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Memory responses of natural killer cells

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Abstract

Natural killer (NK) cells have traditionally been classified as a cellular component of the innate immune system, given their ability to rapidly produce effector cytokines and kill infected or transformed cells without prior exposure. More recently, NK cells have been shown to possess features of adaptive immunity such as clonal expansion, longevity, and robust recall responses. NK cell memory can be broadly divided into two categories: antigen-specific and antigen-independent. In the first case, exposure to certain viral or hapten stimuli endows NK cells with antigen-specific immunological memory, similar to T and B cells. In the second case, exposure of NK cells to specific cytokine milieu can imprint long-lasting changes on effector functions, resulting in antigen-independent memory-like NK cells. In this review, we discuss the various conditions that promote generation of these two categories of memory NK cells, and the mechanistic requirements underlying these processes.

Keywords

NK cell; CMV; immunological memory

1. Introduction

Natural killer (NK) cells were first identified in the 1970s as a novel lymphocyte population capable of mediating cytotoxicity against tumor targets without the need for prior sensitization [1–4]. They express a combination of germline-encoded inhibitory receptors, which recognize self molecules such as MHC class I, and activating receptors that bind stress-induced host molecules, pathogen-encoded ligands, and the Fc portion of antibodies [5]. When activating signals outweigh inhibitory signals, the NK cell's effector functions are triggered, leading to production of pro-inflammatory cytokines such as IFN- γ and TNF, and to target cell cytotoxicity mediated by perforin and granzymes.

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NK cells are critical for control of viral infections in both humans and mice. Patients with rare genetic deficiencies in NK cell numbers or functionality are highly susceptible to viral infections, most notably herpesviruses, including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), and varicella zoster virus (VZV), and human papillomavirus (HPV) [6]. Similarly, mice that are genetically deficient in NK cells or have been depleted of NK cells using antibodies have increased susceptibility to infection with mouse cytomegalovirus (MCMV), HSV-1, and vaccinia virus, among others (reviewed in [7]).

Immunological memory, defined as possessing a quantitatively or qualitatively greater response upon secondary immune challenge, is best characterized in mammalian adaptive lymphocytes, but has recently been demonstrated in invertebrates [8], and in innate immune cells within mammals [9]. For T cells, the well-studied process of memory generation can be delineated into three distinct phases: expansion, contraction, and memory [10]. In the expansion phase, naïve T cells that encounter their cognate antigen (foreign peptides presented on MHC molecules) undergo clonal expansion and differentiation into effector cells. This is followed by a rapid contraction phase, during which the majority of effector cells undergo apoptosis to generate a small pool of surviving “memory” cells. These memory T cells maintain their longevity through self-renewal and persist in both lymphoid and non-lymphoid organs. Upon re-encounter with cognate antigen, they display enhanced effector function and host protection.

Although NK cells have traditionally been considered part of the innate immune system, it is increasingly appreciated that they share many features with adaptive lymphocytes. NK cells develop from the same common lymphoid progenitor that gives rise to T and B cells [11]. Like T and B cells, they require common gamma chain-dependent cytokines for their development and homeostasis [12–14], and their responsiveness is tuned through an “education” or “licensing” process analogous to T cell development in the thymus [15–17]. Most notably, studies over the last decade have demonstrated that mature NK cells possess features of immunological memory. This review will discuss the key findings in support of NK cell memory, and the mechanisms involved in the generation and survival of memory NK cells.

2. Antigen-dependent generation of NK cell memory

2.1. Hapten-specific NK cell memory

The first demonstration of antigen-specific recall responses by NK cells was in the setting of hapten-induced contact hypersensitivity (CHS) [18]. Although previously thought to be T cell-mediated, robust CHS was observed following immunization and challenge of RAG2-deficient mice, which lack B and T cells, with a single chemical hapten such as 2, 4-dinitro-1-fluorobenzene (DNFB) [18, 19]. Specificity during challenge was demonstrated when delayed hypersensitivity occurred only when the same hapten (DNFB) was used for challenge, and not a different hapten (e.g. oxazolone). NK cells were shown to accumulate at the site of hapten administration in the skin, and depletion and transfer studies showed that NK cells were both necessary and sufficient for the hapten-specific secondary response. Interestingly, the recall response was found to be mediated by a subset of hepatic NK cells

