JEM Journal of Experimental Medicine

REVIEW

Immune Memory Focus

Virus-specific NK cell memory

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NK cells express a limited number of germline-encoded receptors that identify infected or transformed cells, eliciting cytotoxicity, effector cytokine production, and in some circumstances clonal proliferation and memory. To maximize the functional diversity of NK cells, the array and expression level of surface receptors vary between individual NK cell "clones" in mice and humans. Cytomegalovirus infection in both species can expand a population of NK cells expressing receptors critical to the clearance of infected cells and generate a long-lived memory pool capable of targeting future infection with greater efficacy. Here, we discuss the pathways and factors that regulate the generation and maintenance of effector and memory NK cells and propose how this understanding may be harnessed therapeutically.

Introduction

Natural killer (NK) cells are innate immune cells capable of mounting a cytotoxic response and/or secreting cytokines upon detection of infected or transformed cells. Detection of such aberrant cells is achieved using a remarkably small number of germline-coded receptors that activate NK cells in response to the presence of "stress" ligands, viral proteins, or a loss of inhibitory ligands such as MHC class I. Humans (and mice) with a defect in NK cell numbers or function are extremely susceptible to certain viral infection and cancers (Morvan and Lanier, 2016; Cerwenka and Lanier, 2016; Mace and Orange, 2019). NK cells were first shown to mount antigen-specific acute and "memory" immune responses in a model of hapten (2,4-dinitrofluorobenzene or oxazolone)–mediated contact hypersensitivity. Treatment of *Rag2*$^{-/-}$ mice, which lack functional B or T cells, with a specific hapten resulted in an NK cell–dependent increased swelling upon treatment of the mouse's ear with the same hapten, which lasted for at least 4 wk (O'Leary et al., 2006).

In a subsequent study, virus-induced NK cell memory was demonstrated in response to mouse CMV (MCMV) infection (Sun et al., 2009a). Ligation of the NK cell activating receptor Ly49H by the virally encoded protein m157 initiates a clonal-like proliferation of this NK cell subset, similar to T and B cells. This expanded population of Ly49H^{+} NK cells, directly and through activation of an adaptive immune response, controls peripheral viremia and drives MCMV into latency, where virus can only be detected in salivary glands. In humans, human CMV (HCMV)–infected individuals similarly possess a population of "adaptive" NK cells (NKG2C^{+}CD57^{+}) that can expand and persist as memory cells (Lopez-Vergès et al., 2011; Gumá et al., 2004).

Finally, antigen-independent activation of NK cells can produce longevity and anamnestic responses through exposure to homeostatic and inflammatory cytokines (Cooper et al., 2009; Sun et al., 2011; Nabekura and Lanier, 2016a). Because antigen-dependent memory NK cell formation has a strong dependency on proinflammatory cytokines, it is possible that NK cell memory can be generated to pathogens other than CMV. Indeed, there have been reports of putative memory NK cell generation in response to hantavirus (Björkström et al., 2011) in humans and influenza, vaccinia, and Friend viruses in mice (Gillard et al., 2011; Littwitz-Salomon et al., 2018; van Helden et al., 2012). Although future studies will elucidate greater details about the mechanisms through which NK cell memory is generated to such other viruses, currently, CMV-generated NK cell memory represents the most well studied and best characterized. This review will summarize the key findings highlighting the memory capability of NK cells, divided into the topics of clonal expansion, long-lived survival, functional importance, and clinical application.

Clonal expansion of NK cells

A hallmark feature of immunological memory during primary infection is the clonal proliferation of antigen-specific lymphocytes. Here, we will discuss the pathways involved in driving proliferation as summarized in Fig. 1 A. During MCMV infection,

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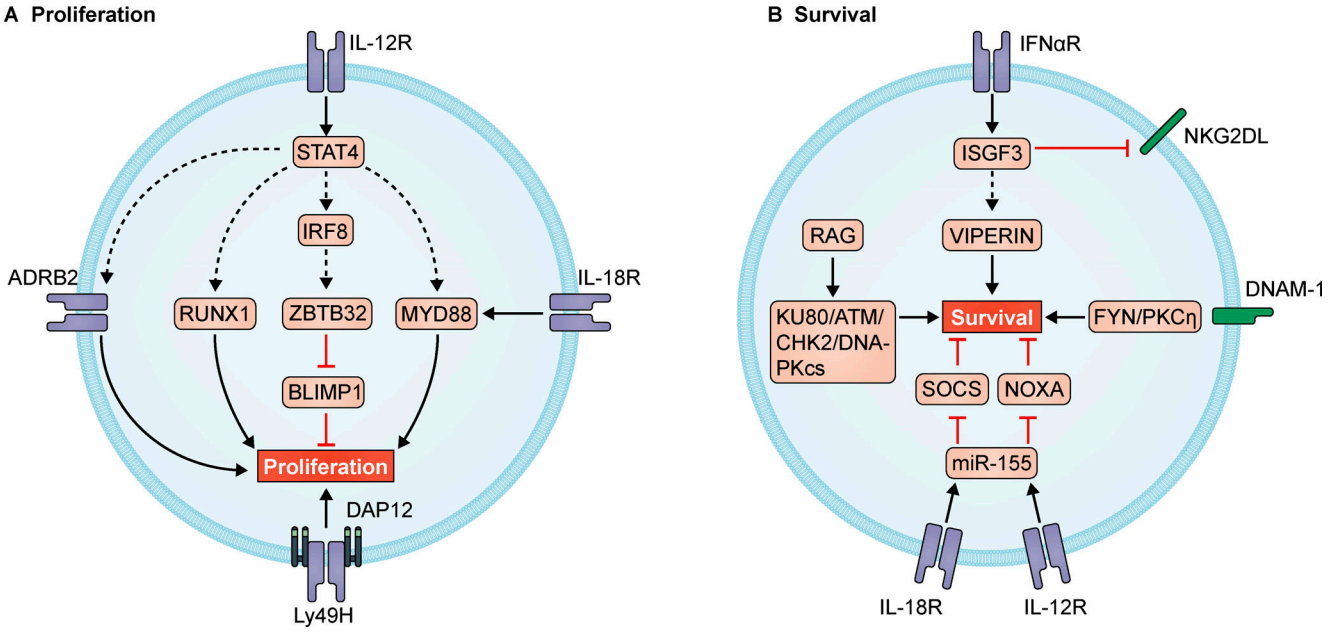

