

# Evidence for Natural Killer Cell Memory Minireview

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Natural killer (NK) cells are generally considered to be part of the innate immune system. Over the past few years, however, evidence has accumulated suggesting that NK cells have certain features that are characteristic of the adaptive immune system. NK cells reportedly respond in an antigen-specific manner to a variety of small molecules and certain viruses, and mediate enhanced responses to these antigens upon secondary exposure. In infections with mouse cytomegalovirus (MCMV), MCMV-specific NK cells undergo clonal expansion, and display increased effector function after the resolution of the infection. In addition, inflammatory conditions resulting from exposure to certain cytokines seem to promote prolonged effector function in NK cells in an antigen-non-specific fashion. Taken together, these studies reveal new aspects of NK biology, and suggest that NK cells, like T and B cells, may carry out memory responses and may also exhibit greater capacity to distinguish antigens than was previously recognized.

## Introduction

The immune system has the capacity to learn from previous encounters with pathogens and to mount a stronger and more rapid response upon secondary infection. This phenomenon has been termed adaptive immunity or immunological memory. It is often thought that the capacity for adaptive immunity goes hand in hand with the somatic antigen receptor diversification mechanisms that operate in T and B cells, which act to generate a large number of cells with different clonal specificities. A key DNA-modifying somatic diversification mechanism relies on the *Rag1* and *Rag2* genes, which encode the subunits of the RAG recombinase that assembles B and T cell receptor genes, generating diversity in the process. After primary exposure to antigen, naïve antigen-specific T or B cells proliferate vigorously in a process known as clonal expansion, and some of them differentiate into memory cells [1]. Following the primary response, most of the effector cells die during the contraction phase, but antigen-specific memory cells are maintained in highly elevated numbers compared with the number of antigen-specific cells in naïve animals. Upon re-exposure to the antigen, individual memory cells exert their functional responses more rapidly than do naïve cells. The expanded number of memory cells combined with their faster response leads to strongly enhanced responses to a second antigen exposure (called the ‘recall response’).

The immune system can also mount another set of rapid responses that do not require pre-sensitization, collectively termed innate immune responses. Innate responses rely on germline-encoded receptors and do not require clonal expansion. The innate immune system comprises many kinds of cells, each capable of performing specific functions. These cells include phagocytic cells, such as macrophages

and neutrophils, which are important for eliminating bacteria and certain parasites, and other cell types, such as dendritic cells, which serve as pathogen sensors capable of alerting and activating other leukocytes. Natural killer (NK) cells are also considered to be innate effector cells, although they are lymphocytes, like T and B cells. NK cells are capable of killing other cells, including cancer cells and cells infected with viruses and other intracellular pathogens. The innate immune system can eliminate some minor infections and delay the development of more serious infections, allowing time for the development of adaptive immune responses.

Although NK cells lack any known somatic diversification mechanisms, it has recently been reported by several groups that they are capable of mediating adaptive immune responses, which were previously thought to be an exclusive property of T and B cells. Here, we provide an overview of the evidence for antigen-specific responses and memory responses of NK cells, both of which are hallmarks of adaptive immunity.

## Key Characteristics of NK Cells

NK cells were so named because they display cytotoxic activity against transformed and virus-infected cells without prior sensitization [2,3]. NK cells can kill target cells by excreting granules containing perforin, which perforates the membrane of target cells, and granzymes, which penetrate the target cell, activate caspases and thus cause target cell apoptosis. NK cells also secrete various cytokines, in particular interferon  $\gamma$  (IFN $\gamma$ ), which has both anti-neoplastic and anti-viral effects [4]. In addition to being activated by tumor cells or virus-infected cells, NK cells can also be activated by cytokines that are secreted by dendritic cells and other cells in response to pathogens. Specifically, NK cells secrete IFN $\gamma$  following stimulation with the cytokines IL-12 and IL-18.

