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Immunity
Review

Natural Killer Cell Memory

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http://dx.doi.org/10.1016/j.immuni.2015.09.013

Natural killer (NK) cells have historically been considered short-lived cytolytic cells that can rapidly respond against pathogens and tumors in an antigen-independent manner and then undergo cell death. Recently, however, NK cells have been shown to possess traits of adaptive immunity and can acquire immunological memory in a manner similar to that of T and B cells. In this review, we discuss evidence of NK cell memory and the mechanisms involved in the generation and survival of these innate lymphocytes.

Introduction

In recent years, natural killer (NK) cells have become appreciated to possess a number of developmental and functional features in common with cells of the adaptive immune system. These similarities include the development from a common lymphoid progenitor cell (Kondo et al., 1997), a requirement for common γ-chain-dependent cytokines (e.g., interleukin-15 [IL-15]) during development and homeostasis (Di Santo, 2006), expression of the recombination-activating genes (RAGs) during ontogeny (Karo et al., 2014), and a developmental education process that is analogous to T cell development in the thymus (Orr and Lanier, 2010; Sun and Lanier, 2011). Moreover, much like their T and B cell counterparts (which use their activating antigen receptors, T cell receptor [TCR] and B cell receptor [BCR], respectively, to recognize antigens), NK cells express germline-encoded activating receptors capable of directly binding to pathogen-derived or stress-induced self-antigens. It has only recently become appreciated that NK cells can also acquire functional qualities commonly associated with immunological memory in response to pathogens and in non-infectious settings. In this review, we discuss the evidence supporting NK cell memory and the mechanisms involved in the generation and survival of these self-renewing, long-lived NK cells.

NK Cell Memory in Response to Viral Infection

The ability of the immune system to respond rapidly and provide enhanced protection of the host against a previously encountered pathogen is defined classically as immunological memory. Long-lived memory cells are generated after initial infection and display heightened responses upon secondary challenge with the same pathogen. The process of memory formation in T cells has been well studied and is generally divided into three distinct phases (Williams and Bevan, 2007) (Figure 1). Upon exposure to cognate antigen, naive T cells clonally expand and differentiate into effector T cells during the "expansion" phase. This first phase is followed by a second "contraction" phase, where the vast majority of effector T cells undergo apoptosis to form a small, but stable, pool of surviving T cells that then enter the third "memory" phase. Memory T cells then persist throughout the host organs and maintain their longevity through self-renewal until subsequent encounters with their cognate antigen, when they exhibit enhanced effector function and host protection.

Because cells of the innate immune system lack the ability to undergo somatic rearrangement of their receptor genes, it was hypothesized that these cells, including NK cells, lack antigen specificity and therefore cannot develop classical immunologic memory (as defined above). However, in C57BL/6 mice, the activating receptor Ly49H is expressed on ~50% of NK cells and binds with exquisite specificity to the mouse cytomegalovirus (MCMV)-encoded glycoprotein m157 expressed on infected cells to drive the expansion of virus-specific NK cells during the acute phase of MCMV infection (Arase et al., 2002; Brown et al., 2001; Daniels et al., 2001; Dokun et al., 2001; Smith et al., 2002). In an experimental system in which Ly49H$^+$ NK cells were adoptively transferred into mice lacking this receptor, these Ly49H$^+$ cells underwent robust antigen-driven expansion after MCMV infection, and the fate of these antigen-experienced cells was determined (Sun et al., 2009, 2010). After control of the infection, expanded effector NK cells undergo a contraction phase to establish a long-lived and self-renewing "memory" or "adaptive" pool of antigen-specific cells that can be recovered many months after infection in a variety of peripheral tissues (Sun et al., 2009) (Figure 1). Compared to naive NK cells, these memory NK cells display a unique transcriptional signature (Sun et al., 2011b), and compared to naive NK cells from uninfected mice, they possess functional attributes commonly associated with memory T cells, such as secondary expansion, enhanced effector function ex vivo, and increased protection against virus challenge (Sun et al., 2009). This expansion and memory formation of virus-specific NK cells is dependent on the interaction with viral antigen, given that MCMV lacking the glycoprotein m157 does not induce Ly49H$^+$ NK cell expansion or development of memory after infection (Sun et al., 2009). Indeed, a recent study demonstrated that MCMV-primed memory NK cells display reduced "bystander" responses after heterologous infections and IL-12- and IL-18-induced activation in the absence of m157 antigen, suggesting that memory Ly49H$^+$ NK cells become specialized for the purpose of controlling MCMV upon re-exposure (Min-Oo and Lanier, 2014). Together, these results demonstrate that NK cells, like CD8$^+$ T cells, undergo activation, expansion, and contraction in an antigen-specific manner to generate long-lived memory cells in response to viral infection.

In addition to evidence supporting NK cell memory formation during MCMV infection, several studies suggest that NK cells

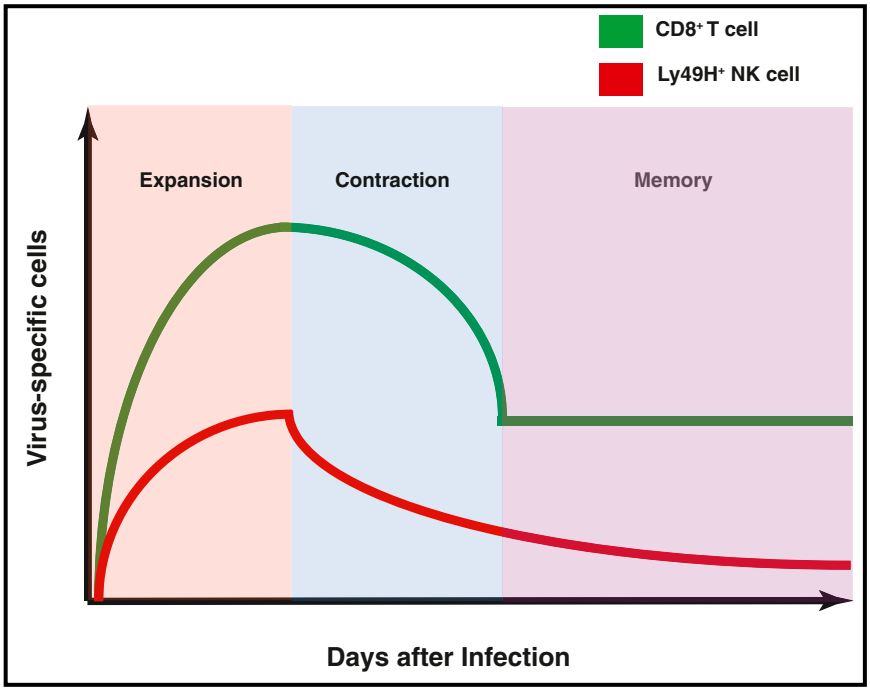


Figure 1. Magnitude and Kinetics of CD8$^+$ T Cell and NK Cell Memory Formation after Infection