expressing the chemokine receptor CXCR6, but not by splenic NK cells. Further studies showed the hapten response is mediated by CD49a⁺DX5⁻ tissue-resident cells [20], and required pro-inflammatory cytokines including IL-12, type I and II IFNs [21] and expression of the transcription factor AHR [22]. Antibody-mediated blockade of NKG2D, or molecules involved in trafficking of NK cells, such as CD18 and P- or E-selectin, prevented development of CHS responses at secondary sites [18]. Thus, hepatic NK cells are capable of generating an antigen-specific recall response to haptens (Fig. 1A). However, many questions remain to be addressed, including the identity of the antigen receptor, the precise nature of the ligand, and whether these cells are best classified as mature NK cells or a distinct subset of ILC1s [23].

2.2. MCMV-specific NK cell memory

Antigen-specific responses to pathogens were long thought to be unique to B and T cells that are able to undergo somatic rearrangement of their antigen receptor gene loci. However, antigen specificity has also been demonstrated for the germline-encoded activating receptor Ly49H, which is expressed on a subset of NK cells in C57BL/6 mice, and uniquely recognizes the MCMV-encoded glycoprotein m157 [24–27]. This receptor-ligand interaction results in the activation and proliferation of Ly49H⁺ NK cells [25, 28]. Following adoptive transfer of Ly49H⁺ NK cells into mice lacking the receptor, this virus-specific NK cell compartment expanded up to 1000-fold within 7 days following MCMV infection, before then undergoing a contraction phase to generate a pool of memory cells [29]. These long-lived memory NK cells were found to persist in both lymphoid and non-lymphoid tissues, and were detectable several months after infection.

2.2.1. Properties of MCMV-specific memory NK cells—Compared to naïve NK cells, memory NK cells possess a unique transcriptional profile [30], and show enhanced effector functions, including heightened production of IFN- γ and degranulation [29]. Thus, although memory NK cells proliferate at a similar rate to naïve cells upon secondary MCMV infection, they provide greater protection against lethal challenge [29]. These studies demonstrate that NK cells, like T cells, differentiate through expansion and contraction phases to generate long-lived memory cells in response to viral infection (Fig. 1B).

MCMV-specific memory NK cells have a more mature phenotype than resting cells, including higher expression of KLRG1, CD11b, Ly6C, CD43 and lower expression of CD27. Within the naïve NK cell pool, it is the KLRG1^{lo} subset that have the greatest capacity to expand and form memory [31]. KLRG1 expression on NK cells has been shown to depend on the host microbiota and the availability of IL-15, since acute antibiotic treatment or the presence of an intact T cell compartment to compete for IL-15 leads to a reduced frequency of KLRG1⁺ NK cells. Additionally, KLRG1 expression is regulated in a cell-intrinsic manner by the activity of the RAG recombinase during ontogeny [32]. However, because all memory NK cells express high levels of KLRG1 and yet are able to expand at a comparable level to naïve cells, this suggests that while KLRG1 may be a marker of heterogeneity within the naïve NK cell pool, its expression alone does not determine the proliferative capability of NK cells. Notably, memory NK cells express higher levels of

Ly49H, but not of other activating receptors relative to naïve cells, and the expression of Ly49H is even greater on secondary memory NK cells [33]. This suggests there may be a selection process in which NK cells with the highest avidity for MCMV-infected cells are more likely to form memory cells.

2.2.2. Mechanisms of MCMV-specific memory formation

2.2.2.1. Signal 1: Memory T cell generation requires a trio of independent signals to promote activation, clonal proliferation, and maximal effector function [10]. Signal 1 is the antigen-specific interaction of the TCR with peptide presented on MHC, signal 2 is co-stimulation through receptors such as CD28, and signal 3 is provided by pro-inflammatory cytokines. As discussed above, the Ly49H-m157 interaction plays a critical role in the generation of MCMV-specific memory NK cells, as infection with an MCMV strain lacking m157 (MCMV m157) does not induce expansion or development of memory NK cells [29], while vesicular stomatitis virus (VSV) expressing m157 promotes greater expansion and memory cell formation than VSV expressing an irrelevant antigen (e.g. OVA) [34, 35]. Thus, similar to T cells, NK cells require this ‘Signal 1’ via Ly49H for full activation of effector function (Fig. 2). Notably, infection with MCMV m157 can drive expansion and memory cell generation when an alternative Signal 1 is provided; this was demonstrated for Ly49D⁺ NK cells following infection with MCMV m157 and injection of splenocytes from BALB/c mice that express H-2D^d which is recognized by Ly49D [36]. Future studies are necessary to determine whether other activating receptors, such as NKP46, can promote memory cell formation.