NK cells do not express the RAG recombinase that mediates DNA recombination of T and B cell antigen receptor genes. Instead, they express various germline-encoded receptors, some of which stimulate the cells to mediate cytotoxicity or produce cytokines, and others that inhibit stimulation. Nevertheless, NK cells defy the conventional innate–adaptive dichotomy in several ways (Table 1) [5]. First, NK cells, together with T and B cells, belong to the lymphoid lineage, based both on cell morphology and their origin in the common lymphoid progenitor [6]. Second, NK cells undergo an education process that prevents them from being autoreactive [7], as do T and B cells. Third, the expression pattern of some of the germline-encoded receptors in NK cells is clonally distributed, and the complement of receptors expressed by each NK cell determines its specificity [8]. Fourth, it was reported early on by Karre’s group [9] that naïve NK cells exhibit very weak functional activities until preactivated with sensitive target cells *in vivo*, suggestive of an adaptive aspect to the NK response. Fifth, at least a subset of NK cells can survive for a prolonged period of time (over six months), in contrast to the relatively brief lifetime of most innate immune cells [10]. In recent years, three independent lines of investigation, summarized below, have provided substantial evidence that NK cells can mount adaptive-like responses, including robust antigen-specific responses to a second challenge with an antigen.

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Table 1. Innate and adaptive characteristics of NK cells.

	Innate immune cell characteristics	NK cell characteristics	Adaptive immune cell characteristics
Cell lineage	Myeloid, lymphoid	Lymphoid	Lymphoid
Genes encoding major known recognition receptors	Germline genes	Germline genes	Rearranging genes
Clonally distributed expression of recognition receptors	No	Yes (in some cases <sup>a</sup> )	Yes
Multiple recognition receptors can independently activate individual cells	Yes	Yes	No
Cells undergo education processes to prevent autoreactivity	Unknown	Yes	Yes

<sup>a</sup>Receptors from the Ly49 and NKG2A-C families of genes, each of which include some activating and some inhibitory members, are expressed in a clonally distributed fashion, whereas receptors such as Nkp46 and NKG2D are uniformly expressed.

### Response of NK Cells to Small Molecules and Viruses

Contact hypersensitivity refers to the immune response to topical administration of certain chemical irritants. It was thought that these reactions are mediated exclusively by T cells until the von Andrian lab [11] demonstrated that mice lacking T and B cells can mount contact hypersensitivity responses to at least three different reactive chemical irritants: 2,4-dinitro-1-fluorobenzene (DNFB), 4-ethoxymethylene-2-phenyl-3-oxazolin-5-one (oxazalone), and picryl chloride. The mice exhibited enhanced recall responses to the same chemical, but not to a different one, demonstrating antigen specificity [11]. Importantly, these contact hypersensitivity responses could be elicited as late as 4 months after the original challenge. Antibody-mediated depletion of NK cells abrogated the response, and the response was absent in mutant mice lacking NK cells as well as T and B cells. Notably, recall contact hypersensitivity responses could be conferred to naïve mice by transferring NK cells from chemically treated mice. It was shown that transfer of a subset of liver-derived NK cells could confer the recall response, whereas transfer of splenic NK cells was ineffective [11].

Later studies added considerably to the story by showing that NK cells could mount recall responses to diverse viral antigens, such as vesicular stomatitis virus (VSV), virus-like particles (VLPs) containing influenza A-derived hemagglutinin and/or matrix protein 1, or VLPs containing the HIV-1-derived Gag protein and/or Env protein [12]. Furthermore, it was shown that, in order to confer the recall response, liver NK cells had to express the chemoattractant receptor CXCR6 (which is expressed on roughly 50% of liver NK cells), and that blocking CXCR6 with antibodies can impair the recall response. Since these initial studies, a few additional groups have also reported that NK cells can respond more vigorously upon secondary stimulation against challenges with additional irritants (such as fluorescein isothiocyanate), as well as the viruses vaccinia, and herpes simplex virus 2 (HSV-2) [13–15].

A central issue that remains unresolved is how NK cells can recognize and differentiate between all of these different antigens, since there are no known VSV-, vaccinia-, HSV-2-, or HIV-1-specific NK receptors. The ability of NK cells to respond to such a wide diversity of distinct antigens, including pathogens that are not endemic to mice, such as HIV-1, is puzzling and suggests that a hitherto unknown RAG-independent receptor diversification mechanism may exist in NK cells.