Upon exposure to cognate antigen, naive CD8$^+$ T cells clonally expand and differentiate into effector cells during the "expansion" phase. This first phase is followed by a rapid "contraction" phase, when the vast majority of effector CD8$^+$ T cells undergo apoptosis to form a small, but stable, pool of surviving cells that then enter the third "memory" phase. Memory CD8$^+$ T cells persist throughout the host organs and maintain their longevity through self-renewal until a subsequent encounter with their cognate antigen, when they exhibit enhanced effector function and host protection. In an experimental system in which Ly49H$^+$ NK cells were adoptively transferred into mice lacking this receptor, these Ly49H$^+$ cells underwent robust antigen-driven expansion after MCMV infection. Similar to activated CD4$^+$ T cells, expanded effector NK cells undergo a slower and sustained contraction phase to establish a long-lived and self-renewing "memory" pool of antigen-specific NK cells that can be recovered many months after infection in a variety of peripheral tissues.

contribute in secondary immune responses to other viral infections. NK cells previously exposed to herpes simplex virus 2 (HSV-2) or vaccinia virus infection display enhanced interferon-γ (IFN-γ) production and protection upon re-challenge in a process that is specific to the priming virus but independent of the adaptive immune system (Abdul-Careem et al., 2012; Gillard et al., 2011). Adoptive transfer of NK cells from influenza-infected mice into naive recipients generates memory-like NK cells that home to the bone marrow and can respond to subsequent influenza infection (van Helden et al., 2012), although no specific ligand-receptor interaction has been implicated in this study. Longitudinal studies in rhesus macaques have suggested that NK cells are associated with the prevention of disease progression in macaques infected with simian immunodeficiency virus (SIV) (Bostik et al., 2009; Takahashi et al., 2014), and acute infection with SIV induces robust NK cell activation and cytotoxicity (Giavedoni et al., 2000). In addition, a recent study found that in comparison to NK cells from uninfected macaques, NK cells from SIV-infected macaques display enhanced cytotoxicity in vitro to Gag- and Env-pulsed dendritic cells (Reeves et al., 2015). This enhanced effector response is dependent on interactions with NKG2A or NKG2C, because the addition of blocking antibodies abolishes the observed cytotoxic response. Furthermore, this response has been found to be durable and antigen specific, because NK cells harvested from macaques vaccinated with adenovirus expressing SIV Gag or Env only display enhanced cytotoxicity to antigen-matched, but not mismatched, targets and could be measured 5 years after vaccination (Reeves et al., 2015). These results are corroborated by findings demonstrating that hepatic NK cells in mice can mediate recall responses to HIV and influenza-like particles (Paust et al., 2010). Together, these studies collectively support recall responses of memory NK cells in several additional viral models but are limited by unknown interactions between NK cell receptors and cognate pathogen-encoded antigens that mediate these responses. Therefore, the identification of viral antigens and their corresponding activating NK cell receptor pairs that mediate enhanced recall responses in these models will further strengthen the concept of antigen-specific NK cell memory.

Mechanisms of MCMV-Induced NK Cell Memory: Activation and Expansion

Several recent studies have focused on understanding the molecular mechanisms controlling the expansion phase of MCMV-induced memory NK cell generation. Acute MCMV infection induces robust production of pro-inflammatory cytokines such as IL-12, IL-18, type I IFNs, and IFN-γ (Biron and Tarrio, 2015). Although IL-12 and the transcription factor STAT4 are required for activation of NK cells and IFN-γ production, IFN-γ does not act in an autocrine manner to drive NK cell expansion or differentiation (Sun et al., 2012). IL-33, IL-18, and MyD88 signaling further optimizes the expansion of virus-specific NK cells but is not required for the generation of memory NK cells or recall responses (Madera and Sun, 2015; Nabekura et al., 2015). In addition, signals from pro-inflammatory cytokines (including IL-12, IL-18, and type I IFNs) are necessary and sufficient to drive the expression of the transcription factor Zbtb32, which is essential for the proliferation and protective function of antigen-specific NK cells during MCMV infection (Beaulieu et al., 2014). Zbtb32 acts as an important molecular cell-cycle checkpoint to promote a pro-proliferative state in activated NK cells by antagonizing the tumor suppressor factor Blimp-1 (Beaulieu et al., 2014). Although the precise mechanisms of how Zbtb32 antagonizes Blimp-1 function in virus-specific NK cells remain to be elucidated, the finding that pro-inflammatory cytokines are essential for maximal Zbtb32 expression provides a mechanistic explanation of how and why inflammatory signals

