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Sweet Is the Memory of Past Troubles: NK Cells Remember

Deborah W. Hendricks, Gundula Min-Oo and Lewis L. Lanier

Abstract Natural killer (NK) cells are important in host defense against tumors and microbial pathogens. Recent studies indicate that NK cells share many features with the adaptive immune system, and like B cells and T cells, NK cells can acquire immunological memory. Here, we review evidence for NK cell memory and the molecules involved in the generation and maintenance of these self-renewing NK cells that provide enhanced protection of the host.

Contents

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1 Introduction

Natural killer (NK) cells were once considered without receptors ("null cells"), "non-specific," homogeneous, and short-lived. Much has changed in the four decades since these cells were named "natural killer" cells in 1975 by Kiessling et al. (1975). Activating and inhibitory receptors that regulate their responses have been discovered, specific ligands for many of these receptors have been identified, distinct subsets have been characterized, and self-renewal of mature NK cells has been documented. Recent studies by Blish and colleagues using multiparameter mass cytometry have estimated the existence of 6000–30,000 phenotypically distinct NK cell types in the blood of a healthy adult (Horowitz 2013). Although the capacity of NK cells to acquire memory has only been appreciated recently, hints for the existence of NK cell immunological memory originated in the 1960s, before NK cells were named or were proven to represent a distinct lineage of lymphocytes. In the classic studies of Cudkowicz and Stimpfling, they noted that *H*2 heterozygous F1 hybrid mice rejected parental bone marrow, but more importantly the rejection occurred more rapidly if the F1 mice received a second graft of parental bone marrow cells (Cudkowicz and Stimpfling 1964). Further, the *H*2 heterozygous F1 mice could be rendered tolerant of the parental bone marrow graft if the F1 recipients were inoculated repeatedly with parental splenocytes—suggesting that NK cells could also acquire immunological tolerance (Cudkowicz and Stimpfling 1964). F1 hybrid resistance later was shown to be mediated by NK cells (Murphy et al. 1987). Here, we review evidence for NK cell memory and the mechanisms involved in generating and maintaining memory NK cells.

2 Antigen-Specific Recall Responses in a Contact Hypersensitivity Model

2.1 Hepatic NK Cells Acquire Memory to Haptens and Viruslike Particles

In the past decade, the ability of NK cells to acquire adaptive immune capacity has become an area of major interest. Von Adrian and colleagues first demonstrated NK cell-mediated antigen-specific recall responses using a model of hapten-induced contact hypersensitivity (O'Leary et al. 2006). In this model, chemical haptens, such as 2,4-dinitro-1-fluorobenzene (DNFB) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone), induce a form of delayed contact hypersensitivity in mice. Initially, sensitization is established by applying the hapten in a solvent directly to a section of mouse skin that has been shaved; 4–5 days later, the recall response to challenge is measured by ear swelling after the application of a non-irritating dose of the immunizing hapten (O'Leary et al. 2006). Contact hypersensitivity of this type, where ear swelling is dependent on an identical sensitization and challenge

hapten, initially was thought to be mediated solely by T lymphocytes (Gorbachev and Fairchild 2001). For recognition of specific antigens, T and B cells generate a unique repertoire of T cell receptors (TcR) and immunoglobulins (Ig), respectively, by non-homologous recombination of V, D, and J genetic segments of the TcR and Ig genes driven by the expression of the recombination-activating genes (Rag) (Schatz and Ji 2011). Surprisingly, von Adrian's work showed that Rag-deficient mice, which are devoid of T and B cells, exhibit robust contact hypersensitivity in response to hapten challenge. This response was mediated by NK cells, which were shown to be both necessary and sufficient (Paust et al. 2010). NK cells mediating contact hypersensitivity could be generated from SCID mice, but not from SCID–beige mice, whose NK cells are defective in lytic granule formation (Majewska-Szczepanik et al. 2013). Accumulation of NK cells was also found at the site of inflammation (O'Leary et al. 2006).

Adoptive transfer experiments revealed that hepatic NK cells, but not splenic NK cells, are capable of mounting a contact hypersensitivity response to haptens (Paust et al. 2010). These hepatic cells were found to be mature, $CD11b^{+}CD27^{-}$ and $Ly49C/I^{+}$ NK cells that had also acquired the Thy-1 marker after activation with the sensitizing hapten. Additional characterization of this subset revealed expression of the chemokine receptor CXCR6, on which they are dependent for function (Paust et al. 2010). CXCR6 recognizes the liver-expressed chemokine CXCL16; this receptor–ligand pair has been implicated in cancer metastases (Deng et al. 2010) and in iNKT cell survival in mice (Geissmann et al. 2005). Blocking experiments using either anti-CXCR6 or anti-CXCL16 antibodies in mice, and studies using CXCR6-deficient mice, abolished the hapten recall response by these NK cells (Paust et al. 2010). $CXCR6^{+}$ hepatic NK cells from hapten-sensitized mice were also shown to mediate cytotoxicity against hapten-modified B cells in vitro. In this case, the chemokine CXCL16 inhibited the NK cell-mediated killing of the hapten-modified B cells (Paust et al. 2010). Furthermore, these $CXCR6^{+}$ hepatic NK cells were found to be not only capable of mediating recall responses to haptens, but could also mount a contact hypersensitivity response to viral antigens in the form of virus-like particles carrying distinct viral proteins from HIV, influenza, and attenuated vesicular stomatitis virus (VSV) (Paust et al. 2010). These recall responses to structurally distinct proteins did not require continued exposure to antigen, but were elicited in a similar manner as was seen with chemical haptens. Although fate-mapping experiments have shown that NK cell precursors do transiently express Rag during development (Borghesi et al. 2004), mature NK cells do not have the capacity for Rag-dependent rearrangement of receptor-encoding gene segments, and therefore, unlike T and B cells, their receptor repertoire is presumably exclusively germline-encoded (Lanier 2005). This condition limits the number and diversity of NK receptors and would argue against the ability of NK cells to specifically recognize such a diverse array of antigens via specific receptors. Although the activating receptor NKp46 has been reported to bind to the influenza protein hemagglutinin (HA) (Gazit et al. 2006; Draghi et al. 2007), Paust et al. (2010) showed that HA was not required for NK cell-mediated contact hypersensitivity recall responses to influenza virus-like particles. No specific receptors have

been identified for HIV or VSV on NK cells, and given that HIV is not a pathogen of mice, there is no selective pressure for the evolution of a germline-encoded receptor for HIV. These studies of NK cell memory in the context of skin hypersensitivity point to a requirement of an inflammatory stimulus (sensitization by the hapten) and liver-specific chemokines (CXCL16) for the development of antigen-specific hepatic memory NK cells; however, the exact mechanism remains unclear.

