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## NK cells and immune “memory”

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### Abstract

Immunological memory is a hallmark of the adaptive immune system. However, the ability to “remember” and respond more robustly against a second encounter with the same pathogen has been described in organisms lacking T and B cells. Recently, natural killer (NK) cells have been shown to mediate antigen-specific recall responses in several different model systems. Although NK cells do not rearrange the genes encoding their activating receptors, NK cells experience a selective education process during development, undergo a clonal-like expansion during virus infection, generate long-lived progeny (i.e. memory cells), and mediate more efficacious secondary responses against previously encountered pathogen – all characteristics previously ascribed only to T and B cells in mammals. This review describes past findings leading up to these new discoveries, summarizes the evidence for and characteristics of NK cell memory, and discusses the attempts and future challenges to identify these long-lived memory NK cell populations in humans.

### Introduction

The innate and adaptive immune systems have traditionally been segregated into well-defined compartments. Innate immunity features short-lived cells that respond rapidly and non-specifically against pathogen exposure. Re-encounter with the same pathogen is thought to result in a qualitatively and quantitatively identical response as the first encounter. In contrast, adaptive immunity consists of T and B cells, which respond more slowly, but with high specificity due to somatically rearranged genes that generate an infinitely diverse set of antigen receptors. The clonal expansion of a pool of antigen-specific effector T and B cells initiated by pathogen exposure results in a population of long-lived “memory” cells that are able to respond quicker and more robustly during subsequent encounters with the same pathogen. The lifespan of many innate immune cells are thought to be on the order of hours or days - relatively short compared to T and B cells, which persist for months to years -

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making immune memory unimportant, or unnecessary, for short-lived cells comprising the innate immune system such as granulocytes and dendritic cells. Although frequently discussed in isolation, these two sides of the immune system rarely act in isolation and the interplay between cells of the innate and adaptive immune arms contributes to the most productive overall responses against pathogen invasion.

### Evidence for innate immune memory

Although immunological memory has been one of the classical hallmarks that distinguishes adaptive immune T and B cells from all other cells of the hematopoietic lineage, evidence has been found in invertebrates (such as crustaceans, flies, beetles, and mosquitoes) and more primitive species (such as tunicates, sea urchins, and sea sponges) that immune “memory” exists independently of lymphocytes possessing rearranged antigen receptors (1–3). Exposure of the copepod *Macrocyclus albidus* (a minute crustacean) to its natural pathogen, the parasitic tapeworm *Schistocephalus solidus*, results in resistance to challenge with an antigenically similar tapeworm (4), providing evidence for the early existence of innate immune memory. *Drosophila melanogaster* injected with a sublethal dose of *Streptococcus pneumoniae* or *Beauveria bassiana* (a natural fruitfly pathogen) also demonstrated resistance against subsequent bacterial challenge compared to flies that were unprimed with bacteria (5). The underlying mechanisms for the observed protection following priming required activation of the Toll pathway in phagocytes, which was suggested to mediate the secondary responses and resistance. Specific priming of resistance against its natural bacterial pathogen *Bacillus thuringiensis* and the common bacterium *Escherichia coli* was observed in the red flour beetle, *Tribolium castaneum* (6). Demonstrating specificity in this innate immune response, beetles previously primed with heat-killed bacteria were more likely to survive a subsequent exposure to the same bacteria that was used in priming, rather than exposure to a heterologous pathogen. Similarly, *Anopheles gambiae* mosquitoes (which are the major vector for malaria spread in Africa) pre-exposed to *Plasmodium falciparum* demonstrate enhanced immunity upon parasite re-infection (7). The protective effect was attributed to circulating granulocytes, whose numbers are rapidly increased upon primary infection. Together, these reports suggest that innate immune cells in many simpler organisms (which lack adaptive immune cells) can be primed by previous infections, and mount stronger secondary responses upon homologous pathogen challenge.