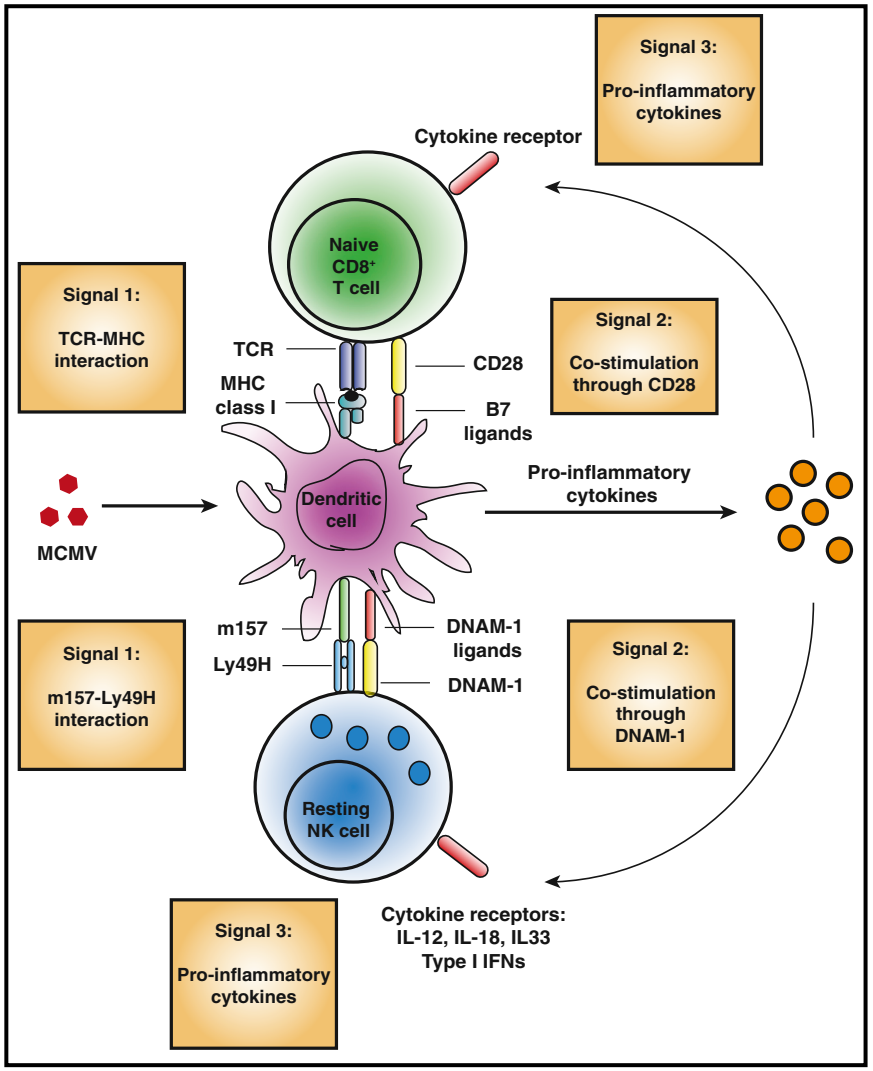


Figure 2. Activation of CD8⁺ T Cells and NK Cells during MCMV Infection

During viral infection, NK cells and CD8⁺ T cells mount specific responses after antigen receptor is triggered. TCR-MHC interactions (signal 1), co-stimulation (including CD28; signal 2), and pro-inflammatory cytokines (including IL-12 and type I IFNs; signal 3) are the three signals thought to promote the activation and clonal expansion of naive CD8⁺ T cells. Similarly, naive NK cells receive signals via activating receptors, co-stimulatory receptors (including DNAM-1), and pro-inflammatory cytokine receptors (including IL-12R, IL-18R, and IL-33R).

are required for the robust proliferation of antigen-specific NK cells during MCMV infection, even when viral antigen is present in high amounts (Sun et al., 2012). This pathway in NK cells might be analogous to "signal 3" in the widely accepted model of T cell activation, which hypothesizes that three independent and coordinated signals from the TCR (signal 1), co-stimulatory receptors such as CD28 (signal 2), and cytokine receptors for IFN-α and IL-12 (signal 3) are required for maximal effector function (Williams and Bevan, 2007) (Figure 2). Indeed, co-stimulatory activating signals are also required for the proliferation of antigen-specific NK cells in the presence of antigen and pro-inflammatory signals, because Ly49H⁺ NK cells lacking the activating receptor DNAM-1 or downstream signaling molecules PKCeta and Fyn fail to expand and form long-lived memory cells after MCMV infection (Nabekura et al., 2014). Thus, the signaling requirements to drive optimal activation and proliferation of antigen-specific NK cells are analogous to their T cell counterparts: receptor engagement with antigen (Ly49H-m157; signal 1), co-stimulatory signaling (DNAM-1; signal 2), and pro-inflammatory cytokine signaling (IL-12, IL-33, IL-18, STAT4, MyD88, and Zbtb32; signal 3) (Figure 2). Whether antigen-specific NK cells require additional transcription factors, cytokines, or co-stimulatory signals for clonal proliferation and memory formation will be an interesting topic for future research.

Mechanisms of MCMV-Induced NK Cell Memory: Contraction and Survival

Induction of apoptosis in effector CD8⁺ T cells after viral clearance is an essential mechanism for preventing immune-mediated pathology by regulating the numbers of cytolytic lymphocytes (Marrack and Kappler, 2004). Therefore, the contraction phase represents a critical determinant in the development of NK cell memory in response to viral infection. During T cell memory formation, mitochondria-associated Bcl-2 family proteins such as Bcl-2 and Bim play contrasting roles in the survival of antigen-specific effector T cells (Grayson et al., 2000; Hildeman et al., 2002; Prlic and Bevan, 2008). Similarly, the majority of effector NK cells downregulate the pro-survival molecule Bcl-2 after MCMV infection (Beaulieu et al., 2014; Min-Oo et al., 2014), and Bim-mediated pro-apoptotic signaling during the contraction phase regulates the size of the memory NK cell pool (Min-Oo et al., 2014). Previous studies have shown that the pro-survival cytokine IL-15 is required for the maintenance of adoptively transferred effector and memory NK cells (Firth et al., 2013). Expression of microRNA-155 functions to suppress Noxa, which has been shown to suppress IL-15-mediated survival by antagonizing Mcl-1 (Huntington et al., 2007), and suppressor of cytokine signaling 1 (SOCS1) during NK cell activation and expansion to enhance survival in response to MCMV infection (Zawislak et al., 2013). However, the survival mechanisms that effector NK cells use to counteract apoptosis during the contraction phase to form memory cells remain poorly defined.

A recent study has demonstrated that most effector NK cells rapidly accumulate dysfunctional mitochondria as a consequence of MCMV-driven proliferation (O'Sullivan et al., 2015), most likely initiating Bim-mediated apoptosis. In contrast, a

small number of persisting effector Ly49H$^+$ NK cells rapidly remove their damaged mitochondria by inducing a self-catabolic process known as autophagy during the contraction phase as a survival mechanism to form a stable pool of memory NK cells (O'Sullivan et al., 2015). Furthermore, the mitophagy-specific receptors BNIP3 and BNIP3L have been found to control the clearance of dysfunctional mitochondria and mitochondria-associated reactive oxygen species (ROS) to promote MCMV-induced memory NK cell generation (O'Sullivan et al., 2015). Given that recent studies have also determined a role for autophagy in the survival of memory B and CD8$^+$ T cells (Chen et al., 2014; Puleston et al., 2014; Xu et al., 2014), it will be of interest to investigate whether mitophagy induction is indeed a hallmark of classic immunological memory formation.

Mechanisms of MCMV-Induced NK Cell Memory: Developmental and Homeostatic Control of NK Cell "Memory Precursors"

During infection, antigen-specific CD8$^+$ T cells are thought to generate a heterogeneous effector population consisting of KLRG1^{hi} short-lived effector cells (SLECs), which are more terminally differentiated, and KLRG1^{lo} memory precursor effector cells (MPECs), which can give rise to long-lived memory cells (Kaech and Wherry, 2007). However, an open question remains as to whether functional heterogeneity or discrete cell subsets exist within effector NK cells or their naive precursors. Recent evidence suggests that compared to KLRG1$^+$ NK cells, KLRG1$^-$Ly49H$^+$ naive NK cells expand to a greater extent and preferentially generate MCMV-specific memory NK cells (Kamimura and Lanier, 2015). The maturation of KLRG1$^-$ to KLRG1$^+$ NK cells can be linked to host-microbiota-derived signals, because acute antibiotic treatment is found to increase the percentage of KLRG1$^-$ naive NK cells. Further analysis of NK cells from T-cell-deficient *Tcrα*$^{-/-}$ and *Rag1*$^{-/-}$ mice has revealed higher expression of KLRG1 on naive NK cells, as well as a reduced capacity for memory cell generation, suggesting that host T cells can also suppress NK cell maturation by limiting IL-15 availability and repressing host NK cell homeostatic proliferation (Kamimura and Lanier, 2015). In addition, other evidence suggests that during ontogeny, RAG has a cell-intrinsic role that confers enhanced cellular fitness of the naive NK cells responding to MCMV infection. In RAG "fate-mapping" mice, NK cells with a history of RAG expression have been found to express lower amounts of KLRG1 and preferentially generate memory NK cells (Karo et al., 2014). Because peripheral Ly49H$^+$ NK cells do not express RAG proteins during homeostasis or MCMV infection, RAG expression during development can introduce functional cellular heterogeneity in the naive NK cell repertoire, favoring memory NK cell precursor generation (Karo et al., 2014). Collectively, these studies support the hypothesis that both cell-intrinsic and environmental factors can select for subsets of naive NK cells with enhanced memory-formation capacity, although it still remains unknown whether effector NK cells also possess heterogeneous phenotypic markers to distinguish their capacity to generate memory cells.