Figure 1. **Regulation of proliferation and survival is critical to memory NK cell formation during MCMV infection. (A)** Proliferation. IL-12 activates STAT4, which drives a cascade of proliferation-promoting transcription factors (e.g., IRF8 and Zbtb32) in NK cells that results in the suppression of BLIMP-1, in addition to up-regulating factors such as MyD88 and the neurotransmitter receptor ADRB2. Along with IL-12 and STAT4 signaling, IL-18 signals via MyD88 in NK cells, and together, these two proinflammatory cytokines drive Ly49H$^+$ NK cell proliferation. **(B)** Survival. IL-12 and IL-18 also cooperate to regulate SOCS1 and NOXA through a microRNA-155 (miR-155)–dependent mechanism. Signaling through IFNαR induces the ISGF3 complex (consisting of STAT1, STAT2, and IRF9), protecting NK cells against NKG2D-mediated "fratricide" and ensuring their survival. Signaling through the costimulatory molecule DNAM-1 also facilitates survival in a PKCη- and FYN-dependent manner. Expression of RAG is critical to induction of DNA damage repair pathways that maintain viability during rapid cell division. Dashed lines represent induction of mRNA. NKG2DL, NKG2D ligand.

the activating NK cell receptor Ly49H recognizes a virally encoded glycoprotein, m157, expressed on the surface of infected cells (Smith et al., 2002; Arase et al., 2002). Similar to peripheral CD8$^+$ T cells triggered through their TCR by cognate foreign antigens, Ly49H$^+$ NK cells have the ability to clonally expand and generate long-lived immune memory after binding to m157 and signaling through the adapter DAP12 (Sun et al., 2009a; Dokun et al., 2001). Further work has dissected this Ly49H$^+$ population to better understand its clonality, identifying the most potent Ly49H$^+$ NK cells responding to MCMV infection as being low and negative, respectively, for the inhibitory receptors KLRG1 and NKR-P1B (Rahim et al., 2016; Kamimura and Lanier, 2015). In humans, NKG2C$^+$ NK cells that express the MHC class I–binding inhibitory receptor CD158b/j$^+$ (KIR2DL2/KIR2DL3/KIR2DS2), but not CD156a/h$^+$ (KIR2DL1/KIR2DS1) or KIR3DL1$^+$ (CD158e), have an expansion advantage in hematopoietic stem cell transplant patients that reactivated CMV and expressed their respective ligands (Foley et al., 2012). This is in agreement with the finding that KIR3DL1 is expressed at a lower frequency on NKG2C$^+$ NK cells than their NKG2C$^-$ counterparts in CMV-seropositive donors who express the KIR3DL1 ligand HLA-Bw4 (Lopez-Vergès et al., 2011). In a recent study where single-cell transfer of color-barcoded Ly49H$^+$ NK cells was performed, the maximum clonal expansion of a given NK cell clone during MCMV infection reached 10,000-fold, on par with the expansion measured in OT-1 CD8$^+$ T cells, demonstrating the remarkable capacity of virus-specific NK cells to undergo clonal expansion (Grassmann et al., 2019).

Recently, ILC1 (the tissue-resident equivalent to the circulating NK cell), abundantly found in organs such as the liver (Peng et al., 2013; Weizman et al., 2017), have also been shown to form a memory population in response to MCMV (Weizman et al., 2019). These pathogen-experienced liver ILC1s possess a transcriptional and epigenetic profile distinct from naive liver ILC1s (Weizman et al., 2019). Distinguished by their expression of IL-18 receptor (IL-18R), these memory ILC1s produced greater levels of IFN-γ ex vivo and were better able to control MCMV in vivo upon virus rechallenge than their naive counterparts (Weizman et al., 2019). Generation of memory ILC1s was dependent on the MCMV-encoded glycoprotein m12, which is a ligand for the activating NKR-P1 receptors (NK1.1 and NKR-P1A encoded by *Klrb1c* and *Klrb1a*, respectively) expressed on mouse NK cells (Aguilar et al., 2017; Weizman et al., 2019).