2.2 *Insights into the Mechanism of NK Cell Memory in Contact Hypersensitivity and Skin Inflammation*

T cell-driven contact hypersensitivity has a well-described mechanism that involves the migration of dendritic cells (DC) from the site of sensitization (shaved skin) to the draining lymph node, priming of hapten-specific T cells, and reactivation of these effector T cells. In this case, a role for Langerin^{+} dermal DCs has recently been elucidated; this specific DC subset can induce potent contact hypersensitivity, but is dependent on proinflammatory cytokines at the site of sensitization for efficient migration of DC (Bursch et al. 2007; Kaplan et al. 2005; Kaplan 2010). After antigen-specific activation and clonal expansion in the lymph nodes, effector T cells migrate to the skin where upon challenge they mediate inflammation and swelling (Gorbachev and Fairchild 2001). At the site of hapten challenge, there is recruitment of neutrophils and the production of proinflammatory cytokines (Dilulio et al. 1999; Kish et al. 2009). Curiously, there was no evidence of neutrophil infiltration or cytotoxic molecules at the site of hapten challenge in the case of NK cell-mediated contact hypersensitivity (Rouzaire et al. 2012).

Recently, Majewska-Szczepanik et al. reported that immunological memory of NK cells to a specific hapten could be generated within 1 h of primary sensitization and detected within 30 min after the adoptive transfer of primed liver NK cells into naïve, non-sensitized mice and challenge with hapten (Majewska-Szczepanik et al. 2013). As few as 4500 sensitized liver NK cells could be transferred to elicit the hapten-specific response. The generation of hapten-specific memory NK cells was dependent on IFN-γ, IFN-α, and IL-12 in the sensitized host (Majewska-Szczepanik et al. 2013). Because 1 h after sensitization is insufficient time for NK cell proliferation, these findings imply that these hapten-specific NK cells must preexist in the host at a high precursor frequency. Further, how these liver-resident memory NK cells traffic to the ear and initiate a response within 30 min after challenge raises questions about the nature of the hapten-specific receptors used for recognition and the mechanisms required for hapten-induced trafficking of the NK cells.

A recent study by Peng et al. (2013) delves deeper into characterizing the hepatic NK cells that mediate memory responses in contact hypersensitivity models, taking another step toward understanding the potential mechanisms of action. Here, the authors defined transcriptional, phenotypic, and functional properties of this

particular subset of NK cells. Most mature peripheral and splenic NK cells express CD49b (DX5), a marker commonly used to identify NK cells; however, a subset of NK cells (or cells sharing characteristics with NK cells) was identified in the liver that do not express DX5 but rather express the receptor CD49a. CD49a, also known as α1 integrin, is expressed on various cells, including T and iNKT cells, and has been associated with inflammation (De Fougerolles et al. 2000). Liver NK cells with the $CD49a^{+}DX5^{-}$ phenotype represent a minority of the liver NK cell population, compared to the prototypic $CD49a^{-}DX5^{+}$ subset, but $CD49a^{+}DX5^{-}$ NK cells were shown to be liver-resident and not present in afferent or efferent blood in the liver (Peng et al. 2013). Furthermore, the authors suggest that this unique subset of NK cells originates from stem cells in the liver, rather than from NK cell precursors in the bone marrow (the site of circulating NK cell production). These studies revealed significant differences in the transcriptional signatures of the $CD49a^{+}$ and $CD49a^{-}$ subsets, with increased expression of certain chemokine receptors and adhesion molecules, as well as increased CD69 expression on the $CD49a^{+}$ subset; interestingly, these $CD49a^{+}$ cells were also shown to express some Treg-associated genes (e.g., *Lag*3, *Ikzf*2, and *Egr*2). Finally, this study also confirmed that the liver-resident $CD49a^{+}DX5^{-}$ subset of NK cells are responsible for hapten-induced NK cell memory (Peng et al. 2013). Follow-up reports showed that development of these NK cells is dependent on T-bet, but not Eomes, in contrast to conventional NK cells developing in the bone marrow (Daussy et al. 2014). In human livers, Marquardt et al. identified a similar $T\text{-}bet^{+}Eomes^{-}CD49a^{+}$ NK cell subset, with similar functional properties (high cytokine output, low degranulation potential) (Marquardt et al. 2015). It remains to be seen whether these human hepatic NK cells represent a memory-like subset and are capable of recall responses involved in contact hypersensitivity. Finally, it is unclear whether these $CD49a^{+}$ cells in liver are derived from the NK cell lineage or are members of the innate lymphoid cell (ILC) lineage, which share several characteristics with NK cells.

3 The Development of NK Cell Memory in Chronic Viral Infections

3.1 NK Cell Memory Following Mouse Cytomegalovirus Infection

The findings of von Adrian and colleagues in contact hypersensitivity models suggested that NK cells can acquire properties of immunological memory, including the ability to respond to rechallenge in an antigen-specific way (Paust et al. 2010). Additional support for this concept came from extensive work in a mouse model of viral infection, where virus antigen-driven proliferation triggered by specific, defined ligand–receptor interactions led to the development of a long-lived and self-renewing memory NK cell population [reviewed in Min-Oo et al. (2013), Marcus and Raulet (2013)].