Human NK Cell Memory in Response to Viral Infections

In humans, NK cells are also essential for controlling viral infections. Patients with rare genetic deficiencies resulting in diminished NK cell numbers or function have an increased susceptibility to infection with herpesviruses, including Epstein-Barr virus (EBV), HSV, human cytomegalovirus (HCMV), varicella zoster virus, and human papillomavirus (Orange, 2002). Interestingly, NK cells expressing the activating CD94-NKG2C receptor are found at a higher frequency in HCMV-seropositive healthy individuals than in HCMV-seronegative individuals (Gumá et al., 2004; Gumá et al., 2006; Lopez-Vergès et al., 2011). One study has documented a T-cell-deficient newborn who is acutely infected with HCMV and in whom greater than 80% of the NK cell population expresses NKG2C as viral loads increase, suggesting a prolific expansion of this subset (Kuijpers et al., 2008). Although the HCMV-induced viral (or host) ligand that drives NK cell proliferation remains unknown, virally induced HLA-E has been shown to be critical for triggering expansion of NKG2C$^+$ NK cells in response to HCMV in vitro (Rölle et al., 2014). Similar to the Ly49H$^+$ NK cell response against MCMV infection, IL-12 produced by inflammatory CD14$^+$ monocytes has been implicated as a critical factor in driving the differentiation and prolific expansion of NKG2C$^+$ NK cells through induction of CD25 in response to HCMV infection (Rölle et al., 2014). Furthermore, NKG2C is most likely important in the recognition of HCMV because human NKG2C$^+$ NK cells robustly expand in allogeneic-transplant patients during acute HCMV infection (Della Chiesa et al., 2012; Foley et al., 2012a; Foley et al., 2012b; Lopez-Vergès et al., 2011). NKG2C and the maturation marker CD57 are also co-expressed at high amounts in a unique subset of NK cells that remain at an increased percentage in HCMV-seropositive individuals and further increase after HCMV reactivation (Foley et al., 2012a; Lopez-Vergès et al., 2010; Lopez-Vergès et al., 2011). In humans with a null allele of the gene (*KLRC2*) encoding NKG2C, NK cell differentiation is compromised during HCMV infection, resulting in altered adaptive immunity and elevated anti-HCMV immunoglobulin G (IgG) titers (Goodier et al., 2014; Muntasell et al., 2013), demonstrating the importance of this receptor in defense against HCMV. In contrast, NKG2C-null humans are still able to control HCMV infection, possibly through expression of activating killer cell immunoglobulin-like receptors (KIR) on NK cells (Béziat et al., 2013) or other receptors associated with non-DAP12 adaptor chains (Schlums et al., 2015), demonstrating compensatory mechanisms that human NK cells can use to combat HCMV infection.

Although NKG2C$^+$ NK cells have been observed to expand in patients with other viral infections such as hepatitis C virus (HCV), hepatitis B virus (HBV), chikungunya virus, or HIV (Béziat et al., 2012; Brunetta et al., 2010; Gumá et al., 2006; Petitdemange et al., 2011) and can rapidly proliferate robustly after hantavirus infection to persist for over a year (Björkström et al., 2011a), this occurs only in individuals who have been previously infected with HCMV. NKG2C$^+$ NK cells have also been reported at higher frequencies in the peripheral blood of HCMV$^+$ children co-infected with EBV than in the blood of children with HCMV infection alone (Saghafian-Hedengren et al., 2013). However, a recent longitudinal analysis of college students has shown that acute EBV infection does not cause an expansion of peripheral NKG2C$^+$ NK cells, regardless of previous infection status with HCMV (Hendricks et al., 2014). Similarly, another study has shown that HSV-2 infection does not significantly alter the NK cell repertoire, given that no specific expansion of NKG2C$^+$ NK

cells was observed (Björkström et al., 2011b). Together, these studies suggest that the expansion of NKG2C$^+$ NK cells might be specific to HCMV infection rather than a generalized response to acute herpesvirus infections. Although it remains unknown whether co-infection with certain viruses can promote subclinical reactivation of HCMV and subsequent expansion of NKG2C$^+$ NK cells, it is evident that prior HCMV infection is necessary for this process to occur.

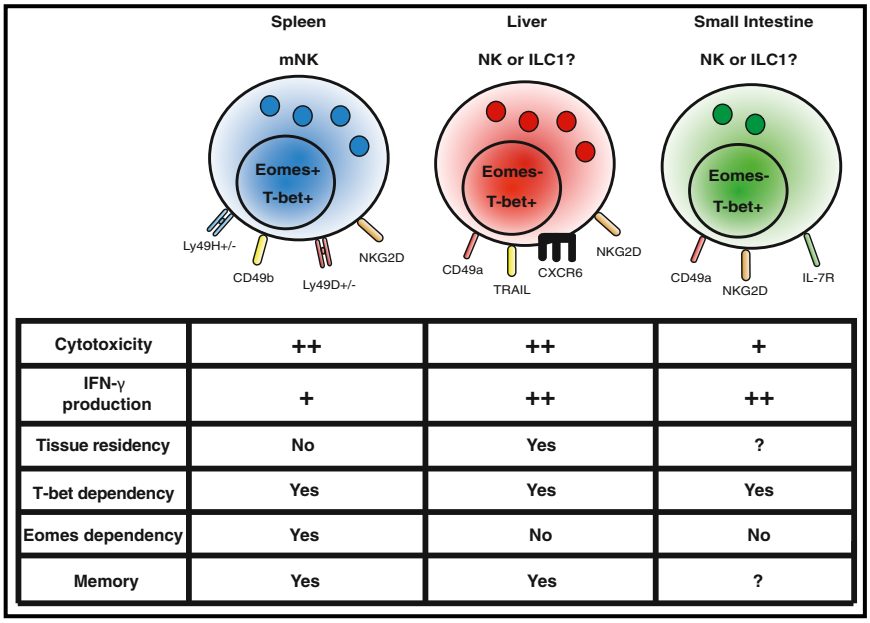
The question still remains as to whether human NK cells have specificity to other viral infections in the absence of HCMV. Many studies have suggested that NK cells differ in their responses to HIV-1 infection depending on the individuals' KIR and human leukocyte antigen (HLA) genotypes (Martin et al., 2007), and HIV-1-encoded proteins can down-modulate surface ligands for certain activating NK cell receptors (Matusali et al., 2012; Shah et al., 2010). Using mass cytometry to assess 41 NK-cell-associated receptors, a recent study showed that human NK cell receptor diversity increased during an anti-viral response to either West Nile virus or HIV-1 in vitro and thus led to a higher frequency of CD57$^+$ cells and higher production of IFN-γ after incubation with HIV-1-infected targets (Strauss-Albee et al., 2015). NK cell receptor diversity has been found to be lower in newborns than in adults and associated with an increased risk of HIV-1 acquisition in adults (Strauss-Albee et al., 2015), suggesting that throughout the lifespan of humans, NK cells might become specialized to previously encountered pathogens, potentially limiting their anti-viral effector functions against newly encountered pathogens. In support of this hypothesis, similar to MCMV-elicited memory NK cells, HCMV-elicited human memory NK cells have been found to have reduced IFN-γ production in response to stimulation with pro-inflammatory cytokines IL-12 and IL-18, most likely through lower expression of these cytokine receptors (Schlums et al., 2015). HCMV-elicited memory NK cells also fail to kill autologous activated immune cells in vitro (Schlums et al., 2015), further suggesting that viral infection can lead to diminished "bystander" activation and functional specification of human memory NK cells for HCMV. Although the NK cell receptor diversity was found to be relatively stable during a 6-month time frame in healthy adults (Strauss-Albee et al., 2015), little is known about how NK cell receptor diversity changes during adolescence or acute viral infections and how or whether this receptor diversity persists after infection during the lifespan of the host. Therefore, understanding the precise mechanisms underlying the specificity and diversity of these human "memory" NK cells might enhance the efficacy of vaccine design against HCMV, hepatitis virus, and HIV.