### NK Cell Response to Mouse Cytomegalovirus

The first clues that NK cells can carry out clonal antigen-specific responses came from the study of the response to

mouse cytomegalovirus (MCMV) by the Yokoyama group [16,17]. A subset of NK cells in the C57BL/6 strain of mice expresses the Ly49H receptor, which binds to the MCMV protein m157 displayed on the surface of MCMV-infected cells. Early reports showed that Ly49H<sup>+</sup> NK cells undergo clonal expansion in mice infected with MCMV, and the proliferation was antigen-specific because infection with a MCMV mutant lacking m157 did not cause expansion of Ly49H<sup>+</sup> NK cells [16,18,19]. Unlike antigen receptors in T and B cells, however, Ly49H is encoded by the germline and is expressed by a very large fraction of NK cells in naïve mice (roughly 40–50% of NK cells). Furthermore, the overall expansion of Ly49H<sup>+</sup> cells in infected mice was relatively modest, and so the relevance of this response to adaptive immunity was unclear. Later experiments demonstrated that germline-encoded MCMV-specific receptors exist in mouse strains other than C57BL/6 (such as BALB/c, NOD, and others), and that clonal expansions of MCMV-specific NK cells could also be observed in those strains [20].

Adding considerably to the story, the Lanier lab subsequently showed that when tiny numbers of naïve NK cells were transferred into mice lacking a functional Ly49H receptor (due to a mutation in the DAP12 protein, which transduces signals from Ly49H), the donor Ly49H<sup>+</sup> NK cells underwent a robust (100–1,000 fold) expansion [18]. Furthermore, the transferred NK cells persisted for more than 2 months, exhibited a more differentiated phenotype and were more responsive than naïve NK cells to restimulation (as measured by IFN $\gamma$  secretion and LAMP-1 expression). Importantly, NK cells from MCMV-infected mice were more effective than NK cells from naïve mice in protecting neonatal mice from MCMV infection, suggesting a type of protective ‘NK memory’. Notably, while IFN $\gamma$  secretion was higher in these NK cells, their proliferation behavior was similar to that of ‘naïve’ NK cells, not only in terms of magnitude of the response but also in the rate of expansion and contraction. These studies established that receptor activation could drive NK cells to undergo clonal expansion and respond in a manner that has several features in common with the adaptive responses of T and B cells. Thus, when experimental manipulations were used to limit the frequency of antigen-specific naïve NK cells, the extent of clonal expansion was substantial in the primary response, and protective memory was established. It remains unclear whether, for any natural NK antigens, the number of antigen-specific clones is highly limiting, or whether substantial clonal expansion occurs in normal conditions.

### Cytokine-induced NK Responses

In an independent approach to the issue of memory NK cell responses, work from the Yokoyama lab has shown that

Table 2. Inducers of NK cell 'memory'<sup>a</sup>.

	Antigen-specific memory			Cytokine-induced memory
	Chemical irritants <sup>b</sup>	Virus-like particles <sup>b</sup>	MCMV	
Receptor(s)	Unknown	Unknown	Ly49H	IL-12R + IL-18R
Clonal expansion	Unknown	Unknown	Yes	Not applicable
Duration	~4 months	~4 months	~70 days	~4 weeks
Degranulation	Enhanced	Unknown	Enhanced	Unchanged
IFN- $\gamma$ secretion	Unknown	Unknown	Enhanced	Enhanced
Protection from viral infection	Not applicable	Enhanced	Enhanced	Unknown

<sup>a</sup>NK cell 'memory' has been reported to be antigen-specific in the case of chemical irritants, virus-like-particles, and MCMV, or alternatively it can be induced by cytokines.

<sup>b</sup>Examples of chemical irritants capable of eliciting contact hypersensitivity responses include DNFB, oxazolone, picryl chloride, and FITC (see text). Both chemical irritants and virus-like particles can elicit NK-cell-dependent contact hypersensitivity reactions that are antigen specific.

activation of NK cells with the cytokines IL-12 and IL-18 has long-lasting effects on NK cell activity [21]. Following adoptive transfer, IL-12/IL-18-activated NK cells were capable of more vigorous IFN $\gamma$  responses for several weeks post-transfer. The heightened responsiveness was observed despite the fact that no phenotypic differences could be detected between naïve and transferred NK cells at time points later than one week after activation, when the NK cells had reverted to a quiescent state. A key point in these studies was that the enhanced responsiveness could also be detected in the progeny of IL-12/IL-18-activated NK cells, providing a possible explanation for the maintenance of the enhanced response over a prolonged time period. A recent study extended these findings and demonstrated that the 'memory' phenotype is maintained even after homeostatic proliferation [22]. A caveat to most of these studies is that the NK cells were stimulated *ex vivo* before injecting them into mice, but a more recent paper by Sun *et al.* [23] showed that the formation of 'memory' NK cells in MCMV-infected mice also depends on IL-12. In sum, these findings suggest that cytokines associated with inflammation can modulate the NK response to secondary activation, although in this case there is no indication of antigen specificity in the recall response.