In humans, HCMV-infected individuals similarly possess a population of NK cells (NKG2C$^+$CD57$^+$) that undergo clonal expansion and persist as memory cells (Lopez-Vergès et al., 2011; Gumá et al., 2004). These memory NK cells express the activating receptor NKG2C but are negative or low for expression of the inhibitory receptor NKG2A (Zhang et al., 2013). Furthermore, these cells have a lower expression frequency of the activating receptor adapter molecule FcRγ than NKG2C$^-$ NK cells (Zhang et al., 2013). The shared ligand for both inhibitory NKG2A and activating NKG2C (which also signals through DAP12) is HLA-E. Sequencing of the HLA-E–presented UL40-derived peptide from HCMV-seropositive individuals identified that the sequence of this peptide impacted the size of the

NKG2C$^+$ NK cell population (Hammer et al., 2018). A similar phenomenon has been reported in mice, with Ly49P and Ly49L controlling MCMV in an MHC haplotype– and MCMV protein–dependent manor, with Ly49L being shown to be capable of driving antiviral proliferation (Pyzik et al., 2011; Kielczewska et al., 2009). In humans, NKG2C$^+$ NK cell activation and clonal proliferation is further promoted by CD2 binding to LFA-3 (CD58; Hammer et al., 2018; Rölle et al., 2016), along with epigenetic changes during differentiation into memory cells (Schlums et al., 2015; Lee et al., 2015; Luetke-Eversloh et al., 2014).

As with antigen-specific B and T cells, Ly49H$^+$ and NKG2C$^+$ NK cells in mouse and human, respectively, undergo avidity selection for NK cell clones that express the greatest amount of virus-specific receptor (Adams et al., 2019; Grassmann et al., 2019). In mouse studies, although both Ly49H^{hi} and Ly49H^{lo} NK cells could undergo avidity maturation by increasing their surface levels of Ly49H during MCMV infection, the Ly49H^{hi} population proliferated in vivo more rapidly and demonstrated greater cytotoxicity ex vivo than their Ly49H^{lo} counterparts by making more productive contacts with m157-expressing target cells (Adams et al., 2019). In contrast, Ly49H^{lo} NK cells were shown to produce higher levels of IFN-γ during MCMV infection (Adams et al., 2019), highlighting a diversification of effector function in the different subsets resulting in a "division of labor" during the antiviral immune response. Further investigation is required to determine the underlying mechanisms by which Ly49H (or NKG2C) signaling drives clonal expansion and memory formation of NK cells.

As rapidly responding innate lymphocytes, NK cells rely heavily on cytokines to activate their antiviral function. Proinflammatory cytokines drive the early effector responses that are a precursor to NK cell memory generation. IL-12 and IL-18 have been shown to be critical for Ly49H$^+$ NK cell expansion in response to MCMV (Madera and Sun, 2015; Sun et al., 2012; Andoniou et al., 2005). Using IL-12R–deficient NK cells in mixed bone marrow chimera or adoptive transfer settings, IL-12–induced STAT4 signaling drove NK cell proliferation, IFN-γ production, and memory formation (Sun et al., 2012). The adaptor proteins CRKII and CRKL have been shown to be required for optimal STAT4 and STAT1 phosphorylation, and mice lacking both these proteins produce less IFN-γ and proliferate less than WT NK cells during MCMV (Nabekura et al., 2018). However, the identification of which NK cell receptors they act downstream of requires further investigation. In co-culture assays of human NK cells with HCMV-infected fibroblasts, CD14$^+$ monocyte-derived IL-12 was demonstrated to be a key driver of CD25 up-regulation and NKG2C$^+$ NK cell expansion (Rölle et al., 2014).

Similarly to IL-12 and STAT4, IL-18–induced MyD88 signaling was shown to be critical for optimal NK cell expansion and IFN-γ production, where the IL-12/STAT4 signaling axis up-regulated *Myd88* gene expression to cooperatively drive effector and memory Ly49H$^+$ NK cells during MCMV infection (Madera and Sun, 2015). Interestingly, the few persisting memory NK cells lacking IL-18R were able to proliferate similarly to their WT counterparts upon secondary transfer into a naive host followed by a rechallenge (Madera and Sun, 2015), suggesting that IL-18 may be more critical during primary infection and cytokine exposure but less so during a recall response.

The hypothesis that naive and memory NK cells may respond differently to the same stimuli during primary and secondary virus infection is consistent with recent findings demonstrating that significant epigenetic changes occur during the differentiation of effector and memory cells, some of which became stable in the long-lived NK cells (Lau et al., 2018). In NK cells activated during MCMV infection, STAT4 bound to many putative gene enhancer regions (e.g., intergenic and intronic) that also increased in chromatin accessibility, as assessed by overlapping STAT4 chromatin immunoprecipitation sequencing and ATAC-seq (assay for transposase-accessible chromatin using sequencing) peaks (Lau et al., 2018). This increased chromatin accessibility resulted in increased gene expression as determined by RNA sequencing, with several genes of critical effector molecules following this pattern, including *Ifng* (a key antiviral cytokine produced by NK cells) and *Fyn* (a critical kinase downstream of NK cell activating receptors, including NKG2D, CD137, and 2B4; Lau et al., 2018; Dong et al., 2012; Lowin-Kropf et al., 2002; Rajasekaran et al., 2013). Consistent with these findings, H3K4me3 chromatin immunoprecipitation sequencing showed that this "permissive" histone modification increases in abundance at the *Irf8*, *Runx1*, and *Runx3* gene loci of NK cells upon IL-12 and IL-18 stimulation and in a STAT4-dependent manner (Adams et al., 2018; Rapp et al., 2017).