Although NK cell receptor-viral-ligand (or virally induced host ligand) interactions that mediate specific responses to human viruses have yet to be identified, human NK cells can also use antibody-dependent cellular cytotoxicity (ADCC) to directly recognize and kill antibody-coated targets via binding of CD16 on the NK cell to the Fc region of the IgG bound to the target cell (Lanier et al., 1989). Could antibody production during viral infection confer memory and enhance functionality to a subset of NK cells? Recently, a subset of CD56$^+$CD16$^+$ NK cells present in 30% of healthy humans was described to lack expression of the intracellular signaling adaptor FcεRIγ, but compared to FcεRIγ NK cells, they displayed superior ADCC to antibody-coated virally infected target cells (Hwang et al., 2012; Zhang et al., 2013), indicating that these cells might be a functionally distinct subset. The presence of these FcεRIγ$^-$ NK cells was strongly associated with CD57 and NKG2C expression, but because FcεRIγ$^-$ NK cells can also be NKG2C$^-$ and found in HCMV-seronegative individuals, this suggests that FcεRIγ$^-$ memory-like human NK cells are not limited only to HCMV-infected individuals (Zhang et al., 2013), and their formation might depend on other non-DAP12-associated receptors (Schlums et al., 2015). These FcεRIγ$^-$ NK cells also possess lower amounts of transcription factor PLZF, DAB2, and adaptor protein EAT-2 while displaying hypermethylation at the promoter regions of several of these genes, most likely maintaining the memory-like phenotype observed in this subset (Lee et al., 2015; Schlums et al., 2015). Epigenetic regulation of enhanced effector function most likely reflects a key feature of human NK memory subsets because a similar epigenetic "imprinting" of the *IFNG* locus was found in human NKG2C$^+$ NK cells after HCMV exposure (Luetke-Eversloh et al., 2014). However, how these NK cells specifically differentiate in response to infection and maintain the longevity of their responses remains unknown.

NK Cell Memory in Response to Haptens

Although antigen-specific recall responses have been shown for NK cells during certain viral infections, the initial demonstration of antigen-specific recall responses by NK cells was reported in a model of hapten-induced contact hypersensitivity (O'Leary et al., 2006). Previously understood to be caused primarily by T cells (Gorbachev and Fairchild, 2001), immunization with a chemical hapten such as 2,4-dinitro-1-fluorobenzene (DNFB) can induce robust contact hypersensitivity in *Rag*-deficient mice, which lack T and B cells, only upon re-challenge with the same hapten (O'Leary et al., 2006; Paust et al., 2010). NK cells have been shown to be both necessary and sufficient to mediate contact hypersensitivity in this model and were found to accumulate at the site of hapten administration on the skin (O'Leary et al., 2006; Paust et al., 2010). Surprisingly, adoptive-cell-transfer experiments have shown that hepatic NK cells, but not splenic NK cells, mediate the contact hypersensitivity response only to the original sensitizing hapten, suggesting an antigen-specific recall response (O'Leary et al., 2006; Paust et al., 2010). These hepatic NK cells express the chemokine receptor CXCR6 and Thy-1 (CD90) (Paust et al., 2010). CXCR6 is critical for mediating contact hypersensitivity because antibody blocking of CXCR6 or its ligand CXCL16, and experiments using CXCR6-deficient mice, eliminates the hapten recall response (Paust et al., 2010). In this model, many questions, including the identity of the antigen receptor, remain unanswered. The nature of the ligand driving NK cell recall responses, and whether the NK cells sense the haptens themselves, a hapten-modified protein (such as major histocompatibility complex [MHC] class I), or an unrelated ligand induced by hapten treatment, also remains unclear. Furthermore, it remains to be elucidated why previously activated NK cells isolated from the liver are the only population from *Rag*-deficient mice that can mediate anamnestic responses against homologous hapten challenge, and how they might traffic from the liver to the site of hapten challenge is unknown.

Several recent studies have investigated the phenotype of these hepatic NK cells in greater detail. Although the majority



	Spleen mNK	Liver NK or ILC1?	Small Intestine NK or ILC1?
Cytotoxicity	++	++	+
IFN-γ production	+	++	++
Tissue residency	No	Yes	?
T-bet dependency	Yes	Yes	Yes
Eomes dependency	Yes	No	No
Memory	Yes	Yes	?

Figure 3. Functional and Phenotypic Heterogeneity of ILC1s

(Left panel) mNK cells co-express the Tbox transcription factors T-bet and Eomes and require both for their development. mNK cells also express the marker CD49b ($\alpha2\beta1$ integrin), but typically not CD49a ($\alpha1\beta1$ integrin), during homeostasis or after adoptive transfer. mNK cells have been found to re-circulate in parabiotic mice and can be found in the spleen and most peripheral organs.

(Middle panel) CXCR6$^+$ NK cells or ILC1s in the liver express T-bet, but not Eomes, during homeostasis or after adoptive transfer. These cells require T-bet, but not Eomes, for their development. Unlike mNK cells, this liver population expresses CD49a, but not CD49b. CXCR6$^+$ liver NK cells have been found to reside in tissue in parabiotic mice and selectively home to the liver after adoptive transfer.