Interestingly, a recent study identified another MCMV-encoded ligand that is recognized by an activating NK receptor. MCMV m12 directly interacts with both activating and inhibitory NKR-P1 family receptors with differing affinities [37]. It will be of great interest to determine if the interaction of m12 with the activating NKR-P1C receptor (more commonly known as NK1.1) can contribute towards the formation of MCMV-specific NK cell memory, either on its own or in combination with the m157-Ly49H interaction.

2.2.2.2. Signal 2: NK cells require the co-stimulatory molecule DNAX accessory molecule-1 (DNAM-1) and the downstream signaling molecules PKC η and Fyn for optimal expansion and differentiation into memory cells [38]. Notably, the DNAM1 ligands CD155 and CD122 are upregulated on dendritic cells and macrophages early after MCMV infection [38]. Recently, it was shown that co-expression of Ly49D promotes the survival and differentiation of Ly49H⁺ memory NK cells [39]. Thus, in this context, it is unclear whether Ly49D behaves as a true ‘Signal 2’, or a second ‘Signal 1’ along with Ly49H. Similarly, NKG2D was found to amplify the proliferation of Ly49H⁺ NK cells, but was unable to drive expansion of NK cells in the absence of Ly49H [40]. Conversely, Ly49H⁺ NK cells that co-express the inhibitory receptors Ly49A or NKR-P1B do not expand as well as MCMV-specific NK cells that lack expression of these inhibitory receptors [39, 41], suggesting that inhibitory receptors may function as brakes to control the extent of NK cell activation and expansion. Together these studies show the importance of a co-stimulatory ‘Signal 2’ for the optimal response of NK cells to MCMV infection (Fig. 2). Future studies are needed to address whether traditional co-stimulatory molecules, such as CD28 and GITR, or

checkpoint molecules, such as CTLA-4 and PD-1, that are known to modulate T cell responses, also play important roles in the formation of MCMV-specific memory NK cells.

2.2.2.3. Signal 3: In addition to providing the viral antigen m157, acute MCMV infection induces production of pro-inflammatory cytokines including IL-12, IL-18, and type I and II IFNs [42]. The IL-12/STAT4 signaling pathway is required for robust expansion of NK cells and for the formation of memory cells [43], serving as an important ‘Signal 3’. Interestingly, although this pathway is required for NK cell production of IFN- γ , IFNGR-deficient NK cells are able to expand and generate memory similar to WT cells, suggesting that autocrine sensing of IFN- γ is dispensable for memory cell formation [43]. In contrast, the ability to sense type I IFN is critical for memory NK cell generation, as the IFNAR/STAT1 signaling pathway protects proliferating NK cells from being killed by other NK cells (in a process known as ‘fratricide’) during the expansion phase [44]. Two members of the IL-1 family of cytokines, IL-18 and IL-33, and their downstream signaling molecule MyD88, are also important for the optimal expansion of MCMV-specific effector NK cells, but are dispensable for the recall response of memory cells to MCMV [35, 45].

