like NK cells. Yokoyama and colleagues have found a modestly higher frequency of memory-like than control NK cells in the lymph nodes at 7 days post-transfer [21]. This trafficking pattern is not explained by expression of CD62L, because both memory-like and control NK cells express similar levels. However, the authors did not look at later time points, so it is unclear whether these memory-like NK cells take up residence in a specific organ. A recent study by Sijts and colleagues has provided some insight [19]. The authors have demonstrated that influenza virus infection triggers migration of NK cells into the airways and proliferation of both immature and mature NK cells in the bone marrow (BM) but not spleen, lung, draining lymph node, and bronchoalveolar lavage. Adoptively transferred NK cells from influenza virus-infected mice survive longer than a few weeks in naïve recipient mice. These long-lived NK cells undergo homeostatic proliferation (Box 2) and secondary expansion in the BM in response to not only influenza virus infection, but also respiratory syncytial virus (RSV) infection. There is some evidence that the activating receptors NKp46 and NKp44 recognize HA of influenza virus [28,29]. However, Sijts and colleagues have seen a similar phenomenon during infection with the unrelated respiratory virus RSV, suggesting that these long-lived NK cells are generated not in response to a virus-specific antigen, but rather to inflammatory cytokines [19]. Respiratory illnesses often generate a 'cytokine

Box 2. The special case of homeostatic proliferation

Mature NK cells undergo homeostatic proliferation when transferred into recipient Rag × Il2rγ-deficient mice. These NK cells persist and are long lived, similar to MCMV-induced memory NK cells [65]. The kinetics of these NK cells undergoing homeostatic proliferation resemble those of Ly49H$^+$ NK cells during MCMV infection. NK cells transferred into an immunodeficient recipient undergo expansion, peaking at day 5, and then go through a contraction phase over the next 2 weeks. Surprisingly, a small but stable pool of NK cells is maintained for up to 180 days, residing in both lymphoid and nonlymphoid tissues [65]. When compared to naïve NK cells from intact C57BL/6 mice, these homeostasis-driven NK cells have increased expression of IFN-γ transcripts at day 7 and enhanced production of IFN-γ and expression of CD107a, a marker of degranulation, in response to Ly49H and NKp46 stimulation. Not only are a higher percentage of these homeostasis-driven NK cells producing IFN-γ, but also they produce more of this cytokine on a per cell basis. Although these NK cells become activated and proliferate in response to MCMV infection even 60 days after transfer, whether they mediate enhanced effector function or protection from secondary challenge has not as yet been addressed.

What are the forces driving homeostatic proliferation and the generation of a long-lived population of NK cells? Homeostatic proliferation of T cells is driven not only by cytokines, such as IL-7, IL-15, and IL-21, but also by the interaction of T cell receptor (TCR) with self-MHC [66]. Although IL-15 is required for NK survival during homeostatic proliferation, there is a dearth of evidence on the other factors necessary for this process [67–69]. One possibility is that NK cells require interaction with self-MHC, much in the same way that T cells do. During development in the BM, NK cells expressing inhibitory receptors that recognize self-MHC class I are 'licensed' to become functional effector cells [70]. NK cells lacking these receptors become hyporesponsive, and are considered 'unlicensed'. Thus, interaction with self-MHC could be another factor driving the formation of these long-lived NK cells during lymphopenia-induced proliferation. Raulet and colleagues, however, have shown that NK cells proliferate similarly when transferred into irradiated wild-type and β2-microglobulin-deficient hosts, suggesting that interaction with self-MHC is not a significant factor in this process [68]. Although naïve T cells require this interaction during homeostatic proliferation, memory T cells do not, suggesting that these long-lived NK cells may indeed be driven solely by cytokine signals. Further research is necessary to tease apart the requirements for generating these homeostatic-expanded NK cells.

storm', and a recent study has measured cytokine levels in the plasma of patients with severe influenza and found elevated levels of IL-12, IFN-γ, and IL-6 during infection [30]. Thus, it is possible that cytokine activation alone is generating these long-lived NK cells. These data provide evidence that the BM may be the long-term residence for these cytokine-induced memory-like NK cells.