The transcription factors IRF8, RUNX1, and RUNX3 have themselves been shown to be key regulators of cell cycle genes critical to the proliferative stage of the antiviral NK cell response resulting in memory (Adams et al., 2018; Rapp et al., 2017). One of the mechanisms by which IRF8 drives clonal proliferation of NK cells in response to IL-12/STAT4 signaling is by promoting the expression of the transcription factor *Zbtb32*, which antagonizes the anti-proliferative factor BLIMP-1 (Beaulieu et al., 2014). Interestingly, STAT4 also up-regulates *Adrb2*, which encodes the adrenergic signaling receptor, and expression of ADRB2 was recently shown to be essential for optimal clonal proliferation and memory formation (Diaz-Salazar et al., 2020). This potential neuron–immune cell crosstalk highlights how novel signals beyond traditional cytokine signaling and activating receptor engagement provide extrinsic inputs that drive productive effector and memory NK cell formation (Diaz-Salazar et al., 2020). Finally, the T-box transcription factors Tbet and Eomes, which play an important role in NK cell maturation, also aid the proliferation of effector NK cells and generation of memory and were found to be STAT4 dependent (Madera et al., 2018; Lau et al., 2018). Thus, IL-12–driven STAT4 signaling plays a major and multifaceted role in driving clonal proliferation of NK cells, critical to memory formation. In addition to these classic inflammatory cytokines, IL-33 signaling through ST2 receptor has also been demonstrated to enhance m157-mediated NK cell proliferation (Nabekura et al., 2015).

In parallel to the formation of NK cell memory driven by Ly49H-m157 interactions, long-lived cytokine-induced memory NK cells are also formed during MCMV infection. Cytokine-

induced NK cells do not undergo the extensive expansion on the order of Ly49H$^+$ NK cells to MCMV infection, but do become KLRG1^{hi}, Ly6C$^+$, and CD11b$^+$, while down-regulating CD27 and DNAM-1 (Nabekura and Lanier, 2016a). Ex vivo treatment of NK cells with IL-12 + IL-18 primes them to proliferate more and produce more IFN-γ when transferred into immunodeficient hosts in comparison to untreated NK cells (Cooper et al., 2009).

In addition to proinflammatory cytokines, homeostatic cytokines (such as IL-2 and IL-15 that are present in greater abundance in lymphopenic mice) can also drive a transient activation of NK cells leading to longevity. The adoptive transfer of NK cells into *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ mice or sublethally irradiated mice caused a rapid homeostatic proliferation of NK cells followed by a contraction and maintenance of memory-like cells (Sun et al., 2011), likely driven by elevated levels of IL-2 or IL-15 in the lymphopenic hosts. The transferred NK cells were more capable of producing IFN-γ in response to activating receptor ligation ex vivo and expressed an elevated level of KLRG1 in addition to a greater propensity to be CD27$^+$, suggesting a differentiation that contrasts with proinflammatory cytokine exposure. Furthermore, even 60 d following transfer, the Ly49H$^+$ population within the transferred NK cells robustly expanded in response to MCMV infection (Sun et al., 2011). Thus, exposure to specific homeostatic and proinflammatory cytokines can promote the formation of long-lived NK cells, a potential therapeutic strategy currently being tested in a variety of disease settings.

Memory NK cell survival

Following the clonal expansion of NK cells during infection, contraction of effector cells is essential to the formation of an effective memory population. Rapid proliferation during the expansion phase of the antiviral NK cell response generates cellular stress that can lead to apoptosis if not properly controlled. In this section, we will discuss the pathways that have been identified to control memory cell survival, as summarized in Fig. 1 B. Loss of the proapoptotic factor BIM (*Bcl2l11*$^{-/-}$) reduced apoptosis in expanded Ly49H$^+$ NK cells, resulting in the generation of a memory pool that was greater in size but possessed a phenotype more similar to naive than memory cells (Min-Oo et al., 2014). Furthermore, *Bcl2l11*$^{-/-}$ NK cells demonstrated a reduced functional ability to protect against MCMV (Min-Oo et al., 2014).

Although apoptosis of the majority of effector lymphocytes is desirable for maintaining lymphoid tissue size and homeostasis following viral clearance, survival of some cells that will go on to form immunological memory is a critical aspect of host immunity against subsequent infection. Unexpectedly, key mediators of NK cell "fitness" are the recombination-activating genes (RAGs), whose expression during NK cell ontogeny was shown to be critical for the induction of DNA damage repair pathways essential to cell viability during rapid division (Karo et al., 2014). Another important protective mechanism during the activation and expansion of NK cells during MCMV infection is the up-regulation of microRNA-155, which was shown to repress the expression of pro-apoptotic Noxa and the STAT signaling repressor Socs1 (Zawislak et al., 2013).