### Evidence for NK cell memory

Natural killer (NK) cells have long been categorized as a component of innate immunity. However, several lines of developmental, phenotypic, and functional evidence suggest that NK cells are closely related to T and B cells mediating adaptive immunity (8–10), with the exception of recombination-activating gene (RAG)-mediated rearrangement of antigen receptor genes. First, similar to the generation of T and B lymphocytes, NK cells are derived from the common lymphoid progenitor (11). Second, like T and B cells, NK cells require cytokines of the IL-2 receptor common-gamma chain family, particularly IL-15, for their development, homeostasis, and survival (12). Third, like thymocytes and pre/pro-B cells, immature NK cells undergo an “education” process whereby only appropriately selected

cells (i.e. tolerant to self) are able to be functional effectors in the periphery (13–17). Fourth, activated NK cells express many of the same surface receptors (CD25, CD43, CD44, CD69, CD122, Ly6C, CD62L, and KLRG1) as activated T cells (18, 19). Fifth, NK cells are functionally similar to T cells in their ability to produce IFN-gamma and TNF-alpha following activating receptor-mediated or cytokine-induced stimulation (18, 19). Sixth, NK cells are functionally related to CTL through their shared ability to mediate cytotoxicity via perforin and granzymes (18, 19). With the exception of antigen receptors generated from somatically rearranged genes, the evidence above suggests that NK cells are evolutionarily similar to T and B cells of adaptive immunity. In fact, immature T cells that lack one specific transcription factor (Bcl11b) have been shown to de-differentiate into NK-like precursor cells (20–22). Taken together, these observations suggest that NK cells may be developmentally and functionally more closely related to adaptive immune lymphocytes than innate immune cells. In line with their lymphocytic nature, recent studies have further demonstrated that NK cells can undergo a clonal-like expansion following virus infection in both humans and mice, and that previously primed NK cells can mediate secondary memory responses.

Early studies suggested the possibility that NK cell memory could exist. In a model of F1 hybrid resistance (where the parental bone marrow graft is rejected by F1 recipient mice), B10 x B10.D2 mice “primed” with parental B10 bone marrow cells rejected a second B10 graft more efficiently (23). When mice were primed with parental B10.D2 or a third party allogeneic bone marrow prior to the parental B10 graft, the rapid rejection was abrogated. At the time, NK cells had not yet been identified as the cells mediating hybrid resistance; however, a retrospective interpretation of these early experiments suggests relevance for NK cell memory in graft rejection. Recent studies have indicated that NK cell priming can also occur non-specifically during inflammation induced by TLR triggering on DCs or exposure to cytokines (24, 25). Thus, although NK cells were initially described by their ability to kill certain tumor cells without previous sensitization (26–30), NK cells become even more potent effectors when stimulated. A study using the MHC class I-deficient RMA-S tumor model showed that NK cells primed by tumors that lacked MHC class I were also more activated and mediated greater effector responses (31). A significant increase in IFN- $\gamma$  secretion and cytotoxic activity was measured in the activated NK cells only following inoculation with RMA-S tumor cells and not with RMA tumor cells expressing MHC class I, suggesting that in addition to inflammatory signals, NK-sensitive tumor cell targets can prime resting NK cells for greater effector potential, similar to T cell priming.

A more recent study demonstrated that NK cells mediate contact hypersensitivity responses to chemical haptens in RAG-deficient mice (32). In mice that lack T and B cells, contact hypersensitivity responses to 2,4-dinitrofluorobenzene and oxazolone persisted for greater than 4 weeks, and the responses were only elicited by the hapten to which mice were originally exposed and not by a different hapten. In the control mice lacking T, B, and NK cells due to a genetic deficiency in the RAG genes and the IL-2 receptor common gamma gene, no contact hypersensitivity was measured in previously sensitized mice. Interestingly, the only population of hapten-specific memory NK cells that could transfer sensitivity resided in the liver. Further studies are required to determine why only liver NK cells mediated the secondary response and the nature of the receptor-hapten interaction driving