Cytomegalovirus (CMV) is a member of the beta-herpes family of viruses and is known to establish lifelong persistent infections in humans and mice (Goodrum et al. 2012). Although T cells are critical in controlling CMV latency and preventing reactivation (Hanley and Bollard 2014), a role for host NK cells has been established in the acute stages of this infection (López-Botet et al. 2014; Tyznik et al. 2014). This DNA virus has been present in the human population for thousands of years and as such has evolved clever mechanisms of immune evasion, while simultaneously leaving an imprint on the human genome with respect to NK cell recognition of virus (Gumá et al. 2004; Vidal and Lanier 2006; Sun and Lanier 2009; Brizić et al. 2014). In a similar manner, mouse CMV (MCMV) has coevolved with its host for long enough to impact the NK receptor repertoire. NK cells from C57BL/6 (B6) mice carry a germline-encoded activating receptor, Ly49H, that specifically recognizes the MCMV-encoded protein m157 (Lee et al. 2001; Arase et al. 2002; Dokun et al. 2001; Smith et al. 2002). The MCMV model has established a critical role for NK cells in controlling host resistance to infection (Scalzo et al. 1992), which is, in part, driven by recognition of m157 by Ly49H. We took advantage of this very well-defined interacting ligand–receptor pair to assess whether NK cell memory could develop in a manner similar to $CD8^+$ T cell memory and whether it also required antigen-driven proliferation and contraction, prior to the establishment of a long-lived pool of antigen-specific memory NK cells (Sun et al. 2009). In B6 mice, $Ly49H^+$ NK cells expand during the acute phase of infection to make up >80 % of the total NK cell population in the periphery and in MCMV target tissues, such as the spleen and liver, by 7 days post-infection (Dokun et al. 2001). We used an adoptive transfer strategy where $Ly49H^+$ NK cells were transferred into Ly49H-deficient or DAP12-deficient hosts; both recipient strains lack $Ly49H^+$ NK cells and thus provided an opportunity to track their expansion and longevity following MCMV infection (Sun et al. 2009). Here, we observed prolific expansion of $Ly49H^+$ NK cells, driven by recognition of m157 on infected cells, followed by contraction of this subset. The remaining $Ly49H^+$ NK cells formed a long-lived memory cell pool and were detected in tissues as late as 70 days post-infection (Sun et al. 2009, 2010), suggesting that they have the potential for self-renewal or increased survival potential. More recent studies indicate that these $Ly49H^+$ memory NK cells may persist in the host for more than 6 months and possibly more than a year. Assessment of the memory properties of this population revealed an ability to undergo secondary expansion in naïve hosts, enhanced effector functions ex vivo, and the ability to protect against MCMV rechallenge, significantly better than naïve $Ly49H^+$ NK cells (Sun et al. 2009).

Following this initial discovery, several studies have focused on understanding the mechanisms governing MCMV-induced memory NK cell generation (Fig. 1). $Ly49H^+$ cells carry an array of other activating and inhibitory receptors, and it was shown that the generation of memory NK cells was also dependent on the activating receptor DNAM-1, which acts via downstream signaling through Fyn and PKCeta to enhance the $Ly49H^-$-driven expansion of NK cells (Nabekura et al. 2014). Moreover, $Ly49H^+$ NK cell expansion does not occur in the absence of an appropriate inflammatory environment. MCMV, like other viruses, induces a robust

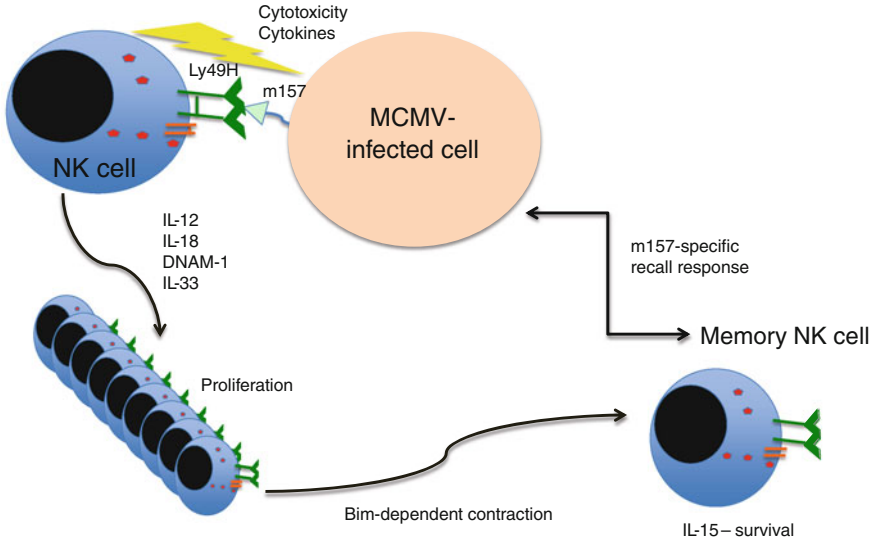

Fig. 1 Generation of MCMV-specific memory NK cells. Schematic representation of the generation of MCMV-specific memory NK cells

cytokine response during acute infection, primarily type I interferons, IL-12, and IL-18, as well as IFN-γ [reviewed in Biron (2015)]. The proliferation phase of $Ly49H^+$ NK cells following MCMV infection is dependent on IL-12 and STAT4 signaling (Sun et al. 2012); on the other hand, IL-15, which is known to be a key NK cell survival factor, was shown to be more critical during the maintenance phase (Firth et al. 2013). IL-18 (Madera and Sun 2015) and IL-33 (Nabekura 2015) are required for an optimal primary response of NK cells to MCMV, but are not required for the generation or maintenance of the memory NK cell population. Moreover, IL-18 and IL-33 are not required for the recall response of $Ly49H^+$ memory NK cells (Madera and Sun 2015; Nabekura 2015). A role for microRNAs in NK cell memory has also been uncovered, as miRNA-155 drives suppression of both Noxa and SOCS1 to allow for proliferation and appropriate expansion of the antigen-specific NK cell subset following MCMV infection (Zawislak et al. 2013).

In a recent report, we established that not all NK cell subsets are equally capable of generating MCMV-specific memory NK cells. $KLRG1^-Ly49H^+$ NK cells preferentially expand, while more mature $KLRG1^+$ NK cells had a reduced capacity for proliferation and memory cell generation (Kamimura and Lanier 2015). The percentage of $KLRG1^+$ NK cells was found to be regulated by host T cells and the microbiota; the mechanism was linked to IL-15 availability, which is in excess in the absence of T cells and limiting in the absence of microbiota and causes a maturation of NK cells from $KLRG1^-$ to $KLRG1^+$ (Kamimura and Lanier 2015). These findings suggest a link between the microbiome and the ability to generate memory NK cells in response to infectious insults.