In addition to the IL-12/STAT4 signaling that induces many proliferative pathways in NK cells during MCMV infection, the type 1 IFN–activated transcription factor STAT1 is a key regulator of NK cell survival during this same expansion phase, protecting cells from "fratricide" via induction of NKG2D ligands (Madera et al., 2016). STAT1, STAT2, and IRF9 represent members of the ISGF3 complex and were shown to be activated and transcriptionally up-regulated by IFN-α signaling in NK cells (Geary et al., 2018). Deletion of any of the three components of the ISGF3 complex impaired the ability of Ly49H$^+$ NK cells to expand in response to MCMV (Geary et al., 2018). Although redundancy in function was observed among all three members of the ISGF3 complex, there was not a complete overlap in the genes they individually appeared to regulate. STAT1 signaling was recently shown to also increase chromatin accessibility of the *Rsad2* locus, encoding the antiviral factor Viperin and aiding the survival of effector Ly49H$^+$ NK cells (Wiedemann et al., 2020).

Interestingly, IFN-γ production by NK cells during MCMV infection was increased in the absence of STAT1 but decreased in the absence of IRF9, whereas the opposite was true for granzyme B, a critical executor of apoptosis in target cells during NK cell–mediated cytotoxicity (Madera et al., 2016; Geary et al., 2018), suggesting individual members of this complex may be regulated by signals independent of IFN-α. IFN-γ, although important for viral clearance, was not found to be required for the clonal proliferation or generation of memory in antiviral NK cells following MCMV infection, and neither was TNF-α (Andrews et al., 2003; Sun et al., 2012), suggesting a lack of requirement for autocrine signals via these cytokines. Furthermore, IL-15 signaling, although critical to NK cell homeostasis, was not essential for the clonal expansion of Ly49H$^+$ NK cells (Sun et al., 2009b), highlighting that proinflammatory cytokines produced by cells other than NK cells appear to be more critical in the generation of robust effector and memory NK cell responses during viral infection.

Receptor signaling can also initiate anti-apoptotic pathways. The costimulatory molecule DNAM-1 that is expressed by a subset of both Ly49H$^+$ and Ly49H$^-$ naive NK cells was shown to play an important role in NK cell survival and viral clearance. Although DNAM-1 expression is up-regulated on Ly49H$^+$ NK cells during MCMV infection, the DNAM-1$^+$ population was more sensitive to induction of apoptosis compared with their DNAM-1$^-$ counterparts (Nabekura et al., 2014). Thus, whereas DNAM-1 expression is clearly critical to clearance of MCMV, loss of its expression increases the chances of an effector NK cell becoming a long-lived memory cell. In these studies, PKCη signaling was essential for both phenotypes during MCMV infection, whereas FYN was only critical for NK cell survival (Nabekura et al., 2014).

In addition to DNA damage, other potential sources of stress for an activated and rapidly dividing NK cell are an inability to meet an increased metabolic demand and potential mitochondrial damage that may occur when trying to do so. mTOR (mechanistic target of rapamycin) signaling, driven in part by IL-15 signaling, is critical for the up-regulation of metabolism during Ly49H$^+$ NK cell expansion (Marçais et al., 2015). Among

the mechanisms by which mTOR achieves this increased metabolism is through activation of the inositol-requiring enzyme 1 and its substrate transcription factor X-box-binding protein 1, which activates c-myc and increases oxidative phosphorylation (Dong et al., 2019). However, this increase in metabolic activity is also accompanied by an increase in mitochondrial-associated ROS and decreased mitochondrial membrane potential, with some mitochondria becoming dysfunctional (O'Sullivan et al., 2015). Clearance of these dysfunctional mitochondria by autophagy was shown to aid the formation of viable memory NK cells (O'Sullivan et al., 2015). Thus, the metabolism of NK cells must be carefully regulated during effector responses and memory formation to provide these highly cytolytic cells with ample energy and key metabolites for cellular processes while avoiding cellular stress.

Functional characteristics of memory NK cells

Memory Ly49H$^+$ NK cells generated during MCMV infection have previously been shown to provide greater protection against challenge with MCMV compared with an equal number of naive Ly49H$^+$ NK cells when transferred into immunodeficient mice (Sun et al., 2009a). This greater protection by memory NK cells is thought to be due to greater degranulation and IFN-γ production (on a per-cell basis) upon activating receptor ligation (Sun et al., 2009a); both functions are enhanced by IL-12 (Min-Oo and Lanier, 2014). Surprisingly, secondary NK cell expansion in response to MCMV was similar in kinetics and magnitude to that of a primary NK cell response (Sun et al., 2009a). Because memory NK cells expressed reduced levels of DNAM-1 and CD27 and higher levels of Ly49H, KLRG1, Ly6C, and CD43 than naive Ly49H$^+$ cells (Sun et al., 2009a; Nabekura and Lanier, 2016a), one might imagine that they function differently than naive NK cells in response to heterologous infections. A summary of these differences is found in Fig. 2.