### Responses of Human NK Cells

Following these mouse studies, human NK cells were tested for their ability to mount recall responses. *In vitro* studies have shown that cytokine stimulation of human NK cells can enhance their responsiveness even after the NK cells return to quiescence, similarly to what has been shown in mice [24]. *In vivo* studies in humans have mostly focused on the response to various infections. Interestingly, NK cells that express the germline-encoded NKG2C receptor are normally absent or rare in the blood of healthy HCMV-seronegative individuals, but appeared in increased numbers in patients undergoing acute HCMV infection, and then diminished partially in numbers after the resolution of the acute phase [25–28]. The increase in NKG2C<sup>+</sup> NK cells was not observed in various other infections, including HSV-2 infection [29], and it has been suggested that NKG2C might be an HCMV-specific receptor analogous to Ly49H in mice. However, more recent studies have also shown an increase in the numbers of NKG2C<sup>+</sup> NK cells in response to infections with Hanta virus or Chikungunya virus [30,31]. In the Hanta virus study, these increases were only seen in HCMV-seropositive individuals, raising the possibility that the increase in NKG2C<sup>+</sup> NK cells reflected reactivation of latent HCMV.

Arguing against that interpretation, no HCMV DNA could be detected in the plasma of these individuals and there was no increase in the absolute numbers of CD8<sup>+</sup> T cells specific for the HCMV pp65 epitope. Notably, in HCMV-infected patients, NKG2C<sup>+</sup> NK cell numbers remained elevated (compared with uninfected individuals) well after the acute infection was cleared, demonstrating that viruses can have a long-term impact on NK cells. It is possible that subclinical levels of HCMV (present in sero-positive individuals) maintain an expanded population of NKG2C<sup>+</sup> NK cells, which can then respond to other infections. An important issue that has not been resolved, however, is whether infections with HCMV, Hanta virus or Chikungunya virus stimulate the clonal expansion of rare NKG2C<sup>+</sup> NK cells, or alternatively induce NKG2C<sup>-</sup> NK cells to express NKG2C.

### Summary and Future Directions

Studies in recent years have provided ample evidence for adaptive features of the NK cell response (see Table 2 for a summary of the characteristics of NK memory). First, studies suggested that NK cells could respond to a much greater diversity of antigens than previously envisaged, but the recognition mechanism for many of these antigens has remained elusive. Second, NK cells reportedly mount antigen-specific recall responses against various antigens. Third, exposure of NK cells to inflammatory cytokines can cause a sustained (albeit non-specific) increase in activity. Fourth, some viral infections have been shown to prime certain sets of NK cells in both mice and humans for a prolonged time period, arguing against the notion that the NK cell response is exclusively innate.

Despite these provocative and interesting findings, the adaptive nature of NK responses is still somewhat hazy, and the physiological relevance of these responses is still far from clear. Impressive clonal expansions were observed in the well-characterized MCMV system only under conditions where the proliferative expansion was artificially amplified by experimentally limiting the number of precursor cells. On the one hand, these experiments elegantly illustrate the capacity of NK cells to undergo specific and substantial clonal amplifications, but whether such large expansions occur during natural responses is unclear. In some of the other systems studied, the specific receptors involved have not been identified. In others, such as the HCMV system, expression of a specific receptor has been correlated with the response, but it remains unclear whether the receptor is HCMV-specific or whether the increased fraction of cells expressing the receptor is due to clonal expansion or

to induction of expression of the receptor. Cytokine pre-activation seems to have a much more prolonged impact on NK cell function than previously realized, but it is unclear what role this phenomenon plays in the response to natural infections. Furthermore, the three lines of evidence supporting the adaptive nature of NK cell responses are divergent and have yet to be integrated into a cohesive framework.

Clearly, an in-depth molecular understanding of all these responses is needed in order to truly understand the role of adaptive mechanisms in the NK cell response. NK cells are still mysterious in numerous respects, but an understanding of the mechanisms underlying the responses discussed here may lead to new NK-based therapeutic interventions against infectious agents and cancer.

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