The proliferation phase of Ly49H$^+$ NK cells responding to MCMV infection bears a striking resemblance to CD8$^+$ T cell expansion during viral infections (Prlic et al. 2007). The contraction of a clonal T cell response is a critical step in the generation of memory T cells. Apoptosis of effector T cells is a key regulatory function to control unhampered immune responses and subsequent immune-based pathology (Kurtulus et al. 2010). Moreover, the reduction in the pool allows for selection of highly functional memory T cells, with increased expression of the prosurvival molecule Bcl-2 (Grayson et al. 2000). Work by a number of groups has identified Bim-mediated apoptosis as the mechanism underlying contraction of T cells after resolution of an infection (Zehn et al. 2014; Hildeman et al. 2002; Pellegrini et al. 2003). Recently, we have demonstrated that this mechanism is also important in the contraction of the Ly49H$^+$ NK cell pool following acute MCMV infection. In competitive cotransfer models, Bim-deficient NK cells showed an identical capacity to proliferate in response to MCMV, but were severely hampered in contraction following peak expansion (Min-Oo et al. 2014). At 30 days post-infection, Bim-deficient memory NK cells composed >90 % of the total memory cells. However, these cells showed a less mature phenotype and were thus unable to functionally respond as well as wild-type NK cells to challenge. This is similar to Bim-deficient CD4$^+$ T cells, where memory functions were impaired due to inappropriate contraction and lack of selection of highly functional CD4$^+$ T cells (Jay et al. 2013).

Although the finding that the Ly49H-m157 interaction in MCMV can generate memory NK cells is intriguing, identifying other models in which antigen-driven proliferation NK cell memory can be validated using defined receptors and ligands remains challenging. Aside from viral proteins, NK cells can also be stimulated and respond to alloantigens, which is the basis for the F1 hybrid resistance in hematopoietic stem cell transplantation, as described previously. Ly49D specifically recognizes H2-D^d, and prior studies had shown that Ly49D$^+$ NK cells can reject allogeneic bone marrow grafts (George et al. 1999). In recent studies, we demonstrated the ability of Ly49D$^+$ NK cells to expand following alloantigen stimulation, in a similar fashion to m157-Ly49H-driven expansion, and like in the case of MCMV infection, inflammatory cytokines, such as IL-12, were essential to expand and generate Ly49D$^+$ memory NK cells (Nabekura and Lanier 2014). This study validated the findings of antigen-specific proliferation driven through Ly49H signaling in a distinct context, here alloantigen presentation versus viral infection. These intriguing findings in mouse models prompted us to explore whether human NK cells also acquire immunological memory.

3.2 *NK Cell Memory Following Human Cytomegalovirus Infection*

Over a decade ago, Guma et al. observed that CMV seropositivity was associated with an increased proportion of NK and T cells that express the activating receptor CD94-NKG2C (Gumá et al. 2004). This subset of NK cells was negative for the

inhibitory receptor CD94-NKG2A and had lower expression of the activating NKp30 and NKp46 receptors. These findings provided the first hint that CMV infection could selectively impact the NK cell repertoire in humans. Subsequently, we provided evidence that expanded on these findings and support the proposition that these CD94-NKG2C^{+} NK cells represent a NK cell memory population in humans (Lopez-Vergès et al. 2011). Our studies have shown that CMV seropositivity is significantly associated with an increase in NKG2C^{high}CD57^{+} NK cells in healthy adults. More importantly, this CD94-NKG2C^{+} NK cell population preferentially expands during acute CMV infection in solid organ transplant patients, but not in individuals who do not reactivate CMV. This NK cell population was persistent and could be detected more than 250 days after onset of viremia. In another study of patients undergoing allogeneic hematopoietic stem cell transplantation, CMV reactivation also led to the expansion of this NKG2C^{high}CD57^{+} NK cell subset (Foley et al. 2012). These cells displayed a mature phenotype, with preferential acquisition of CD57 [a carbohydrate antigen previously shown to be induced on effector CD8^{+} T cells (Focosi et al. 2010)] and KIR. This subset of NK cells continued to expand up to a year after the first detection of CMV viremia. Collectively, these data provide strong evidence that in humans, as in mice, NK cells can be long-lived and may mediate specific recognition of CMV infection.

The expansion of CD94-NKG2C^{+} NK cells has also been observed during other viral infections in humans, leading to the question: Can other viral infections lead to NK cell memory development in humans? Expansion of NK cells expressing CD94-NKG2C has been observed in individuals with chronic hepatitis B or hepatitis C virus (Béziat et al. 2012), in aviremic HIV-1-infected patients (Gumá et al. 2006), and in individuals following hantavirus infection (Björkström et al. 2011). A transient expansion and persistent survival of NKG2C^{+}CD57^{+} NK cells is also seen in individuals infected with chikungunya virus (Petitdemange et al. 2011). Importantly, however, in all these studies, expansion of the CD94-NKG2C^{+} NK cell population occurs **only** if the individual had experienced prior CMV infection. It is possible that infection with certain viruses leads to subclinical reactivation of CMV, although no overt CMV viremia could be detected in the studies of HBV and HCV infection and in hantavirus infection (Béziat et al. 2012; Björkström et al. 2011b). By contrast, in a study of college students experiencing acute infectious mononucleosis caused by Epstein–Barr virus infection, we did not detect an expansion of the CD94-NKG2C^{+} NK cell subset in CMV-seropositive individuals (Hendricks et al. 2014). Taken together, these findings suggest that infection with CMV is absolutely necessary to generate this population of CMV-specific CD94-NKG2C^{+} NK cells, which respond during reactivation of CMV.

These findings in studies of CMV infection lend strong support to the concept of a specific CD94-NKG2C^{+} memory NK cell population in humans. There have been a limited number of studies that have implicated the ability of other pathogens to induce expansion of specific NK cell subsets. In a study of individuals receiving the influenza virus vaccine, the frequency of NK cells expressing high amounts of 2B4 receptor (CD244) increases, while NKp46 expression is downregulated (Jost et al. 2011). This phenomenon is also observed in vitro, with influenza-infected PBMCs.

In individuals infected with *Mycobacterium tuberculosis*, a population of NK cells expressing the T cell memory-associated marker CD45RO was found in the pleural fluid, but not in peripheral blood (Fu et al. 2011). In response to IL-12 stimulation, these cells were more cytotoxic and a higher frequency expresses IFN-γ compared to $CD45RO^{-}$ NK cells. Finally, chronic HIV-1 patients have higher frequencies of mature $CD57^{bright}$ NK cells (Hong et al. 2010).