These cytokines promote activation and expression of numerous signaling molecules and transcription factors that contribute to the NK cell response to MCMV. Notably, both IL-12 and type I IFN signaling promote expression of Zbtb32, a BTB-ZF transcription factor that was shown to be critical for the proliferative burst of Ly49H⁺ NK cells by antagonizing the anti-proliferative factor Blimp-1 [46]. Pro-inflammatory cytokine signaling also induces expression of a microRNA, miR-155, which promotes anti-viral NK cell expansion and memory formation by limiting expression of Noxa and Socs1 [47]. Noxa is known to mediate apoptosis of resting NK cells by antagonizing the pro-survival factor Mcl-1 downstream of IL-15 signaling [48]. This pathway is likely to be similarly important during the NK cell response to MCMV infection, as effector or memory NK cells adoptively transferred into IL-15 deficient hosts disappeared just as rapidly as naïve NK cells [34]. Thus, several pro-inflammatory cytokines, analogous to ‘Signal 3’ for T cells, are required for maximum formation of NK cell memory (Fig. 2); however, future studies will fully elucidate the downstream pathways and their roles in generating long-lived memory NK cells. Additionally, it will be of interest to determine if inhibitory cytokines, such as IL-10 or TGF- β , play important regulatory roles in controlling the proliferative or effector response of NK cells during MCMV infection.

2.2.2.4. Additional factors mediating contraction and memory: During the generation of memory T cells, Bcl-2 family proteins including Bim and Bcl-2 have opposing functions in regulating the survival of effector cells [49–51]. Likewise, following MCMV infection, the majority of effector NK cells downregulate the pro-survival molecule Bcl-2 [46, 52], and NK cells deficient in Bim are impaired in their ability to trim the effector pool, leading to a larger but less functional pool of cells at memory time points [52]. Bim-mediated apoptosis is likely driven by the rapid accumulation of dysfunctional mitochondria in effector NK cells, as Bcl-2 family members generally operate in the context of the mitochondrial membrane [53]. However, NK cells that are able to clear the damaged mitochondria through

autophagy mediated by Atg3, BNIP3, and BNIP3L, will persist as long-lived memory cells [54].

2.3. HCMV-specific NK cell memory

Similar to the expansion of Ly49H⁺ NK cells following MCMV infection in mice, NK cells expressing the activating heterodimeric CD94/NKG2C receptor are present at higher frequency in HCMV-seropositive healthy individuals relative to donors that are seropositive for other herpesviruses or HCMV-seronegative [55–57]. One report showed that during acute HCMV infection of a T cell-deficient infant more than 80% of the NK cell population expressed CD94/NKG2C, suggesting this subset in humans also possesses prolific proliferative capacity [58]. Furthermore, several studies have described an increase in CD94/NKG2C⁺ NK cells following HCMV reactivation or infection in patients receiving hematopoietic stem cell or solid organ transplant [57, 59–61]. The expanded NKG2C⁺ NK population persisted for months in the absence of detectable viremia [57, 60], and in some healthy individuals was stably maintained for years at elevated frequency [62, 63]. Future studies are needed to determine if sub-clinical or tissue-restricted HCMV reactivation events contribute to the maintenance of the NKG2C⁺ NK pool in different anatomical locations, and whether there are cell intrinsic mechanisms that keep the population stable similar to mouse Ly49H⁺ NK cells or memory CD8⁺ T cells.

2.3.1. Properties of HCMV-specific NKG2C⁺ memory NK cells—The CD94/NKG2C-expressing NK cell subset often co-expresses the terminal maturation marker CD57 but does not express the inhibitory receptor NKG2A, suggesting antigen experience and maturity [57]. During acute infection, NKG2C⁺ NK cells produce more IFN- γ in response to target cells than NKG2C⁻ NK cells from the same donor [60], and have enhanced degranulation when cultured with infected macrophages and HCMV-specific antibodies [64]. Furthermore, NKG2C⁺ NK cells from HCMV seropositive donors expanded more during HCMV reactivation compared to NKG2C⁺ NK cells from seronegative donors, and had enhanced IFN- γ production in response to target cells [65], indicative of a secondary response upon antigen re-exposure. This improved effector cytokine production by memory NK cells is likely mediated by epigenetic remodeling at the *IFNG* locus [62].