the responses. Following this report, our group used the well-characterized NK cell response to mouse cytomegalovirus (MCMV) to determine whether immune memory could exist in virus-specific NK cells (33). NK cells in C57BL/6 mice bearing the Ly49H receptor have been shown to specifically recognize MCMV-infected cells expressing the viral glycoprotein m157 and undergo a “clonal-like” expansion (34–36). This proliferation was antigen-specific because infection of mice with a mutant MCMV lacking m157 could not drive Ly49H<sup>+</sup> NK cell expansion (33). Using an adoptive transfer system, small numbers of Ly49H<sup>+</sup> NK cells were observed to proliferate 100–1000 fold in lymphoid and non-lymphoid tissues in the recipient following MCMV infection, resulting in a long-lived pool of memory NK cells (Fig. 1A). This self-renewing population of NK cells was able to undergo secondary and even tertiary expansion following several rounds of adoptive transfer and virus infection (37). The memory NK cells recovered from previously infected mice several months later exhibited more robust effector functions *ex vivo* and were far more effective at protection against viral challenge compared to an equal number of resting NK cells from naïve mice, demonstrating a qualitatively different secondary response in NK cells that had previously encountered viral antigen (33). More recently, another report showed that memory-like NK cells could be induced *in vitro* by exposure to inflammatory cytokines such as IL-12 and IL-18 (38). Following adoptive transfer into recipient mice, these cytokine-activated NK cells were found to respond more robustly several weeks later (measured by IFN- $\gamma$  production following activating receptor triggering) compared to resting NK cells. Altogether, these studies demonstrate that NK cells can persist for far longer than previously estimated (39–41), and have the potential to contribute alongside memory T and B cell responses during subsequent encounters with the same pathogen.

### Characteristics of NK cell memory

How do memory NK cells differ qualitatively from resting NK cells? We know that CD8<sup>+</sup> T cells undergo a programmed differentiation during virus infection that results in dramatic changes in their gene expression profile at each stage (42). Similarly, we have recently used gene array technology to profile transcription in Ly49H<sup>+</sup> NK cells at different stages following MCMV infection: resting, acute activation, expansion, contraction, and memory maintenance phases (Fig. 1B). Although much work remains to determine the relative contributions of specific genes during each particular phase of the NK cell response, the global signature confirms that the mRNA transcriptional profile of resting NK cells is different from that of memory NK cells (Fig. 1B). Furthermore, the profiles of resting and memory NK cells differ greatly from both early activated and clonally expanded Ly49H<sup>+</sup> NK cells, demonstrating that NK cells at each phase of the response to MCMV exhibit a gene profile that is unique and stage-specific (Fig. 1B). The gene array “roadmaps” that immunologists studying memory T cell have generated (42–44), along with the identification of specific transcription factors for T cell differentiation, homeostasis, and survival (45), are useful tools that can guide the search for the factors that govern differentiation of NK cells following activation.

T cell studies have shown that during effector-to-memory cell differentiation different subsets of memory cells are generated, characterized by their anatomical location and expression of cytokine and homing receptors (46–48) (Fig. 2). Can the central memory

versus effector memory T cell paradigm be found in memory NK cells as well? Memory NK cells generated during virus infection were all found to highly express KLRG1 (33), an inhibitory receptor recognizing e-cadherins, also predominantly expressed on effector memory CD8<sup>+</sup> T cells (Fig. 2). Perhaps memory NK cells resemble this more terminally differentiated subset of memory T cells (Fig. 2). Unlike the overall maintenance of memory CD8<sup>+</sup> T cell numbers, which “plateau” following contraction (49), the memory NK cell population contracts similarly to memory CD4<sup>+</sup> T cell responses during LCMV infection (50). Although memory NK cells have been shown to self-renew (measured by BrdU uptake over the course of several days) (37), absolute numbers steadily decline and in the absence of secondary infection, memory NK cells in mice are difficult to detect 6 months after MCMV infection; however, this represents a relative time span equivalent to decades in humans (J.C.S. and L.L.L., unpublished observations). Whether the overall long-lived NK cell population experiences preferential survival of the progeny of a subset of memory cells remains to be addressed.