(Right panel) NK cells or ILC1s found in the small intestine lamina propria neither express Eomes nor require its expression for their development. These cells require T-bet for their development and co-express CD49a and the IL-7 receptor (IL-7R). In the steady state, ILC1s are less cytotoxic than mNK cells but produce IFN-γ after cytokine stimulation. Whether these tissue-localized NK cells or ILC1s reside in tissue or can acquire the property of long-lived memory remains unknown.

of splenic and peripheral mature NK (mNK) cells express the marker DX5 (CD49b [$\alpha2\beta1$ integrin]), a subset of liver NK cells lack expression of DX5, similarly to "immature" NK cells in the bone marrow, and instead express $\alpha1\beta1$ integrin (CD49a) (Yokoyama et al., 2013) (Figure 3). Experiments in parabiotic mice have found these CD49a$^+$DX5$^-$ NK cells to reside in the liver (Peng et al., 2013), and they might represent the CXCR6-expressing NK cells able to specifically mediate hapten recall responses after adoptive transfer (Peng et al., 2013). Another report suggests that these liver-resident CD49a$^+$DX5$^-$ NK cells might be a separate and stable lineage of cells distinct from immature or mNK cells and similar or equivalent to the recently described group I innate lymphoid cells (ILC1s) found in the small intestine lamina propria. The ILC1s in the small intestine have been reported to not express Eomes, which is expressed by mNK cells, but are dependent on T-bet for their development (Klose et al., 2014). Consistent with this hypothesis, liver CD49a$^+$DX5$^-$ NK cells do not express Eomes during homeostasis or after stimulation with IL-12 in vitro and have been found to be dependent on T-bet for their development (Daussy et al., 2014; Gordon et al., 2012). In Eomes reporter mice, liver DX5$^-$Eomes$^-$ NK cells have been found to express CXCR6 and specifically home back to the liver while maintaining their phenotype after adoptive transfer into lymphoreplete hosts, although their trafficking to other peripheral tissues has not been determined (Daussy et al., 2014). Conversely, DX5$^+$Eomes$^+$ mNK cells in the liver do not express CXCR6 and retain expression of both DX5 and Eomes after recovery in both the spleen and liver 2 weeks after transfer (Daussy et al., 2014). These results have been corroborated by findings that Eomes expression is lower in liver DX5$^-$CD49a$^+$ NK cells than in liver DX5$^+$CD49a$^-$ mNK cells (Sojka et al., 2014) and that liver DX5$^-$CD49a$^+$ NK cells retain their phenotype after adoptive transfer into sublethally irradiated recipients (Peng et al., 2013). Because both DX5$^-$CD49a$^+$ and DX5$^-$Eomes$^-$ NK cells have been found to have unique gene-expression profiles in comparison to DX5$^+$ mNK cells in the spleen and do not express DX5 during either homeostasis or hapten challenge following adoptive transfer (Daussy et al., 2014; Peng et al., 2013), they most likely represent the same mature stable subset that is distinct from mNK cells.

Recently, it has been appreciated that NK cells are only one member of a family of innate lymphoid cells (there are three such families: ILC1, ILC2, and ILC3). Although NK cells are considered a member of the ILC1 family (Artis and Spits, 2015), currently there are no definitive criteria to discriminate NK cells from other NKp46$^+$ ILC1-type cells. Eomes, CD49a, CD49b, CXCR6, and TRAIL have been reported to be expressed by different subsets of cells within different tissues (e.g., liver, small intestine, salivary gland, spleen, etc.) at steady state (Cortez et al., 2014; Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014); however, it is unknown whether these are stable traits or whether they can be modulated by the particular microenvironment in the tissue or altered by the activation state of the cells. In summarizing our current knowledge, we will refer to DX5$^+$Eomes$^+$ cells as mNK cells, refer to liver cells with a Nkp46$^+$CXCR6$^+$Trail$^+$CD49a$^+$DX5$^-$Eomes$^-$ phenotype as CXCR6$^+$ NK cells (although these cells might be ILC1s), and refer to small intestine cells with a NKp46$^+$CXCR6$^-$IL-7R$^+$CD49a$^+$DX5$^-$Eomes$^-$ phenotype as ILC1s (Figure 3). Regardless of lineage derivation, these studies collectively support the hypothesis that phenotypically distinct CXCR6$^+$ NK cells can mediate hapten-specific recall responses and can acquire certain facets of immunological memory, although it remains to be determined whether these memory cells are functionally superior to their naive counterparts and precisely how they recognize haptens and maintain their longevity. Given the similarities between NK cells and ILC1s found in the small intestine (Robinette et al., 2015), future studies are

necessary to determine whether other members of the ILC lineage can also acquire memory-like properties.

Although the phenotype of these CXCR6$^+$ liver NK cells has been studied in detail, much less is known about how they mediate memory responses to haptens. Adoptive-transfer experiments in mice have demonstrated that CXCR6$^+$ hepatic NK cells primed with virus-like particles derived from vesicular stomatitis virus (VSV), influenza A, and HIV can also mediate antigen-specific recall responses (Paust et al., 2010), suggesting that these structurally diverse antigens might bind distinct activating receptors capable of recognizing these viral proteins. It has been reported that the activating receptor NKp46 binds to influenza hemagglutinin (HA) (Draghi et al., 2007; Gazit et al., 2006), but HA was not required for NK-cell-mediated contact-hypersensitivity recall responses to influenza particles (Paust et al., 2010). Because the CXCR6$^+$ liver NK cells are not known to undergo rearrangement of receptor-encoding gene segments, their capacity to recognize a diverse spectrum of antigens through specific activating receptors is most likely limited by a germline-encoded receptor repertoire (Lanier, 2005). Although the specificity of the known activating NK cell receptors in mice is not limited to viral proteins, as is the case with Ly49D$^+$ NK cells, which can expand after H-2 alloantigen stimulation and form memory NK cells in the presence of inflammation (Nabekura and Lanier, 2014), CXCR6$^+$ liver NK cells possess fewer known receptors than do mNK cells. These CXCR6$^+$ liver cells do not express Ly49H or Ly49D and express the inhibitory Ly49A, C/I, I, F, and G2 receptors at much lower frequencies than do splenic mNK cells (Sojka et al., 2014). Therefore, it is unlikely that CXCR6$^+$ liver NK cells specifically recognize VSV or HIV proteins through known germline-encoded receptors, because no receptors expressed on mNK cells have been identified for these viruses and because there is no selective pressure for the presence of a specific germline-encoded receptor for HIV given that HIV is not a natural mouse pathogen. Whether an alternative ligand-sensing system exists in NK cells or ILC1s, where antigen receptor genes can be modified somatically by a deaminase (analogous to AID activity during B cell somatic hypermutation) or other DNA-modifying enzymes, remains to be determined.