2.3.2. Mechanisms of HCMV-specific NKG2C⁺ memory formation—Although the molecular requirements and specific ligand(s) driving expansion of NK cells in response to HCMV infection are still active areas of investigation, there is evidence that Signals 1, 2 and 3 may be important for this response, as they are for the response of LY49H⁺ NK cells to MCMV (Fig. 2). ‘Signal 1’ via CD94/NKG2C receptor engagement of HLA-E was shown to drive expansion of NK cells *in vitro*, and blocking this interaction using antibodies against CD94 or NKG2C, or knocking down HLA-E with a short hairpin RNA, greatly impaired NK cell expansion [66]. Recent studies have suggested that a co-stimulatory ‘Signal 2’ may be provided through the interaction of CD2, which is highly expressed by adaptive NK cells [67], and its cognate ligand CD58, which is upregulated by HCMV-infected fibroblasts [68]. This interaction enhanced production of IFN- γ and TNF- α in response to HCMV-infected fibroblasts [68] or CD16-crosslinking [67]; however, its role in promoting expansion or generation of memory-like properties *in vivo* has not been determined. Finally, IL-12

production by CD14⁺ monocytes may represent a ‘Signal 3’, as it was shown to be important for optimal expansion of NKG2C⁺ NK cells in a co-culture system with HCMV-infected fibroblasts [66]. Future studies are needed to address whether these molecules and interaction are crucial for the NK cell response *in vivo*.

The expansion of NKG2C⁺ NK cells has also been observed in response to certain other viral infections, including hepatitis C, hepatitis B, chikungunya, HIV, and hantavirus [56, 69–72]; however, because this only occurs in HCMV seropositive individuals, these findings suggest that superinfection with other viruses may be driving reactivation of HCMV from latency, and thus triggering the proliferation of NKG2C⁺ NK cells. Interestingly, influenza, YFV vaccination, and HSV-2 and EBV persistent infections, do not appear to elicit an expansion of NKG2C⁺ NK cells regardless of HCMV infection history [73–76]. Additional studies are needed to clarify if these instances of NKG2C⁺ NK expansion in the absence of acute HCMV are due to cross-reactive antigens (either viral or viral-induced host) between CMV and these other infections, to the specific inflammatory milieu elicited, to (subclinical) CMV reactivation, or to some combination of these factors. Several studies have suggested that CMV reactivation and the subsequent expansion of NKG2C⁺ NK cells may improve clinical outcomes for leukemia patients [77–80], and a recent study found that among CMV-seropositive patients newly infected with HIV, a higher frequency of NKG2C⁺CD57⁺ NK cells correlated with lower HIV viral load and more rapidly reaching undetectable viremia levels after combination antiretroviral therapy [81]. It will be of great interest to determine what clinical benefits there may be to patients that experience an expansion of the NKG2C⁺ NK cell population in the setting of antiviral immunity against additional infections.

2.3.3. NKG2C-independent memory formation—Despite the level of association between NKG2C and NK responses to HCMV, there is increasing evidence for CMV-elicited memory NK cells generated independently of NKG2C. Approximately 4% of individuals carry a homozygous null allele of *KLRC2*, which encodes the NKG2C receptor [82, 83], and this genotype is associated with higher anti-HCMV IgG titers in children but not in adults [84], suggesting the existence of alternative NK cell-mediated mechanisms for control of HCMV infection. Accordingly, expansion of NK cells expressing other activating receptors (i.e. specific KIRs) has been observed in response to HCMV infection in these individuals [63], or following HCMV reactivation in patients receiving umbilical cord transplants from NKG2C null donors [85]. Furthermore, several studies have identified a novel subset of NK cells associated with exposure to HCMV [86, 87]. Interestingly, this subset is characterized by its lack of expression of FcεRIγ, a high-affinity IgE receptor subunit that commonly associates with the Fc receptor CD16, and was previously thought to be expressed by all NK cells [5]. The FcεRIγ[−] NK cells express high levels CD16 and CD57, but can be either NKG2C positive or negative [86, 87]. This population has lower expression of several signaling molecules (SYK, EAT2) and transcription factors (PLZF, IKZF2) relative to FcεRIγ⁺ NK cells, thought to be maintained by epigenetic modification of these genes [88]. Compared to FcεRIγ⁺ cells, FcεRIγ[−] NK cells showed reduced degranulation and cytokine production when directly exposed to tumor targets, but produced enhanced IFN-γ and TNF-α in response to CD16 crosslinking, and greater degranulation and cytokine production in response to HCMV-infected cells in the presence of HCMV-

specific antibodies [86, 87]. FcεRIγ- NK cells expanded robustly *in vitro* when co-cultured with infected cells and plasma containing virus-specific antibodies against both HCMV and influenza [88]. Notably, the frequency of FcεRIγ- NK cells was increased in HIV⁺ individuals and was correlated with HCMV-specific antibody titers in HIV⁻ individuals [89]. Further mechanistic studies are required to address the underlying pathways promoting the generation and expansion of FcεRIγ- NK cells, and whether there is a clinical relevance for these cells in the context of HCMV infection.