Indeed, memory Ly49H$^+$ NK cells generated by MCMV infection do not up-regulate CD69 or proliferate as efficiently as naive Ly49H$^+$ NK cells in response to challenge with a different pathogen, such as influenza or *Listeria monocytogenes* (Min-Oo and Lanier, 2014). Furthermore, IFN-γ production by memory NK cells is also reduced in response to *L. monocytogenes* infection when compared with naive NK cells (Min-Oo and Lanier, 2014). However, against reexposure to MCMV, the memory Ly49H$^+$ NK cells demonstrated a superior degranulation, IFN-γ production, and cytotoxicity (Min-Oo and Lanier, 2014), suggesting that these long-lived cells have dedicated themselves toward this specific herpesvirus while ignoring heterologous infection and bystander inflammation.

Interestingly, MCMV-induced memory NK cells do show increased effector function against NKG2D ligand–expressing tumor cells (Nabekura and Lanier, 2016a), suggesting that NKG2D triggering may have occurred alongside Ly49H signals in NK cells during primary exposure to MCMV. NKG2D is down-regulated during the peak of MCMV-driven NK cell expansion (Nabekura et al., 2017), even though MCMV inhibits expression of NKG2D ligands on infected cells as an evasion mechanism (Slavuljica et al., 2010); thus, it is not clear what drives NKG2D down-regulation. In mutant viruses that cannot block NKG2D ligand expression, NKG2D can promote Ly49H-driven NK cell proliferation but cannot drive expansion in the absence of Ly49H (Nabekura et al., 2017). In humans, CMV-activated NKG2C$^+$ NK cells that express a single self-reactive inhibitory killer cell Ig-like receptors (KIR) produced more IFN-γ in response to stimulation with K562 (Foley et al., 2012). Similarly, in mice where H-2D^d (the ligand for the activating receptor Ly49D) is expressed, Ly49H$^+$ NK cells that coexpress Ly49D produce more IFN-γ in response to MCMV infection and outcompete their Ly49H$^+$ Ly49D$^-$ counterparts during expansion and memory formation by maintaining expression of the anti-apoptotic gene *Bcl2* (Nabekura and Lanier, 2016b). Higher BCL-2 levels have also been reported in HCMV-driven memory NK cells, suggesting a similar mechanism may control the longevity of human memory NK cells (Zhang et al., 2013). Additional mechanisms underlying the potency of memory NK cells in response to activating receptor signaling remain to be elucidated.

In NK cells activated through HCMV infection, several changes in the expression of receptor signaling molecules have been reported. Some HCMV-driven memory NK cells lack expression of FcRγ, the adaptor molecule that stabilizes the expression of CD16 and natural cytotoxicity receptors and delivers downstream signals (Zhang et al., 2013). A reduction in the expression of natural cytotoxicity receptors such as NKp46 and NKp30 was observed, in part explaining their reduced IFN-γ production and degranulation in response to K562 and 721.221 tumor cell lines (Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015). However, the ability of human memory NK cells to perform antibody-dependent cell-mediated cytotoxicity in response to cells infected with HCMV, flu, or HSV (along with virus-specific antibodies) was enhanced (Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015). Although CD16 expression was slightly reduced in the absence of FcRγ, it is thought that CD16 signaling via its alternative adapter molecule, CD3ζ, which contains three immunoreceptor tyrosine-based activation motif (ITAM) domains compared with one in FcRγ, may amplify the downstream signaling of CD16 (Hwang et al., 2012; Shah et al., 2018). Furthermore, expression of CD2, which synergistically increases phosphorylation of ERK and S6RP, may amplify the activation of adaptive NK cells triggered through NKG2C or CD16 (Liu et al., 2016).

In addition to FcRγ down-regulation during HCMV infection, NKG2C$^+$CD57$^+$ memory NK cells can also lose expression of tyrosine kinase SYK (spleen-associated tyrosine kinase) and the signaling molecules DAB2 and EAT-2 (Lee et al., 2015; Schlums et al., 2015), resembling cytotoxic CD8$^+$ T lymphocytes that also lack expression of these proteins (Schlums et al., 2015). Loss of these signaling proteins was suggested to be due to hypermethylation of their promoter regions, accompanied by a loss of expression of the transcription factor promyelocytic leukemia zinc finger (PLZF; Lee et al., 2015; Schlums et al., 2015). These memory NK cells express less IL-18R and do not phosphorylate STAT4 as efficiently in response to IL-12 and IL-18 stimulation and thus produce less IFN-γ in response to stimulation with these cytokines (Schlums et al., 2015; White et al., 2014), similar to findings in mice where memory NK cells become more