3.3 Antibody-Dependent Memory-Like NK Cells

One important function of NK cells is antibody-dependent cellular cytotoxicity (ADCC), in which NK cells directly kill antibody-coated target cells via binding of CD16 on the NK cell to the Fc region of IgG bound to the target cell. In humans, CD16 associates with two signaling adapter proteins, CD3ζ and FcεRIγ, expressed intracellularly as either homodimers or heterodimers (Lanier et al. 1989, 1991). Binding of CD16 to IgG initiates the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3ζ and FcεRIγ, facilitating recruitment of the tyrosine kinases, Syk and ZAP70, and resulting in NK cell-mediated cytotoxicity and cytokine production (Lanier 2003). Recently, Kim and colleagues have described a subset of $CD56^{+}CD16^{+}$ NK cells in healthy individuals that lack expression of the signaling adaptor FcεRIγ and have reduced expression of the activating receptors NKp46 and NKp30 (Hwang et al. 2012). Approximately 30 % of healthy individuals possess this NK cell subset, and it is strongly associated with CMV seropositivity (Zhang et al. 2013). These $FcεRIγ^{neg}$ cells are functionally distinct, exhibiting diminished direct killing of targets, but surprisingly superior ADCC compared to $FcεRIγ^{pos}$ NK cells (Hwang et al. 2012; Zhang et al. 2013). In the presence of virus-specific antibodies, a greater frequency of $FcεRIγ^{neg}$ NK cells degranulates (CD107a+) and expresses IFN-γ and TNF-α than $FcεRIγ^{pos}$ NK cells. Lack of FcεRIγ by these NK cells is associated with CD94-NKG2C and CD57 expression, although there are also $CD94\text{-}NKG2C^{-}$ NK cells that are $FcεRIγ^{neg}$. Lee et al. (2015) and Schlums et al. (2015) have shown that these $FcεRIγ^{neg}$ memory-like NK cells also possess distinct transcription factors and signaling proteins, as well as distinct epigenetic profiles. These NK cells express lower levels—both transcripts and cell surface protein—of ITGA6, SIGLEC-7, CD7, PECAM1, and TIM-3, while ILT2, CD2, and FAS are expressed at higher levels (Lee et al. 2015). Expression of Syk is also variable; lack of Syk is associated with CMV seropositivity and is mostly observed in the $FcεRIγ^{neg}$ NK cell subset (Lee et al. 2015). Deficiencies in the expression of the transcription factors PLZF and DAB2 and the EAT-2 adapter protein also identify these $FcεRIγ^{neg}$ memory-like NK cells (Lee et al. 2015; Schlums et al. 2015), while expression of IKZF2 is variable. Hypermethylation at the promoter of several of these genes likely drives the phenotype observed and imparts stability to the subset (Lee et al. 2015; Schlums et al. 2015). These $FcεRIγ^{neg}$ memory-like NK cells exhibit distinct effector function from "conventional" NK cells and epigenetically closely resemble

their $CD8^+$ cytotoxic T cell counterparts (Lee et al. 2015; Schlums et al. 2015). In terms of specificity, these $Fc\varepsilon RI\gamma^{neg}$ memory-like NK cells can respond to a broad range of pathogens, because the specificity is conferred through the antibodies rather than by NK receptors for these pathogens.

3.4 Specificity of NK Cell Memory in Mice and Humans

The discovery of MCMV-induced memory NK cells led to the obvious question: Are these NK cells antigen-specific in their enhanced response to challenge, as was seen in the contact hypersensitivity model? Alternatively, $Ly49H^+$ memory NK cells could have acquired a more generally enhanced functional state. Addressing this question would give insight not only into the mechanisms of MCMV-induced memory, but also into whether these cells could offer any cross-protection to heterologous infections. We interrogated this issue by generating MCMV-induced memory NK cells and assessing their response to secondary infection by unrelated pathogens (Min-Oo and Lanier 2014). Surprisingly, the functional response of MCMV-induced memory NK cells was diminished after stimulation with various cytokines, compared directly to naïve NK cells, and this was governed by reduced signaling through the IL-12-induced STAT4 and IFN-induced STAT1 pathways (Min-Oo and Lanier 2014). This was contrary to the enhanced response MCMV-induced memory $Ly49H^+$ NK cells showed when stimulated through their Ly49H receptor in vitro, producing more IFN-γ and degranulating to a greater extent than naïve $Ly49H^+$ NK cells. These cytokines do play a key role in the recall response to MCMV, but in this case, signals through Ly49H synergize with response to these cytokines. Recently, $CD8^+$ T cells have been revealed to show innate-like and antigen-independent responses to IL-12 during acute MCMV infection (Suarez-Ramirez 2014). By contrast, our results indicate that MCMV-induced memory NK cells do not respond as robustly to challenge with either influenza or *Listeria monocytogenes*, compared to naïve $Ly49H^+$ NK cells, either in persistently MCMV-infected mice or in naïve recipients when adoptively transferred together with naïve $Ly49H^+$ NK cells (Min-Oo and Lanier 2014). These findings reveal that $Ly49H^+$ memory NK cells are highly specialized to respond to MCMV and, in fact, dampen their response to general cytokine stimulation, potentially as a mechanism to avoid uncontrolled NK cell responses to bystander infections in latently infected individuals.

T and B cell memory is antigen-specific, and previously, it was thought that innate immune cells are incapable of memory because these cells express only germline-encoded receptors and were considered “non-specific.” However, it is now clear that CMV infection can expand a population of $Ly49H^+$ NK cells in mice and $CD94\text{-}NKG2C^+$ NK cells in humans. Ly49H specifically recognizes the m157 MCMV protein, and Ly49H has no other known ligands. As yet, the ligand driving expansion of $CD94\text{-}NKG2C^+$ NK cells in humans has not been identified. The CD94-NKG2C receptor binds with low affinity to HLA-E, which is broadly

expressed on healthy cells throughout the body. We suspect that infection with CMV alters the peptide repertoire of HLA-E, generating a high-affinity HLA-E ligand for CD94-NKG2C that drives expansion of these NK cells. Either CMV-derived peptides or new host-derived peptides induced in infected cells serve to generate a high-affinity ligand for CD94-NKG2C.