Several questions about these cytokine-induced memory-like cells remain. It is unclear whether this represents the *in vivo* situation during infection or other inflammation. Indeed, in the Yokoyama study, initial cytokine activation occurred *in vitro*, and cells were only again restimulated for cytokine production *ex vivo* [21]. Future experiments should interrogate the responses by these memory-like NK cells in an *in vivo* infection model. In addition, it is unclear whether activation by cytokines *in vivo* could generate and maintain such a population. In terms of host protection during infection, does it make sense to generate a population of non-specific memory-like NK cells?

Whether this phenomenon is physiological may not be the most important issue at hand. Adoptive immunotherapy utilizing purified NK cells is now being tested in patients with cancer and those undergoing hematopoietic stem cell transplantation [31,32]. Cerwenka and colleagues have demonstrated that cytokine-induced memory-like NK cells cooperate with $CD4^+$ T cells to mediate effective antitumor activity *in vivo* [33]. Moreover, Fehniger and colleagues have asked whether cytokine activation could induce human NK cells to exhibit memory-like function [34]. Using their newly developed method for long-term culture of human NK cells *in vitro*, they have found that a higher frequency of pre-activated human NK cells produces IFN-γ upon restimulation with cytokines or K562 targets, even after cell division. The authors saw this enhanced function in both the $CD56^{bright}$ and $CD56^{dim}$ NK cell populations. This functional enhancement is not exhibited by NK cells that have been preactivated by coculture with K562 targets or by cross-linking of CD16 alone, suggesting that activation by cytokines uniquely imprints this memory-like enhancement of function. Similar to mouse memory-like NK cells, cytotoxicity is not enhanced. In contrast to mouse NK cells [21], human memory-like NK cells exhibit a distinct phenotype compared to their naïve counterparts, including higher expression of CD94, NKG2A, NKp46, and CD69 [34]. Expression of these markers, as well as NKG2C, correlates with the expression of IFN-γ by preactivated $CD56^{dim}$ NK cells. Although the authors have not described a mechanism, they have not ruled out a contribution by IL-12 receptor and phosphorylated STAT signaling. Additionally, as with mouse NK cells, they found no increase in IFN-γ transcript levels between preactivated and control NK cells. Thus, it remains to be determined what drives enhanced IFN-γ production by memory-like NK cells in both mice and humans. Despite this, however, these studies have provided insight into ways of enhancing NK cell function (Figure 1, center panel). It is a promising new approach that may lead to better NK cell immunotherapy.

Liver-restricted memory NK cells

Von Adrian and colleagues have demonstrated immunological memory in NK cells using a model of hapten-induced contact hypersensitivity (CH), which is a form of delayed-type hypersensitivity (DTH) induced by chemical haptens such as 2,4-dinitro-1-fluorobenzene (DNFB) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone) [27]. Mice are sensitized on the shaved abdomen or back with hapten, followed by challenge (referred to as elicitation) on the ear 4–7 days later with a nonirritant dose of hapten (no swelling without sensitization) and monitoring of ear swelling thereafter. This ear swelling can be detected only when the haptens used for sensitization and challenge are identical. CH was previously thought to be mediated only by T cells, but new findings suggest that NK cells in Rag-deficient mice can mount a CH response against haptens [35] (Figure 1, right panel). Interestingly, adoptive transfer of NK cells to naive Rag × Il2rγ-deficient mice has revealed that CH-mediating NK cells can only be isolated from the liver of hapten-sensitized mice. Further studies have demonstrated that $Thy1^+CD11b^+CD27^-$ and $Thy1^+Ly49C/I^+$ hepatic NK cells mediate CH [36]. $Thy1^+$ NK cells are known to be immature cells recently derived from BM [37], but NK cells can acquire the expression of Thy1 after activation [38,39]. These data indicate that mature $CD11b^+CD27^-$ and $Ly49C/I^+$ NK cells can be activated after sensitization by haptens; however, unlike MCMV-induced expansion of $Ly49H^+$ NK cells, stimulation by haptens does not induce preferential proliferation of this subset [27]. They also showed that hapten-modified B cells are preferentially killed by hepatic NK cells isolated from mice sensitized with the identical hapten [35]. These observations clearly indicate that hapten-specific NK cells are able to recognize hapten-modified molecules on the surface of B cells. Interestingly, hapten-specific NK cells are dependent upon chemokine CXC receptor (CXCR)6 for their function [35]. Chemokine CXC ligand (CXCL)16, a unique ligand for CXCR6, is constitutively expressed on liver sinusoidal endothelium and known to promote the survival of invariant NKT (iNKT) cells [40]. CXCL16 itself inhibits cytotoxicity against hapten-modified B cells *in vitro*, and NK cells from Cxcr6-deficient mice or mice treated with anti-CXCR6 or anti-CXCL16 monoclonal antibody fail to mount CH responses and are unable to kill hapten-modified B cells [35]. Surprisingly, they show similar DTH responses against not only haptens, but also virus-like particles of HIV, influenza, and UV-attenuated vesicular stomatitis virus [35]. How many diverse antigens can NK cells recognize? NKp46, an activating receptor expressed by all NK cells, has been reported to recognize the HA protein of influenza virus. However, these authors have shown that virus-like particles of HA-deficient influenza are still able to vaccinate NK cells [35]. In their initial report, treatment with anti-NKG2D at challenge efficiently suppressed NK cell-mediated CH to haptens [27]; however, NKG2D does not generate diversity via gene recombination or alternative splicing. Curiously, $CXCR6^+$ NK cells (or $Thy1^+CD11b^+CD27^-$ or $Thy1^+Ly49C/I^+$ NK cells) can be found in spleen or liver of unsensitized mice, but they are unable to mediate CH or kill hapten-modified B cells *in vitro*. These results indicate that dual stimulations – liver specific cytokines or chemokines (including CXCL16) and inflammatory stimuli associated with sensitization by haptens or immunization by virus-like particles – are a prerequisite for the induction