Consistent with a requirement for pro-inflammatory cytokines in MCMV-driven NK cell memory, hapten-specific recall responses in *Rag*-deficient mice are similarly dependent on IFNs and IL-12 (Majewska-Szczepanik et al., 2013). Hapten-specific NK cell memory can be generated within 1 hr of sensitization after adoptive transfer of hepatic NK cells (Majewska-Szczepanik et al., 2013). Given that this time frame is not sufficient for NK cell proliferation, these cells would presumably have to reside at the site of hapten challenge at a reasonable precursor frequency in order to elicit an antigen-specific recall response. Because DX5$^-$Eomes$^-$ liver NK cells produce robust IFN-γ upon stimulation with IL-12 and IL-18 (Daussy et al., 2014) and hapten administration results in pro-inflammatory cytokine production (Kish et al., 2009), it is possible that these hapten-specific liver NK cells use cytokine receptors to augment memory responses similar to those of virus-specific Ly49H$^+$ NK cells (Madera and Sun, 2015; Sun et al., 2012). The questions remain as to how CXCR6$^+$ NK cells sense hapten-induced inflammation or antigens in the skin and how they are able to traffic to peripheral sites after adoptive transfer. Because DX5$^-$CD49a$^+$ NK cells have been found in the skin of mice (Sojka et al., 2014), it is possible that these cells can traffic to the liver after hapten sensitization and that adoptive transfer of these cells allows for their rapid infiltration into the skin of naive mice prior to sensitization; however, it is not known whether DX5$^-$CD49a$^+$ NK cells isolated from the liver and those isolated from the skin share the same functional properties or phenotypic stability. Thus, further characterization of these tissue-specific NK cells and ILC1s is needed for determining their lineage relationship and functional trafficking capacity during inflammatory settings and thus elucidating the mechanisms by which NK cells can mediate memory responses at peripheral tissue sites.

Cytokine-Induced NK Cell Memory

Even though NK cells can undergo specific recall responses to viruses and haptens (Figure 4), pro-inflammatory cytokines are required for the generation of memory in these models. Could pro-inflammatory signals alone be sufficient to generate NK cells with memory-like properties in the absence of an antigen-driven response? A previous study demonstrated that stimulation of mouse splenic NK cells with IL-12, IL-15, and IL-18 in vitro can generate cells with memory-like properties after adoptive transfer into *Rag1*$^{-/-}$ mice (Cooper et al., 2009). Specifically, cytokine-activated NK cells displayed enhanced IFN-γ production after stimulation ex vivo with either cytokines or activating receptor ligation. This enhanced IFN-γ production could be detected for as long as 4 weeks, and to a lesser extent 12 weeks, after adoptive transfer into naive hosts. Enhanced effector function has been demonstrated to be cell intrinsic, and additional evidence suggests that the enhanced IFN-γ production of the pre-activated cells is also inherited by their progeny, as shown by carboxyfluorescein succinimidyl ester (CFSE) dilution (Cooper et al., 2009; Keppel et al., 2013). Unlike MCMV-induced memory NK cells, cytokine-activated NK cells do not display enhanced cytotoxicity upon re-stimulation (Keppel et al., 2013). These results suggest that cytokine-induced memory-like NK cells are functionally distinct from MCMV-induced memory cells but that prolonged longevity and enhanced IFN-γ production can nonetheless be generated by cytokine stimulation alone (Figure 4). Is there evidence that cytokine stimulation alone can induce memory-like NK cells in a physiologic setting? Indeed, during influenza infection, mouse NK cells are recruited to the lung but have been shown to specifically proliferate in the bone marrow (van Helden et al., 2012). Adoptive transfer of NK cells from infected mice into naive recipients generated memory-like NK cells that homed to the bone marrow and responded to subsequent influenza infection (van Helden et al., 2012). These memory-like NK cells are most likely generated through cytokine activation, because influenza-generated memory-like NK cells also respond to respiratory syncytial virus, suggesting that the recall response is not specific to influenza antigens (van Helden et al., 2012). Future studies are needed to define the specific infectious or inflammatory settings in which cytokine-induced memory-like NK cells can be generated and whether the long-term consequences of such enhanced effector function are deleterious to the host.

Generation of memory-like NK cells via exposure to pro-inflammatory cytokines might also prove to be advantageous for

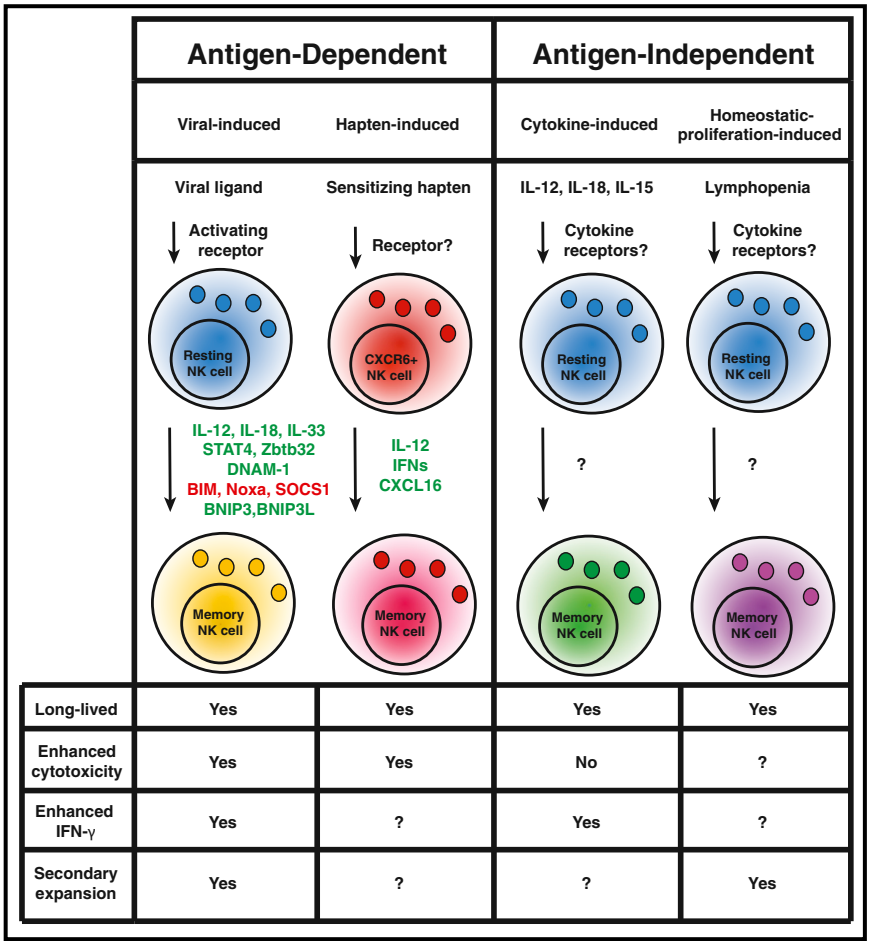


Figure 4. Antigen-Dependent and -Independent Mechanisms of NK Cell Memory Formation