The selective expansion of CD94-NKG2C$^+$ NK cells in response to infection with CMV has been documented in numerous studies by several independent laboratories. Therefore, CMV-induced CD94-NKG2C$^+$ NK cells provide the most compelling evidence that antigen-specific memory NK cells exist in humans. Although expansion of CD94-NKG2C$^+$ NK cells has been observed during other viral infections [e.g., hepatitis (Béziat et al. 2012), hantavirus (Björkström et al. 2011a), and HIV (Gumá et al. 2006)], this only occurred in individuals who are infected persistently with CMV. Of note, in a longitudinal study of college students at University of Minnesota with Dr. Kristen Hogquist (Odumade et al. 2012), we demonstrated that acute infection with EBV did not expand CD94-NKG2C$^+$ NK cells in CMV-seronegative students. Importantly, EBV infection did not drive expansion of the CD94-NKG2C$^+$ NK cells present in students previously infected with CMV (Hendricks et al. 2014). Coinfection with EBV and CMV did elicit an increased frequency of NKG2A$^+$CD57$^+$ NK cells (that did not express CD94-NKG2C receptors) in the blood of EBV-infected individuals that persisted into latency. It is unclear whether this subset of cells is responding specifically either to viral infection or to the inflammatory cytokines generated during these infections. In another study, herpes simplex virus 2 (HSV-2) infection did not induce the expansion of a specific subset of NK cells (Björkström et al. 2011b). However, the CMV serological status was not available for any of the subjects, and the study was conducted with patients with recurrent genital herpes infection and with asymptomatic HSV-2-positive individuals and did not include HSV-2-negative individuals. Collectively, these findings indicate that like mouse CMV-specific memory NK cells, the human CD94-NKG2C$^+$ NK cells appear devoted to response to CMV and not heterologous pathogens.

4 Cytokine-Driven Memory-Like NK Cells

The ability of NK cells to generate antigen-specific memory is likely limited, if our knowledge is correct that they express only germline-encoded receptors. NK cells that have been activated by cytokines alone have been reported to display memory-like properties. This cytokine-driven memory has been described for both mouse and human NK cells and may provide a useful method for generating these memory-like cells for immunotherapy.

4.1 Cytokine-Driven Memory-Like NK Cells in Mice

While MCMV-specific memory NK cells were being uncovered (Sun et al. 2009), Yokoyama and colleagues described an in vitro method for generating cytokine-driven NK cells with adaptive properties using IL-12, IL-15, and IL-18 (Cooper et al. 2009). NK cells in Rag1-deficient mice were activated with cytokines in vitro and then adoptively transferred into Rag1-deficient recipient mice. After recovery from the recipient mice at a later time, the in vitro cytokine-activated and untreated donor NK cells were phenotypically similar, with comparable expression of CD69, CD11b, CD11c, gp49B, B220, CD122, IL-15Rα, IL-12Rβ1, and CD127. However, in response to restimulation ex vivo with either cytokines or through their activating receptors Ly49H or NK1.1, IFN-γ production by the cytokine-primed NK cells was significantly more robust than their non-activated counterparts. This enhanced response could be detected even three weeks after adoptive transfer into naïve hosts. Interestingly, preactivation of NK cells with cytokines did not lead to enhanced cytotoxicity upon restimulation, making them functionally distinct from MCMV-induced memory NK cells.

The mechanisms driving the generation of these cytokine-induced memory-like NK cells are unclear. While MCMV-specific memory NK cells require exposure to specific antigen, as well as proinflammatory cytokine signaling (Sun et al. 2009, 2012), cytokine-induced memory-like NK cells require cytokine activation alone (Cooper et al. 2009). In this respect, MCMV-specific memory NK cells are similar to B and T cells, which require antigen receptor signaling, costimulation, and cytokines (Zehn et al. 2012). One possible explanation is that activation with cytokines induces epigenetic changes at certain cytokine loci, similar to what has been described for memory induction and maintenance in T cells (Youngblood et al. 2013). NK cells are already poised for rapid IFN-γ production with constitutive production of mRNA transcripts (Stetson et al. 2003), so the question remains as to whether epigenetic modification at the *Ifng* locus would impact the generation of these memory-like NK cells. Epigenetic regulation could, however, provide a plausible explanation for why the progenies of the cytokine-activated NK cells also exhibit enhanced functions (Cooper et al. 2009). These daughter cells were not themselves exposed to cytokines in vitro, suggesting that the mechanism driving these memory-like properties is heritable.

The question remains as to whether these cytokine-induced memory-like NK cells take up residence in a specific organ—as hapten-induced memory NK cells do in the liver (O'Leary et al. 2006)—or whether they are found in both lymphoid and non-lymphoid tissues, as are MCMV-specific memory NK cells (Sun et al. 2009). While a higher frequency of cytokine-activated than non-activated NK cells was described in the lymph nodes at 7 days post-transfer, CD62L expression was similar between the two NK cell populations. Later time points were not analyzed, so the trafficking patterns of these cytokine-induced memory-like NK cells require further study.

Van Helden et al. (2012) suggested that cytokine-induced memory-like NK cells might reside in the bone marrow after influenza infection. While influenza infection induces migration of NK cells into the airways, proliferation of NK cells occurs almost exclusively in the bone marrow. When NK cells from influenza-infected mice were adoptively transferred into naïve recipient mice, a proportion of the cells were long-lived, taking up residence in the bone marrow, undergoing homeostatic proliferation, and expanding in response to subsequent respiratory viral infection. These long-lived NK cells were likely generated through cytokine exposure alone. Although there are reports that the activating receptors NKp46 and NKp44 mediate antigen-specific recognition of influenza virus hemagglutinin (Mandelboim et al. 2001; Arnon et al. 2001), these influenza-generated long-lived NK cells also respond to the unrelated respiratory syncytial virus, suggesting that cytokines—and not viral antigens—are driving these memory-like properties (van Helden et al. 2012). Respiratory viral infection elicits strong production of cytokines. Elevated levels of the proinflammatory cytokines IL-12, IL-6, and IFN-γ are found in the plasma of patients with severe influenza (Heltzer et al. 2009), providing evidence that cytokine-induced memory-like NK cells might be generated in vivo during certain viral infections.

The generation of cytokine-induced memory-like NK cells, which theoretically could respond with enhanced function during any pathogenic insult, might also be problematic to the host. An enhanced response of cytokine-induced memory-like NK cells to subsequent infections without any specificity for the pathogen might result in increased pathology. While this phenomenon could be deleterious to the host in some situations, the ability to generate in vitro NK cells with enhanced function presents an exciting new avenue for NK cell-based immunotherapies. Using mouse NK cells activated with IL-12, IL-15, and IL-18, Cerwenka and colleagues found that these adoptively transferred memory-like NK cells had enhanced efficacy against established tumors when combined with radiation therapy in a mouse model (Ni et al. 2012). This effect was due to their cooperation with $CD4^+$ T cells, which provided the source of IL-2 that drove the rapid proliferation of these NK cells. As has been described for other types of memory NK cells, these cytokine-induced memory-like NK cells were long-lived, persisting for up to 3 months in vivo. Thus, these findings provide compelling evidence that cytokine-induced memory-like NK cells may be beneficial for therapeutic applications.