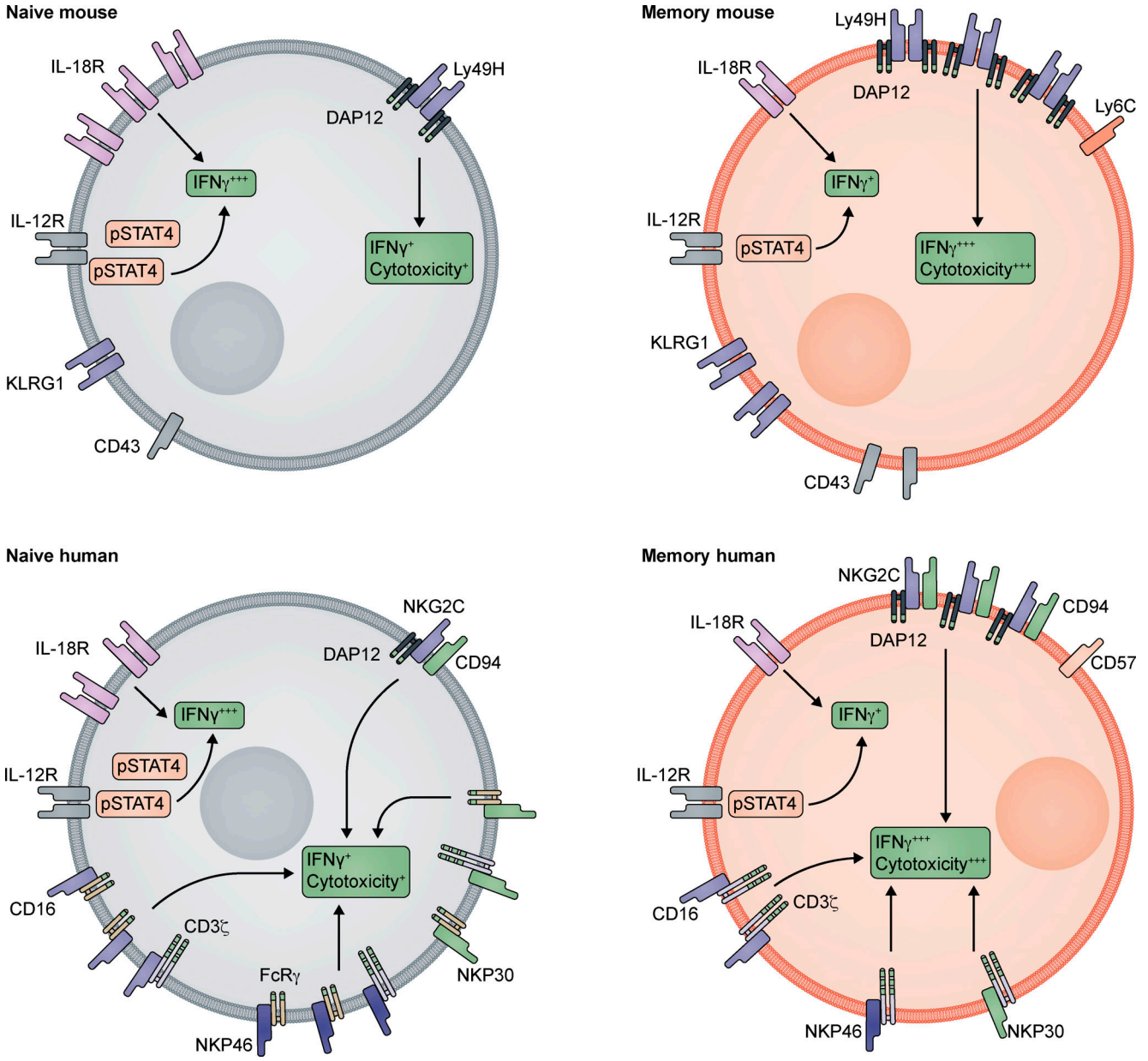

Figure 2. **Human and mouse memory NK cell traits.** As NK cells differentiate from naive to memory cells during viral infection, they become less responsive to certain cytokines but more responsive to activating receptor engagement. This transition can be attributed in part to changes in the overall expression of receptors, adaptor molecules, and signaling proteins regulated at the epigenetic and transcriptional levels.

"antigen focused" following CMV exposure. These studies also highlight how NK cells (and their receptors) can be specifically targeted for therapeutic applications, particularly in instances where receptors and ligands are well defined or potent antibody-dependent cell-mediated cytotoxicity–inducing antibodies exist.

In light of the finding that cytokines can induce memory-like features in NK cells (Cooper et al., 2009), there has been a strong incentive to develop in vitro protocols that use cytokine stimulation to enhance the functionality or longevity of NK cells for therapeutic purposes. Many of these current protocols incorporate low doses of IL-15 to maintain NK cell viability, along with short exposure to high doses of IL-12 and IL-18 to mimic exposure to a viral infection (Romee et al., 2012). The effector function of NK cells was enhanced irrespective of the expression of inhibitory KIRs that have engaged a cognate ligand; however, expression of these receptors does still confer a greater potency in response to CD16 stimulation (Wagner et al., 2017). The increased IFN-γ production of these cytokine-exposed NK cells (in vitro with human cells or in vivo with mouse cells) in response to restimulation or incubation with tumor cell lines was maintained for a number of weeks but diminished over time (Romee et al., 2012; Keppel et al., 2013). Thus, this treatment appears to more similarly mimic the long-lived mouse NK cells generated in lymphopenic mice (Sun et al., 2011) rather than the memory NK cells from mice or humans infected with CMV.

One strategy to overcome the lack of receptor engagement in cytokine-activated NK cells has been to incubate the cells ex vivo with tumor lines or their lysates, thus generating tumor-activated NK cells (Sabry et al., 2011; North et al., 2007). These tumor-activated NK cells up-regulate CD69, CD132, and CD25 driven by the binding of CD2 to its ligand CD15 on the tumor cells, a process that can be amplified by NKG2D, NKp80, and CD16A ligation (Sabry et al., 2011; Pahl et al., 2018). CD69 expression increases NK cell cytotoxicity against some CD69L-expressing tumor cells (North et al., 2007). CD25 and CD132 expression increases sensitivity to IL-2 and IL-15, respectively, increasing proliferation in response to these cytokines. Thus, NK cell memory of prior tumor cell membrane engagement appears to share a dependence on CD2 engagement with the memory generated during CMV infection.