Like memory T cells, memory NK cells have been shown to both self-renew and mount multiple rounds of expansion and contraction in numbers (37). Future studies will determine whether differences exist in the maintenance of memory NK cells compared to T cells, and which cytokine signals (e.g. IL-7 and IL-15) and stromal cell interactions play an important role in the survival of long-lived NK cell populations. Many other outstanding tasks remain, the foremost of which is to identify a reliable and stable marker (or set of markers) to define memory NK cells, as has been identified for memory T cells (Fig. 2). This will allow direct analysis of long-lived NK cells within the endogenous NK cell population and circumvent the need for adoptive transfer to track antigen-experienced NK cells. Furthermore, we have observed that memory NK cells reside in both lymphoid and non-lymphoid tissue, but determining which soluble factors (cytokines and chemokines) modulate organ- and tissue-specific distribution and phenotype of memory NK cells is warranted. The contribution of stromal elements and other leukocytes (such as dendritic cells and CD4<sup>+</sup> T helper cells) towards the generation of memory NK cells requires further elucidation. Lastly, the transcriptional control of NK cell differentiation and memory generation has not been well characterized, in contrast to the case with T cell differentiation. As more pathogen ligands recognized by NK cell receptors are characterized, additional infectious disease models will allow further analysis of the broader principles of NK cell activation, expansion, and memory.

## Do memory NK cells exist in humans?

In humans, NK cells also play a crucial role in the response against CMV. In an early study, a patient selectively lacking NK cells, but having normal B and T cells, was found to suffer a life-threatening illness after infection with human CMV (HCMV) (51). Other reports of specific NK cell deficiencies (or NK cell functional deficiencies) in humans (52, 53) similarly describe overwhelming fatal infections during childhood or adolescence due to HCMV and other herpesviruses such as VZV and EBV, demonstrating the importance of NK cells in the immune response against certain viral infections. Functional Ly49 genes do not exist in humans, and thus far there is no evidence that members of the killer-cell immunoglobulin-like receptor (KIR) family, which encode human NK cell receptors



CD56 and expression of CD16 (low-affinity Fc $\gamma$  receptor IIIA, Fc $\gamma$ RIIIa), with CD56<sup>dim</sup>CD16<sup>bright</sup> cells comprising ~90% and CD56<sup>bright</sup>CD16<sup>neg/dim</sup> cells constituting ~10% of NK cells in the blood (69). Human NK cells, however, are a heterogeneous population with respect to the expression of KIR, NKG2A, and natural cytotoxic receptors (NCR). Recently, our group and others have examined expression of CD57, a carbohydrate antigen that is expressed on subsets of human NK cells and T cells (70). CD57 is expressed only on a minor fraction of NK cells in fetal tissues or cord blood (which represent the most naïve NK cells in humans), and the percentage of NK cells and T cells expressing CD57 increases with age (71–73). A recent study showed that CD57 is a marker linked to cellular maturity of CD8<sup>+</sup> T cells and NK cells, and that it correlates with high cytolytic potential (74). CD56<sup>dim</sup>CD57<sup>-</sup> NK cells can become CD56<sup>dim</sup>CD57<sup>+</sup> after stimulation *in vitro*, during the reconstitution of the immune system in a humanized mouse model and in patients undergoing hematopoietic stem cell transplantation (75, 76). These studies suggest that as mature NK cells differentiate from CD56<sup>bright</sup> to CD56<sup>dim</sup> they lose expression of NKG2A, NCRs, CD27, and CD62L, while acquiring CD16, LIR-1 (also named CD85J and LILRB1), Siglec-9, and KIRs, and the final stage of activation involves the acquisition of CD57 (75–77).