4.2 Cytokine-induced Memory-Like NK cells

The concept of generating memory NK cells with cytokines provides a means to use NK cells in the treatment of cancer (Levy et al. 2011; Passweg et al. 2006). Ni et al. (2012) and Romee et al. (2012) have developed in vitro culture methods for NK cells, similar to those used in mouse studies. By briefly activating purified human NK cells with IL-12, IL-15, and IL-18 and then resting them for an extended period in the absence of cytokines, these groups generated NK cells with a distinct

phenotype and enhanced function. Compared to naïve NK cells, a higher frequency of the cytokine-induced NK cells produced IFN-γ after stimulation with cytokines or target cells, whereas cytotoxicity was not enhanced. As with the cytokine-induced mouse memory-like NK cells (Sun et al. 2009), this enhancement of function was seen in the progeny of the cytokine-induced cells (Romee et al. 2012). Both the $CD56^{bright}$ and $CD56^{dim}$ NK cell populations exhibited this enhanced function, which was generated through cytokine stimulation alone and could not be recapitulated by cross-linking of CD16 by antibody-coated tumor cells (Romee et al. 2012). Thus, these findings suggest that cytokine preactivation has a distinct impact on the memory-like properties of NK cells. These cytokine-induced memory-like NK cells had higher expression of CD69, NKp46, CD94, and NKG2A, and enhanced function was associated with a $CD94\text{-}NKG2A^{+}$ $CD57^{-}KIR^{-}CD69^{+}$ phenotype (Romee et al. 2012). Despite enhanced IFN-γ production, amounts of *Ifng* mRNA transcripts remained similar between preactivated and untreated NK cells, as were IL-12R and STAT phosphorylation (Romee et al. 2012). Thus, as in mouse, a mechanism for the function of these cytokine-induced human memory-like NK cells remains unclear, although epigenetic regulation is a likely possibility.

5 Generating NK Cell Memory Through Homeostatic Proliferation

NK cells do not undergo homeostatic proliferation when transferred into NK cell-replete hosts (Prlic et al. 2003). In NK cell-deficient hosts, however, adoptively transferred NK cells do proliferate (Prlic et al. 2003; Jamieson et al. 2004), and the kinetics of their expansion and contraction mirror those of the $Ly49H^{+}$ NK cell subset during MCMV infection (Sun et al. 2011). These homeostatically expanded mature NK cells also persist in the host, and a small population can be recovered up to 6 months post-transfer. These long-lived cells reside in both lymphoid and non-lymphoid tissues and activate rapidly in response to MCMV infection. It is unclear whether these homeostatically expanded NK cells will exhibit enhanced function in response to MCMV challenge. However, in vitro at least, these NK cells produce more IFN-γ and show greater cytotoxic potential compared to naïve NK cells from intact mice. Taken together, these findings suggest that homeostatic proliferation may generate NK cells with memory-like properties.

IL-15 is crucial not only for NK cell development, but also for survival during homeostatic proliferation (Prlic et al. 2003; Jamieson et al. 2004; Ranson et al. 2003). Other factors that may drive this process and the generation of a long-lived population of NK cells are still unknown. It is possible that much like their naïve T cell counterparts (which require cytokines as well as TcR:MHC interactions), naïve NK cells require interaction with self-MHC for full maturation (Boyman et al. 2012). During development, NK cells are "educated" through interaction between

their inhibitory receptors and self-MHC class I, in a process known as licensing (Elliott and Yokoyama 2011). Licensed NK cells become fully competent effector cells upon engagement of their activating receptors. In contrast, NK cells lacking inhibitory receptors that recognize self-MHC are unlicensed and are hyporesponsive upon stimulation ex vivo. Raulet and colleagues have shown that adoptively transferred NK cells expand similarly in irradiated wild-type and β2-microglobulin-deficient hosts, suggesting that self-MHC class I is not required for homeostatic expansion of NK cells (Jamieson et al. 2004). However, this study did not assess the persistence of the transferred cells, performing analysis only one week after transfer. Further research is necessary to understand the requirements for generating these long-lived NK cells through homeostatic expansion.

6 Do NK Cells Form Memory After Acute Viral Infection?

In addition to CMV infection, clonal NK cell expansion and memory have been reported in other infection models, leading to the intriguing possibility that antigen-driven memory NK cells may be generated through other ligand–receptor interactions. Mice primed with vaccinia virus were able to mount a protective response to challenge in the absence of B and T cells; $Thy1^+$ NK cells mediated this activity. In this model, $Thy1^+$ primed memory NK cells could also provide protection against lethal challenge following adoptive transfer (Gillard et al. 2011). Similarly, a mouse model of genital herpes infection revealed that NK cells primed by exposure to HSV-2 could mount a stronger functional response to HSV antigens ex vivo (Abdul-Careem et al. 2012). Finally, a report by Van Helden et al. showed that acute infection with influenza virus in mice leads to migration of mature NK cell to the bone marrow to undergo proliferation, thereby generating a long-lived, influenza-experienced subset of NK cells (van Helden et al. 2012).

Viral respiratory infections are responsible for significant morbidity and mortality worldwide, especially among vulnerable populations, such as the very young, the elderly, and those who are immunocompromised (Wendt and Hertz 1995; Vento et al. 2008; Hall 2001). Therefore, it is not surprising that a substantial body of work has been dedicated to interrogating the role that NK cells play during acute respiratory infections [reviewed in Culley (2009), Schultz-Cherry (2015)]. There has been no conclusive evidence for the existence of antigen-driven memory NK cells following these infections. CMV is a persistent virus, and it is possible that virally induced, antigen-specific memory NK cells require continual exposure to antigen to be maintained. To address the question of whether NK cells can mount an antigen-specific memory response to acute respiratory infection, we have investigated the response of NK cells in mice infected with influenza virus and Sendai virus.