2.4. Other infections

The ability of NK cells to specifically recognize infections other than CMV, and subsequently form memory, is an active area of research. Several studies have demonstrated an important role for NK cells in control of HIV infection, and specific combinations of KIR and HLA haplotype have been implicated in protection against primary infection or progression to AIDS [90–95]. Studies in rhesus macaques have also shown associations between NK cell phenotypes and disease progression following SIV infection [96, 97]. Notably, a recent study found that NK cells from SIV-infected macaques demonstrated increased cytotoxicity against Gag- or Env-pulsed dendritic cells, and this enhanced functionality required NKG2A and NKG2C [98]. Furthermore, this enhanced cytotoxicity was found to be both antigen-specific and long-lived, as NK cells from vaccinated macaques were able to efficiently kill antigen-matched but not heterologous cell targets 5 years following vaccination [98]. Intriguingly, mouse NK cells isolated from the liver following vaccination with viral-like particles (VLPs) containing HIV antigens were similarly able to mediate a hypersensitivity response after transfer into *Rag2*^{-/-} × *Il2rg*^{-/-} mice and challenge with HIV-VLPs but not Influenza A-VLPs [19]. Hepatic NK cells from immunized mice have also been shown to protect against subsequent challenge with Influenza A or VSV in a virus-specific manner [19]. In other studies, NK cells from mice previously exposed to HSV-2 or vaccinia virus produced more IFN-γ upon stimulation and protected against lethal re-challenge in the absence of T and B cells [99, 100]. Influenza infection of mice led to the generation of a long-lived pool of NK cells that homed to the bone marrow and proliferated in response to subsequent infection with influenza or RSV [101]. In humans, influenza vaccination led to enhanced IFN-γ production by NK cells in response to both heterologous and antigen-matched influenza strains [76, 102]. In total, these studies support the idea of memory NK cells and recall responses to multiple pathogens beyond CMV. However, future studies are required to elucidate the NK cell receptors involved, the precise nature and identity of the ligands (viral-encoded or host), and the mechanistic requirements for these responses.

3. Antigen-independent generation of NK cell memory

3.1. Cytokine-induced memory-like NK cells

In addition to being necessary for expansion and recall responses in antigen-specific settings, pro-inflammatory cytokines by themselves have been described to be sufficient to generate NK cells with memory-like properties in the absence of antigen [103] (Fig. 1C). Specifically, *in vitro* stimulation of mouse splenic NK cells with IL-12 and IL-18 (with or without IL-15) prior to transfer into naïve hosts generated a pool of cells with enhanced

IFN- γ production following *ex vivo* stimulation with cytokines, activating receptor ligands, or tumor targets [103, 104]. This increased cytokine production was detectable for several weeks after transfer; however, the cytokine-induced NK cells did not demonstrate any enhanced cytotoxicity [104]. Cytokine-treated NK cells had increased expression of CD25, the high-affinity α -chain of the IL-2 receptor, and IL-2 was required *in vivo* for their proliferation [105]. Likewise, human NK cells displayed similar memory-like properties following overnight culture with IL-12/15/18 [106]. Cytokine-induced memory-like human NK cells demonstrated greater IFN- γ and TNF production, increased expression of granzyme B and perforin, and superior cytotoxicity in response to K562 tumor targets *in vitro* [107]. Using xenograft models of leukemia or melanoma, human memory-like NK cells were found to provide enhanced protection [107, 108]. Lastly, in a Phase 1 clinical trial, following adoptive transfer of cytokine-activated NK cells into AML patients, donor NK cells expanded *in vivo* (and responded robustly to leukemia targets *ex vivo*), and 5 of 9 patients showed clinical responses [107]. Altogether, these studies demonstrate the potential of utilizing memory NK cells for anti-cancer therapeutics.