Is the CD57 activation marker on human NK cells the equivalent of KLRG1 on the mouse NK cells that have become activated and differentiated into effectors? Because CD8<sup>+</sup> T cells expressing CD57 were reported to possess shorter telomeres than CD57<sup>-</sup> cells and express an effector/memory phenotype (78, 79), it is possible that NK cells expressing CD57 might also represent NK cells that have previously been driven into clonal expansion by encounters with pathogens. The frequency of NK cells expressing CD57 varies in different adult blood donors. Moreover, the frequency of NK cells expressing CD57 within a given NK cell subset in an individual is not uniform; the percentages of CD57<sup>+</sup> and CD57<sup>-</sup> NK cells within the KIR2D, KIR3D, and NKG2A NK cell subsets in a single individual vary considerably, suggesting that NK cells within these subsets exist at different stages of activation or differentiation, likely as a consequence of different exposure to environmental pathogens (75, 76). Further studies are necessary to determine whether the CD94-NKG2C<sup>+</sup> NK cells that specifically expand during HCMV infection will upregulate CD57 expression, and whether CD57 will serve as a marker for NK cells that can form a long-lived memory cell population that respond against subsequent HCMV exposure.

As mentioned previously, memory NK cells in mice generated following MCMV infection produce higher amounts of cytokines and degranulate more robustly compared with resting NK cells (33). Surprisingly, the CD57<sup>+</sup> NK cell population produced less IFN $\gamma$  than CD57<sup>-</sup> NK cells in response to activation by cytokines (i.e. IL-12 and IL18), but had higher amounts of granzymes (75). Although they degranulated similarly in response to stimulation via the majority of activating receptors, CD57<sup>+</sup> NK cells responded better to stimulation through CD16 compared to CD57<sup>-</sup> cells (75). It remains to be determined how CD57<sup>+</sup> NK cells bearing a virus-specific receptor such as CD94-NKG2C will behave *in vivo* during a secondary infection or HCMV reactivation. Thus, although CD57 is a good candidate as a marker of highly mature NK cells that may have been driven to expand in response to

pathogens, further studies are necessary to determine if CD57<sup>+</sup> NK cells represent a long-lived memory cell population in humans.

## Challenges and barriers

The study of human NK cells poses many challenges and barriers, not least of which are the logistical difficulties. First, trafficking of NK cells may contribute to their maturation, function, and longevity, but there is a dearth of information about human NK cells residing in different organs or at the site of infection as most human studies use NK cells from the peripheral blood. Second, there is high variability in the expression of NK cell receptors between individuals, in addition to the extensive polymorphism in the KIRs and their HLA ligands (80–82), thus making the number of donors or patients analyzed in any study crucial. Third, most of the functional data is obtained from *ex vivo* experiments or with cytokine-activated NK cells, which may alter surface receptor expression and cell function. Finally, assessing NK cell numbers and function in response to infection with pathogens is complicated by the fact that in most cases the dose, route, and time of pathogen exposure are unknown. In addition, treatment of infections with antibiotics and antiviral agents may adversely influence immune responses and NK cell responsiveness.

To demonstrate that a specific human NK cell population responds to a pathogen and that antigen-specific NK cells persist after infection, longitudinal studies are required. For HCMV, like most herpes viruses, the majority of the infections are asymptomatic and occur during childhood or adolescence, therefore determining when infection was initiated is difficult. HCMV persists in approximately 60% of the human population and once infected, the virus is never eliminated, but must constantly be restrained by the immune system. Although HCMV causes only subclinical disease in healthy humans, it can be life-threatening in newborns and immunocompromised or immunosuppressed individuals (83–85). In solid-organ transplant patients, where immunosuppressive drugs are used to prevent graft rejection, more than half suffer from clinical manifestations of CMV infection if they are not treated prophylactically with antiviral drugs (83). Therefore, given that many (~30% for the bone marrow transplant patients) of these people will demonstrate re-activation of HCMV, transplantation patients could pose an interesting cohort for longitudinal study of NK cells in response to HCMV infection *in vivo*. Even so, caveats due to immunosuppressive therapeutics administered to the transplant patients and/or the immature differentiation state of the NK cells in the bone marrow transplant patients will likely influence the NK cell response to reactivation or infection with HCMV, potentially confounding interpretation of such studies. Moreover, these patients are susceptible to opportunistic infections in addition to HCMV that may influence the NK cell response.