(Left panels) Antigen-dependent memory NK cells. (Far left) Viral-induced NK cell memory is generated during MCMV infection after the cognate recognition of the MCMV protein m157 on infected cells by the activating Ly49H receptor. Memory generation is promoted by IL-12, IL-18, and IL-33 receptor signaling and co-stimulatory signals by DNAM-1, whereas memory NK cell survival is controlled by BNIP3- and BNIP3L-mediated mitophagy (shown in green). Survival of memory NK cells is negatively regulated by BIM, Noxa, and SOCS1 (shown in red). (Middle left) Sensitization with haptens or specific antigens, in conjunction with CXCL16, IFNs, and IL-12, is necessary for generating CXCR6$^+$ memory NK cells. These phenotypically distinct cells might be selected by the cognate recognition of the hapten, a hapten-modified self-protein, or virus-like particle and develop into memory NK cells, although the receptors responsible for the antigen specificity have not been identified.

(Right panels) Antigen-independent memory NK cells. (Middle right) Cytokine-induced memory NK cells are generated after exposure to IL-12, IL-15, and IL-18. These cells apparently do not require activating NK receptor triggering for their longevity or enhanced IFN-γ production upon restimulation; however, the mechanisms governing their longevity are unknown. (Far right) mNK cells undergo homeostatic proliferation when transferred into recipient *Rag2* × *Il2rg*-deficient mice. Similar to MCMV-induced memory NK cells, these NK cells persist and are long lived. The mechanisms driving NK cell homeostatic proliferation and survival in this model remain unknown.

the use of NK cells in the treatment of cancer (Levy et al., 2011; Passweg et al., 2006). A recent study demonstrated that adoptively transferred mouse NK cells pre-activated with IL-12, IL-18, and IL-15 display enhanced anti-tumor efficacy when combined with radiation treatment (Ni et al., 2012). These memory-like NK cells persist in the host for up to 3 months, suggesting that NK cells might provide durable anti-tumor function in therapeutic settings (Ni et al., 2012). Human NK cells pre-activated with IL-12, IL-18, and IL-15 also demonstrate enhanced functionality, consisting of enhanced IFN-γ production after stimulation with cytokines or tumor targets but no apparent increase in cytotoxic potential (Romee et al., 2012). Similar to mouse cytokine-stimulated memory-like NK cells, the progeny of human cytokine-induced cells show enhanced IFN-γ production, suggesting possible epigenetic regulation of the memory-like phenotype in human NK cells (Romee et al., 2012). In line with this hypothesis, NKG2C$^+$ NK cells from HCMV-seropositive individuals have displayed epigenetic "imprinting" at the *IFNG* locus that resembles the same locus in CD8$^+$ memory T cells and T helper 1 cells (Luetke-Eversloh et al., 2014), although it remains unknown whether cytokine pre-activation alone causes epigenetic changes in the *IFNG* locus to enhance memory-like NK cell effector function. Future studies will be needed to elucidate the consequences of epigenetic regulation on human NK cell memory-like effector function and whether other loci are affected during this process.

Homeostatic Proliferation-Induced NK Cell Memory

NK cells do not undergo homeostatic proliferation if they are transferred into lymphoreplete or "normal" hosts (Prlic et al., 2003). However, in NK-cell-deficient hosts, adoptively transferred NK cells rapidly proliferate (Jamieson et al., 2004; Prlic et al., 2003; Ranson et al., 2003) and contract to form a small stable pool that can be maintained for greater than 6 months in both lymphoid and non-lymphoid tissues (Sun et al., 2011a), where they mirror the longevity of Ly49H$^+$ NK cells after MCMV infection. Lymphopenia-driven NK cells produce more IFN-γ on a per-cell basis and show greater cytotoxicity than do naive NK cells when they are stimulated ex vivo 10 days after transfer; however, after the homeostatic expansion and contraction phases, long-lived NK cells regain a quiescent phenotype comparable to that of resting NK cells (Sun et al., 2011a). Although it remains unknown whether homeostatically expanded NK cells have enhanced functionality after contraction or in response to MCMV infection, they can rapidly proliferate in response to MCMV (Sun et al., 2011a), demonstrating that homeostatic proliferation produces longevity without loss of proliferative capacity (Figure 4). Together, these results suggest that homeostatic

proliferation might generate NK cells with memory-like properties; however, the mechanisms that drive memory-like NK cell generation after homeostatic proliferation are not well studied. In naive T cells, homeostatic proliferation can be driven by common gamma-chain cytokines (IL-15, IL-7, and IL-21) and self-MHC interaction through the TCR (Boyman et al., 2012). Given that IL-15 is important not only for NK cell development but also for their survival in vitro in the absence of proliferation, it is not surprising that IL-15 has been found to be critical for the survival of NK cells during homeostatic proliferation (Huntington et al., 2007; Jamieson et al., 2004; Prlic et al., 2003; Ranson et al., 2003). Other factors might drive this process to generate long-lived NK cells. Thus, future studies will be needed to elucidate the requirements for generating homeostatically expanded long-lived NK cells and to further determine the functional similarities and differences between antigen-dependent and -independent memory NK cells (Figure 4).

Concluding Remarks

A plethora of evidence supporting the generation of NK cell memory in both mice and humans now exists in the literature, and the cellular and molecular mechanisms are rapidly being elucidated. Studies on MCMV infection collectively support a model in which NK cells display antigen-specific expansion to form long-lived memory cells that exhibit functional recall responses. The generation of NK cell memory is not limited to pathogenic settings, and pro-inflammatory cytokine signals are generally required for the production of memory-like qualities in NK cells both in the presence and in the absence of receptor-cognate antigen interactions. However, many unresolved questions regarding NK cell memory remain. What are the molecular mechanisms behind hapten-induced NK cell memory? Can other innate lymphoid cell lineages or myeloid cells acquire memory-like properties in response to infection or cytokines? Future studies will be needed to address whether memory NK cells can be generated during viral infections when other activating receptors encounter their cognate ligands and whether functional heterogeneity exists within effector NK cells to specifically give rise to memory cells in a manner similar to that of $CD8^+$ T cells. With promising studies emerging in macaque models, both mouse and human studies are needed to determine whether the NK cell compartment can be harnessed in immunization strategies against viral pathogens for which no vaccine or cure currently exists. Studies elucidating the mechanisms of NK cell memory formation might also prove to be beneficial for therapeutic use in infectious diseases and cancer.

ACKNOWLEDGMENTS

This work was supported by the American Cancer Society, Searle Scholars Program, Cancer Research Institute, and grants from the NIH (AI085034, P30CA008748, and AI100874). L.L.L. is an American Cancer Society Professor and funded by NIH grants AI066897 and AI068129.

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