of antigen-specific NK cells or the expression of putative antigen-specific receptors in hepatic NK cells.

Another question to be addressed is the mechanism of NK cell-mediated CH. Traditionally, T cell-mediated CH is classified into three steps. The first step involves the migration of dendritic cells (DCs) from the site of hapten application to the draining lymph node (LN). Recent studies have suggested that Langerin$^+$ dermal DCs, which are distinct from Langerhans cells, are the most potent subset for the induction of CH [41–44]. In hapten-sensitized skin, proinflammatory cytokines produced by mast cells are crucial for the efficient migration of DCs [45,46]. The second step is the priming of hapten-specific T cells. In the draining LNs, several co-stimulatory molecules, such as CD86 and OX40 ligand, are responsible for the priming of hapten-specific T cells [47,48]. The third step is the reactivation of effector T cells. Previously primed effector T cells can induce edema at the skin upon secondary hapten exposure. This effect is dependent on cytotoxic granules and inflammatory cytokines such as IFN-γ and IL-17 [49–52]. These cytokines induce CXCL1 and CXCL2 and recruit neutrophils to hapten-challenged skin [49]. Although little is known about the mechanism of NK cell-mediated CH, Rouzaire and colleagues have reported that neither mononuclear cells, neutrophils, nor cytotoxic molecules such as granzyme B, tumor necrosis factor (TNF)-α, Fas ligand nor TNF-related apoptosis-inducing ligand (TRAIL) are detected in the inflammatory skin of NK cell-mediated CH [53]. Moreover, in T cell-mediated CH responses, the swelling of ear skin increases upon repeated challenge with hapten, but curiously no enhancement is observed in NK cell-mediated CH.

Concluding remarks

Here, we have reviewed the three major paths leading to the generation of memory NK cells that have been recently described in the literature. The major underlying definition that links these diverse viewpoints is that memory NK cells are long lived and exhibit a recall response. Beyond these points, each model has shown distinct requirements for tissue distribution, effector function, and antigen specificity (Figure 2). Thus far, it has not been clearly elucidated what drives the formation or maintenance of the NK memory pool; there are likely a variety of factors (cytokines, chemokines, and co-stimulatory molecules) that are required. It is also important to address whether there is overlap between these three distinct types of memory NK cells. For example, can MCMV-induced memory NK cells residing in liver mediate DTH against MCMV? Are MCMV-induced memory NK cells protective against heterologous infections or highly antigen specific as in the CH model? Is IL-12 also important for liver-restricted memory NK cells? Can the addition of IL-12 (and IL-15 plus IL-18) boost MCMV-induced memory or DTH responses? In addition, it is of interest to assess whether certain subsets of NK cells (memory precursor) preferentially generate memory NK cells and whether they can be classified into central and effector memory, as is the case for CD8$^+$ T cells. Future studies are also required to define more clearly the markers of a memory NK cell subset; to investigate other types of receptor–ligand-driven NK cell memory generation; and to elucidate the molecular mechanisms and specificity underlying the generation of memory NK cells.