6.1 NK Cells in Influenza Infection

Despite widespread vaccination campaigns, influenza continues to be a major global health concern, with seasonal epidemics causing high rates of morbidity and mortality among the elderly and young (http://www.who.int/topics/influenza/en/). As an RNA virus, influenza undergoes rapid mutation and frequent gene reassortment, which allows it to escape immune recognition even by previously exposed or vaccinated hosts and can lead to zoonotic viruses that infect humans (Herfst et al. 2014). Influenza is not a natural pathogen of mice, and mouse models of influenza infection rely on mouse-adapted viruses (O'Donnell and Subbarao 2011; Van den Brand et al. 2014); H1N1 has been shown to cause significant immune-mediated pathology in humans and mice, while H3N2 results in milder disease (Bouvier and Lowen 2010). In B6 mice, infection by a lethal form of PR8 H1N1 results in rapid viral replication and strong inflammatory responses in the airways, with the production of cytokines and chemokines (Buchweitz et al. 2007). The early host response involves rapid infiltration of neutrophils into the lungs. We have observed significant recruitment of NK cells into the lungs, peaking by days 5–6. By 8–10 days following infection, a strong $CD8^+$ T cell response was noted, but mice generally succumbed from significant lung pathology (Min-Oo, unpublished).

Although the role of NK cells in host response to influenza has been controversial and inconclusive (Schultz-Cherry 2015; Jost and Altfeld 2013), it has been reported previously that the activating receptor, NKp46, is critical for the control of influenza virus and ultimate outcome to influenza infection in a mouse model. Gazit et al. (2006) reported that mice bearing a homozygous loss-of-function mutation in *Ncr*1 (NKp46) succumbed to infection earlier than wild-type mice, with higher viral burdens. The interaction between NKp46 and influenza surface protein hemagglutinin (HA) in vitro has also been described (Draghi et al. 2007). We examined whether NKp46 recognition of influenza-infected cells could drive the proliferation of NK cells and generate influenza-specific NK cell memory. Using a competitive adoptive cell transfer system and a mixed bone marrow chimera strategy, we assessed the proliferation of wild-type ($NKp46^+$) versus $Ncr1^{-/-}$ ($NKp46^-$) NK cells following H1N1 or H3N2 infection. Our results showed no evidence of NKp46-mediated proliferation of NK cells in the lungs or airways following infection; moreover, we found no evidence of any NK cell proliferation at the site of infection. In contrast, NK proliferation following influenza infection occurs in the bone marrow, as previously reported (van Helden et al. 2012), and these cells are recruited into the lungs by chemokine-induced trafficking. Given that influenza replication is restricted to the airways, it is likely that the systemic cytokine storm is the driving force behind NK cell proliferation in the bone marrow. These findings suggest that antigen-driven memory NK cells would not be generated in a manner similar to that seen with MCMV, as recognition of virally infected cells is not driving the expansion of NK cells. It does not exclude the possibility of long-lived NK cells primed by cytokines following influenza infection. Further, we observed no

difference in viral titers or mortality in *Ncr*1$^{-/-}$ and wild-type C57BL/6 mice infected with influenza virus strains PR8 or X31, indicating that NKp46 is not involved in the NK cell-mediated responses to flu (Min-Oo et al., unpublished data).

6.2 NK Cells in Sendai Virus Infection

Sendai virus (SeV), also known as mouse parainfluenza virus (PIV) type 1, is a natural mouse pathogen, belonging to the *Paramyxoviridae* family of viruses, that causes respiratory infection and pneumonia (Faísca and Desmecht 2007). SeV has coevolved with its host and is closely related to human PIV type 1, suggesting its utility as a relevant mouse model for human PIV (Gorman et al. 1990). In B6 mice, NK cells become activated and accumulate in the lungs and airways of SeV-infected mice (Hendricks et al., unpublished data). Although NK cells are not required for survival, they are required for control of infection, as mice depleted of NK cells have significantly higher titers than mice with an intact NK cell compartment. Interestingly, the activating receptor NKG2D is significantly upregulated on all NK cells during infection and remains elevated even as viral titers diminish. A thorough analysis of receptor expression indicates that the only other receptor modulated during infection is the inhibitory Ly49G2 receptor. This is not unexpected, however, because the Ly49G2^{+} NK cell subset is expanded during both *Listeria monocytogenes* and MCMV infection and it appears that Ly49G2 serves as a marker for NK cell activation (Barao et al. 2011). The increase in NKG2D expression suggested that this receptor might be specifically recognizing SeV-infected cells expressing NKG2D ligands, much as Ly49H^{+} NK cells specifically recognize the m157 protein from MCMV. However, in mixed bone marrow chimeric mice, reconstituted equally with wild-type and *Klrk*1$^{-/-}$ bone marrow, there was no preferential expansion or accumulation of NKG2D-expressing cells during the course of SeV infection, either systemically or at the site of infection. In addition, the primary sites of NK cell proliferation are the bone marrow and the spleen, not the lungs or airways, which are the sites of infection. Finally, NK cells transferred into NK cell-deficient mice did not undergo significant clonal expansion during SeV infection, as do Ly49H^{+} NK cells in MCMV infection. Taken together, these findings strongly suggest that NK cells do not exhibit specific recognition of SeV and thus are unlikely to form antigen-specific memory. The formation of cytokine-induced memory-like NK cells cannot be ruled out however, and further study is necessary to address that question.

7 Conclusions and Implications

Several general principles have been learned from studies of MCMV-specific memory NK cells. It appears that the expansion and generation of NK cell memory requires recognition of antigen by a receptor that transmits signals through an

ITAM-based signaling pathway, similar to the signals transmitted by surface Ig in B cells and TcR in T cells. NK cell memory also critically requires cytokines, in particular IL-12, that work in concert with antigen receptor-dependent signaling to drive the expansion of the specific NK cells and requires IL-15 for the maintenance of memory NK cells, as also required for the maintenance of $CD4^+$ and $CD8^+$ memory T cells (Ku et al. 2000; Purton et al. 2007). Also like T cells, NK cells undergo contraction after control of an infection by a Bim-dependent process and generate a subset of self-renewing memory NK cells that persist for months. There are a number of unresolved questions about NK cell memory for future investigation. Foremost are questions about the molecules responsible for hapten-specific NK cell memory and whether NK cells can be vaccinated to enhance host protection by deliberate immunization. Studies to reveal the mechanisms for NK cell memory may provide insights into memory mediated by other innate immune cells, as well as opportunities for the therapeutic use of NK cells in infectious diseases and cancer.

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