One lingering question in the field is whether cytokine-induced memory NK cells are elicited endogenously during infection, as cytokines such as IL-12 and IL-18 are abundantly produced [42]. A recent study addressed this question using an inducible reporter system to track endogenous NK cell populations following MCMV infection in mice [109]. The authors demonstrated that both Ly49H⁺ and Ly49H⁻ NK cells are long-lived following infection, indicating that NK cells lacking Ly49H can differentiate into cytokine-activated memory-like cells following MCMV infection, and that a single infection can drive multiple subsets of memory NK cells [109]. Whereas the Ly49H⁺ memory NK cells displayed enhanced effector function and anti-tumor activity *in vivo*, the Ly49H⁻ memory NK cells expressed higher levels of Bcl2 and persisted better following transfer into naïve hosts [109]. Notably, the Ly49H⁻ memory NK cells exhibited some functional differences as compared with *in vitro* cytokine-induced memory cells, suggesting that an *in vivo* infection-induced cytokine milieu may imprint unique features on the resulting memory cells. Additional studies are needed to directly compare activated NK cells in the two settings.

The generation of both antigen-specific and -independent memory NK cells during the same immune response may represent an important strategy to balance protection against persistent or repeated exposure to the same virus with protection against heterologous challenges. Although they provided enhanced protection against secondary MCMV challenge [29] and responded better antigen receptor triggering, Ly49H⁺ memory NK cells showed reduced proliferation and cytokine production in response to heterologous infectious challenge with influenza or *Listeria monocytogenes*, and made less IFN- γ than naïve NK cells following stimulation with IL-12 and IL-18 [110]. Similarly, Fc ϵ RI γ ⁻ HCMV-elicited memory NK cells produced less IFN- γ when stimulated with IL-12 and IL-18 compared to Fc ϵ RI γ ⁺ NK cells, and have reduced degranulation in response autologous activated T cells [111]. The consequences of the reduced bystander activation in antigen-specific memory NK cell population require further investigation, as it could be highly relevant for the design of future therapeutic strategies aimed at expanding and harnessing memory NK cell responses.

3.2. Lymphopenia-driven long-lived NK cells

When transferred into lymphopenic hosts (e.g. *Rag2*^{-/-} × *Il2rg*^{-/-} or sub-lethally irradiated mice), NK cells were shown to undergo robust proliferation to fill the empty ‘niche’ [112–114]. Following this homeostatic proliferation, the NK cell pool contracted to generate a long-lived population that could be detected for at least 6 months after transfer [115] (Fig. 1D). Lymphopenia-driven NK cells demonstrated a transient enhanced IFN- γ production and degranulation after transfer [115]. Although the effector capacity of these cells following contraction is unclear, they were able to expand robustly following MCMV challenge months later [115]. In naïve T cells, homeostatic proliferation requires common gamma chain cytokines and TCR/self-MHC interactions [116]. Similarly, IL-15 was required for the survival of NK cells following lymphopenia-driven expansion, but self-MHC interactions with Ly49 receptors was not found to be necessary [112–114]. Induction of autophagy, mediated by Atg3 and Atg5, was also required for survival of long-lived NK cells during homeostatic proliferation [117]. Future studies will elucidate the additional molecular requirements of lymphopenia-driven expansion and determine the functional similarities and differences relative to MCMV-, hapten-, or proinflammatory cytokine-elicited memory NK cells.

4. Memory of other ILCs?

NK cells have recently been recognized to be part of a larger family of innate lymphoid cells (ILCs) that also includes other T-bet-expressing group 1 ILCs, Gata3-expressing group 2 ILCs, and ROR γ t-expressing group 3 ILCs [118]. Because memory-like features can be induced by exposure of NK cells to cytokines alone, it is of great interest to determine whether cytokine stimulation can likewise promote longevity and recall responses in other ILC populations. Notably, one recent study examined the potential recall capability of ILC2s in the lung, and found that ILC2s persisted at increased frequency for months after *in vivo* stimulation with IL-33 or papain, and demonstrated enhanced cytokine production following *in vivo* or *in vitro* secondary stimulation [119]. Although the memory properties of ILC1s and ILC3s have not been directly interrogated (at the time of writing this review), liver ILC1s (defined as Lin-NK1.1⁺NKp46⁺DX5-Eomes⁻) were recently found to undergo homeostatic proliferation and be detectable at least one month after adoptive transfer into *Rag2*^{-/-} × *Il2rg*^{-/-} mice [117], suggesting the capacity of this innate lymphocyte subset to possess longevity. Future studies aimed at determining whether ILCs that have undergone cytokine- or lymphopenia-driven expansion possess increased effector function in settings of host immunity will be of great interest.