## Conclusions

The study of NK cell memory in mice and humans is just beginning. The identification of these long-lived NK cells in mice opens up the possibility that similar populations exist in humans. As specific NK cell responses against many viruses (including herpesviruses, poxviruses, HIV, and influenza) have been described and more NK cell receptor-viral ligand interactions are being elucidated, NK cell subsets can be considered in the design of



adoptive immunotherapy regimens against acute viral infection. Given the current interest in developing strategies to apply NK cells as therapeutic agents against a broad range of malignancies (86, 87), and approaches to augment NK cell function during chronic viral infections (such as HIV-1 and hepatitis C virus), NK cells have the potential to be exploited as another branch of immunity that can confer long-term protection through vaccination.

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## Abbreviations used in this paper

<b>MCMV</b>	mouse cytomegalovirus
<b>HCMV</b>	human cytomegalovirus
<b>LCMV</b>	lymphocytic choriomeningitis virus
<b>VZV</b>	varicella zoster virus
<b>EBV</b>	Epstein Barr virus
<b>HIV</b>	human immunodeficiency virus
<b>KIR</b>	killer-cell immunoglobulin-like receptor
<b>NCR</b>	natural cytotoxicity receptor
<b>MHC</b>	major histocompatibility complex
<b>HLA</b>	human leukocyte antigen

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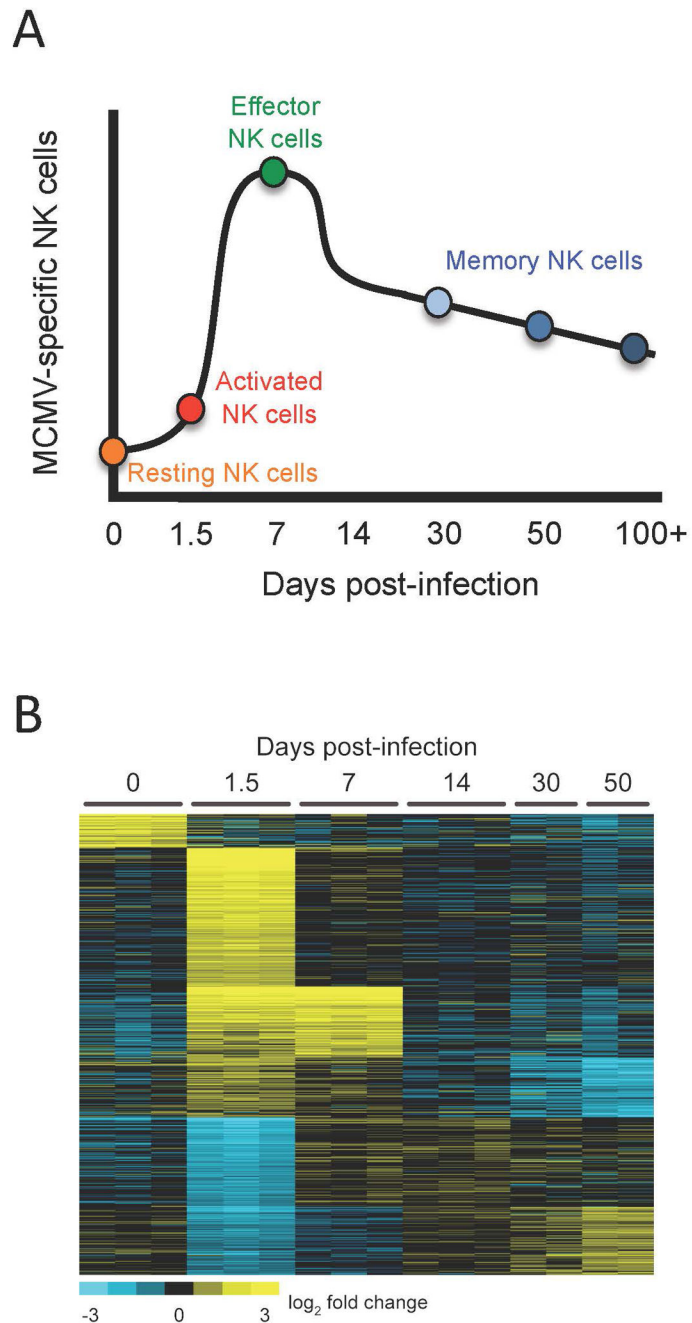
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**FIGURE 1.**