Clinical importance of memory NK cells

Recent studies report that HCMV latency/reactivation can provide protection during leukemia treatment with a hematopoietic cell transplantation by inducing NK cell activation and expansion (Foley et al., 2012; Elmaagacli and Koldehoff, 2016; Cichocki et al., 2016; Jin et al., 2017; Bigley et al., 2016; Yoon et al., 2016; Horowitz et al., 2015; Inagaki et al., 2016). When HCMV is reactivated following hematopoietic cell transplantation, the adaptive NKG2C$^+$ subset of human NK cells has been shown to expand and be a potent producer of IFN-γ (Foley et al., 2012). Additionally, these NK cells were shown to be resistant to CD112- and CD155-mediated myeloid-derived suppressor cell–dependent contact inhibition (Sarhan et al., 2016). In contrast, a subset of PD-1$^+$ NK cells in HCMV-seropositive healthy donors and ovarian carcinoma patients were described to be less responsive to cytokine- and activating receptor–mediated stimulation (Pesce et al., 2017). These superficially conflicting findings highlight the need for further and detailed investigations into the impact of CMV on the antitumor function of NK cells in order to determine how best to harness or therapeutically recreate the ability of CMV to promote NK cell–mediated tumor clearance.

Attempts are now being made to incorporate the above-mentioned strategies of in vivo or in vitro cytokine stimulation for the generation of memory-like NK cells to treat cancer. These memory NK cells express higher granzyme B than naive NK cells and have demonstrated increased IFN-γ secretion and cytotoxicity against the leukemia cell line K562 and primary acute myeloid leukemia (AML) cells in vitro (Romee et al., 2016). Furthermore, such NK cells express the high-affinity IL-2 receptor CD25, causing them to proliferate and increase in functionality in patients in response to low and tolerable doses of IL-2 (Leong et al., 2014; Romee et al., 2016). In an MHC class I–deficient lymphoma (RMA-S) mouse model, tumors could be better controlled by injection of cytokine-induced memory NK cells following radiation treatment, provided that CD4$^+$ T cells were present to support the NK cells by production of IL-2 (Ni et al., 2012).

In a phase 1 clinical trial, when cytokine-induced NK cells were transferred into lymphodepleted and relapsed/refractory AML patients, remission was observed in some individuals (Romee et al., 2016). Interestingly, these memory NK cells did not cause the graft versus host disease (GvHD) associated with T cell–focused therapies (Romee et al., 2016). In a separate study where donor NK cells were primed overnight with the lysate of the leukemia cell line CTV-1 (in the absence of recombinant cytokines) and used to treat AML, a complete remission was reported in four out of seven patients (Kottaridis et al., 2015). A second study using the same NK cell activation strategy (with tumor cell lysates) reported remissions that lasted >30 mo in 3 out of 12 AML patients (Fehniger et al., 2018). These promising data highlight the potential of NK cell–based therapies and how larger clinical studies (and more precise phase 2 trials) need to be conducted to further demonstrate increased patient survival dependent upon NK cells.

The precise conditions required for priming NK cells in various disease settings are currently being refined. For example, NK cells may benefit from a period of "rest" in low-dose IL-2 and/or IL-15 following priming by proinflammatory cytokines, tumor cells, or tumor components (Pahl et al., 2018). In addition to understanding how NK cell memory can be used to improve NK cell transfer therapy, a detailed analysis of the molecular components and signals required for long-term cancer remission is warranted. Lastly, because effective vaccination of the NK cell compartment has been shown to be dependent upon CMV serostatus (Goodier et al., 2016; Darboe et al., 2017), this additional consideration should be carefully assessed in future clinical studies.

Conclusions and future directions

The discovery and study of memory NK cells has taught us much about the heterogeneity and functionality of this innate lymphocyte subset. The importance of proinflammatory cytokines and downstream transcription factors in driving NK cell expansion and memory through epigenetic imprinting appears conserved between mice and humans. This differentiation process in NK cells is currently being harnessed for therapeutic purposes against cancer and infectious diseases. Given the potential of these cytotoxic cells in the treatment of a wide range of diseases, we need a greater understanding of how cytokines and ligand engagement impact effector and memory formation in vitro in order to replicate the in vivo processes of NK cell activation, differentiation, and memory formation.

Acknowledgments

We apologize to those whose important contributions to this field we could not discuss owing to space limitations. We thank members of the Sun laboratory and Dr. Nadia Guerra for their critical reading and discussions of this manuscript.

J.C. Sun was supported by the Ludwig Center for Cancer Immunotherapy, the American Cancer Society, the Burroughs Wellcome Fund, and the National Institutes of Health (grants AI100874, AI130043, and P30CA08748).

Author contributions: S. Sheppard and J.C. Sun conceived and wrote the manuscript.

Disclosures: The authors declare no competing interests exist.

Submitted: 5 October 2020
Revised: 25 November 2020
Accepted: 17 December 2020

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