5. Conclusions

The evidence supporting the generation of NK cell memory in mice, humans, and non-human primates continues to grow, and the molecular mechanisms that govern NK memory cell formation are rapidly being uncovered. Long-lived memory or memory-like NK cells can now be observed in both infectious and noninfectious settings, and can be generated in both antigen-specific and antigen-independent manners. Although multiple mechanistic insights have been achieved using the MCMV infection model, future studies are necessary

to determine if these requirements are shared with memory NK cells generated in other settings, in particular human infectious diseases. Many questions circulating around generation, maintenance, and harnessing of NK cell memory remain unresolved. Is there functional heterogeneity in the effector NK cell pool that determines which cells will become memory cells? Are there epigenetic determinants in differentiating NK cells that promote or restrict memory? What are additional receptors that can drive NK cell memory, and what are their ligands? Do memory NK cells become tissue-resident like T cell subsets, or remain circulating? Does the expansion and longevity of human NK cells provide clinical benefits for heterologous infections or cancers? Can other lineages of ILCs acquire memory-like properties following inflammation, injury, or infection, and can they similarly be harnessed towards disease treatment or prevention? Addressing these questions will guide therapeutic strategies utilizing the power of these potent innate lymphocytes to combat infectious diseases and cancer.

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Highlights

- NK cell memory can be antigen-specific or antigen-independent
- Molecular mechanisms underlying NK cell memory revealed by the MCMV model
- Increasing evidence supports CMV-specific NK cell memory in humans

Antigen-dependent NK memory responses

Antigen-independent NK memory-like responses

Figure 1. Antigen-dependent and -independent pathways that generate memory NK cells

A–B Antigen-specific memory NK cell formation. **A** Following hapten sensitization, CXCR6⁺ liver-resident NK cells mediate contact hypersensitivity responses. This process requires pro-inflammatory cytokine signaling, and molecules involved in NK cell trafficking. **B** During MCMV infection, Ly49H⁺ NK cells undergo a proliferative burst driven by pro-inflammatory cytokines and signaling through activating receptors including Ly49H and DNAM1. Cytokine signaling promotes expression of Zbtb32 and miR-155, which boost the expansion of effector NK cells. Following viral clearance, autophagy pathways and BIM control the contraction of the NK cell population, resulting in a pool of long-lived, MCMV-specific memory cells. **C–D** Antigen-independent memory-like NK cell formation. **C** In

vitro exposure of NK cells to IL-12, IL-15, and IL-18 prior to transfer into naïve hosts generates a population of long-lived cells that make more IFN- γ upon stimulation. These cells depend on pro-survival cytokines, but other factors that promote their generation are unknown. **D** NK cells undergo homeostatic proliferation following transfer into *Rag2*^{-/-} $\times$ *Il2rg*^{-/-} mice to generate long-lived cells. Formation of these long-lived NK cells is promoted by IL-15 and the autophagy machinery. Factors shown in green are known positive regulators of NK cell memory; factors shown in red are negative regulators.

Figure 2. Activation of mouse and human NK cells during CMV infection

NK cells require multiple signals for optimal activation and generation of memory cells. (upper) In mouse, these include signals through activating receptors, such as Ly49H interacting with its ligand m157 (analogous to TCR-MHC interaction, 'Signal 1'); signals through co-stimulatory receptors, such as DNAM-1 interacting with CD155 (analogous to T cell CD28-B7 ligand interactions, 'Signal 2'); and pro-inflammatory cytokines receptors ('Signal 3'). (lower) Maximal expansion of human NK cells *in vitro* required NKG2C/CD94 and the cognate ligand HLA-E, and the pro-inflammatory cytokine IL-12. The interaction of CD2 with its ligand, CD58, may provide a co-stimulatory signal to further support activation of human NK cells.