(A) The virus-specific NK cell response to MCMV infection. During MCMV infection, resting Ly49H<sup>+</sup> NK cells become activated and undergo an expansion phase resulting in the generation of more effector cells. The expansion phase is followed by the contraction of effectors resulting in long-lived memory NK cells months after initial infection. (B) The gene array profile of different stages of the Ly49H<sup>+</sup> NK cell response to MCMV infection. Congenic Ly49H<sup>+</sup> NK cells were adoptively transferred prior to MCMV infection, as previously described (33). The transcriptional signature of naïve (day 0), activated (day 1.5),

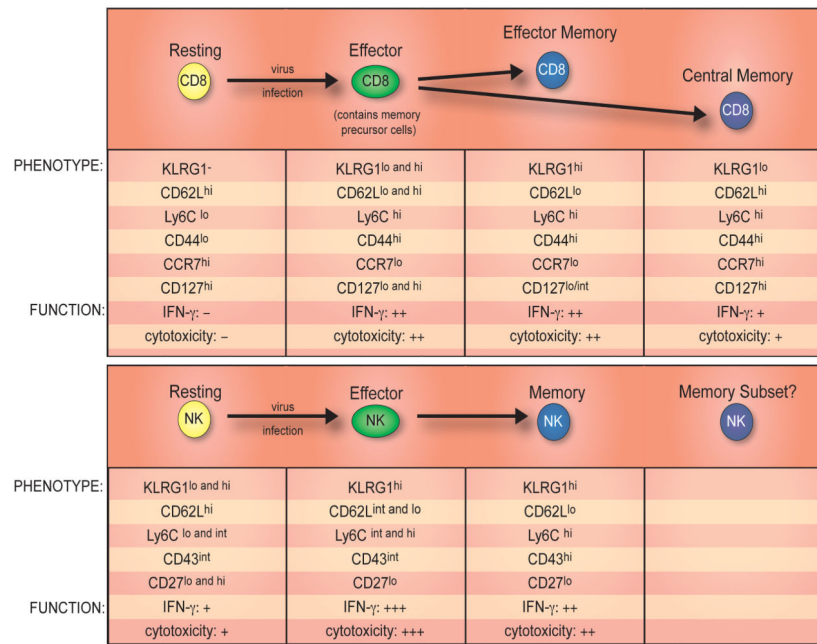
effector (day 7), contracting (day 14), and memory (day 30 and 50) NK cells is unique at each time point following MCMV infection. Ly49H<sup>+</sup> NK cells from 3 separate mice at each time point were individually sorted on a FACS Aria for RNA isolation (except for day 30 and day 50 time points done in duplicate). Samples were hybridized on the MEEBO microarray platform against reference mouse RNA, as previously described (88). All microarray data are available through the Gene Expression Omnibus under accession number GSE25672.

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**FIGURE 2.** A comparison of CD8<sup>+</sup> T cell and NK cell differentiation and memory generation following viral infection. Phenotypic and functional descriptions of resting, effector, and memory T and NK cells are shown. (lo, low; int, intermediate; hi, high)