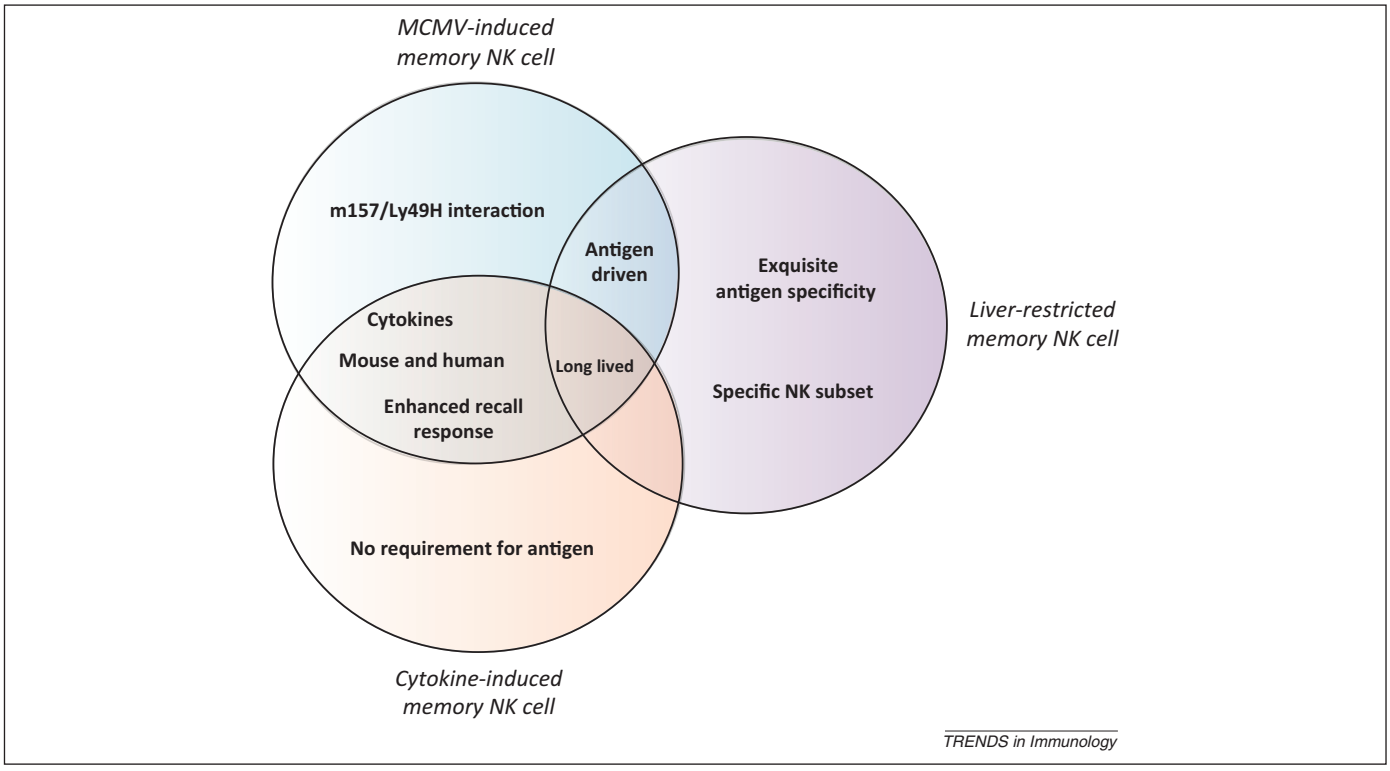

Figure 2. Differences and similarities between the three paths to memory. The Venn diagram illustrates the three major perspectives of natural killer (NK) cell memory that have recently emerged. It is clear that the current definitions of NK cell memory are distinct and show little overlap; this underlines the need to delineate more clearly the mechanisms underlying the formation of memory by NK cells.

Acknowledgments

L.L.L. is an American Cancer Society Professor and supported by National Institutes of Health grants AI066897, AI068129, and CA095137. G.M-O. is a Canadian Institutes of Health Research Bisby Fellow; T.N. is supported by the Uehara Memorial Foundation, the Natio Foundation, Mochida Memorial Foundation for Medical and Pharmaceutical Research, and Japan Society for the Promotion of Science; and D.W.H. is supported by an American Lung Association Senior Research Training Fellowship.

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