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MINIREVIEW

Redefining Memory: Building the Case for Adaptive NK Cells

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ABSTRACT Classically, natural killer (NK) cells have been defined by nonspecific innate killing of virus-infected and tumor cells. However, burgeoning evidence suggests that the functional repertoire of NK cells is far more diverse than has been previously appreciated, thus raising the possibility that there may be unexpected functional specialization and even adaptive capabilities among NK cell subpopulations. Some of the first evidence that NK cells respond in an antigen-specific fashion came from experiments revealing that subpopulations of murine NK cells were able to respond to a specific murine cytomegalovirus (MCMV) protein and that in the absence of T and B cells, murine NK cells also mediated adaptive immune responses to a secondary challenge with specific haptens. These data have been followed by demonstrations of NK cell memory of viruses and viral antigens in mice and primates. Herein, we discuss different forms of NK cell antigen specificity and how these responses may be tuned to specific viral pathogens, and we provide assessment of the current literature that may explain molecular mechanisms of the novel phenomenon of NK cell memory.

KEYWORDS immune memory, innate immunity, natural killer cells

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Cellular components of the innate immune system are typically characterized as using a finite number of germ line-encoded pattern recognition receptors to sense pathogens, neoplastic cells, and other tissue damage (1–3). In contrast, the adaptive immune system, which includes T and B cells and their effector functions, relies on recombinase-activating gene (RAG)-dependent nonhomologous end-joining of chromosomal DNA and its recombination to generate a substantial T and B cell receptor repertoire capable of antigen-specific recognition (4). Activation of T and B cells by their cognate antigen leads to activation, proliferation, and the selection of high-affinity effector and memory cells and results in accelerated and enhanced recall responses by memory T and B cells upon reexposure (1–3). Medically, we exploit the ability to elicit memory immune cells via vaccination, as optimal vaccine strategies are designed to induce long-lived, antigen-specific memory T and B cells that mediate rapid, high-affinity recall responses upon encounter of the actual pathogen. In contrast, innate cells have been thought of solely as a nonspecific first line of defense against pathogens that may also serve to augment or tune adaptive responses but do not generate memory in their own right.

Natural killer (NK) cells are primary effector cells of the innate immune system that can rapidly eliminate tumor and virus-infected cells. Although NK cells have not traditionally been thought to carry adaptive capabilities or require antigen priming, they do encode a complex array of receptors to recognize specific ligands on target cells, and the tuned integration of these signals results in cytokine secretion and

cytolysis or, alternatively, tolerance (5). In humans, the largest group of NK cell receptors belong to the killer cell immunoglobulin (Ig)-like receptor (KIR) family, which consists of type I integral membrane proteins that form a polymorphic family within the Ig superfamily (6, 7). In mice, a similar group of NK cell receptors are type II integral membrane, C-type lectin-like molecules belonging to the Ly49 family (8). Both KIRs and Ly49s are germ line encoded, highly polymorphic receptors and selectively expressed on most naive NK cells, but NK cells can express one or more receptors (6, 7). KIRs and Ly49 receptors recognize host-derived major histocompatibility complex class I (MHC-I) molecules, contribute to the processes of "licensing" and "education" which occur during NK cell development, and ensure that only NK cells capable of engaging self-MHC with their inhibitory receptors are allowed to become functionally competent but are also restrained from killing healthy cells (9–13). Other receptor families in both mice and humans are leukocyte immunoglobulin-like receptors (LIRs), C-type lectin-like receptors (LLRs), tumor necrosis factor (TNF) superfamily receptors, and natural cytotoxicity receptors (NCRs), including members of the NKG2 family, and the common NK cell receptors NKp30, NKp46, and NKp80 (7, 14–20). Interestingly, NK cells and T cells share a progenitor. Both express NCRs, and many effector functions, such as gamma interferon (IFN-γ) release and perforin/granzyme-mediated killing, overlap significantly between NK cells and cytotoxic T lymphocytes (CTLs) (21). Indeed multiple cellular and noncellular components of the innate and adaptive immune system have been conserved in vertebrates for hundreds of millions of years, making it tempting to speculate that evolutionary pressures may have led to the development of an adaptive immune system from its innate counterpart in higher-order vertebrates (22). Over the past 10 years, a multitude of independent studies have revealed that subsets of murine and primate NK cells are capable of antigen-dependent expansion and long-lived immunological memory. Together, these data suggest that NK cell function may traverse both innate and adaptive immune systems, thus representing a third lineage of lymphocytes capable of antigen specificity. Here, we summarize and discuss current knowledge of NK cell-mediated adaptive immunity, its origins, and potential clinical applications (Fig. 1; Table 1).

ADAPTIVE NK CELL-MEDIATED IMMUNE RESPONSES TO ALTERED SELF-ANTIGENS

Exposure to haptens, self-proteins altered by the addition of a chemical moiety (23), induces a classical contact hypersensitivity (CHS) response, and typically the first exposure to hapten results only in sensitization. A second exposure to the same hapten generates an immune reaction resulting in a characteristic itchy rash, fluid-filled blisters, and hives. Human examples of hypersensitivity disease include asthma, rhinoconjunctivitis, otitis, rhinosinusitis, urticaria, angioedema, eczema, food allergy, drug allergy, insect allergy, occupational allergic diseases, and anaphylaxis (24). The CHS response is commonly used to investigate sensitization and antigen recall and was thought to be mediated primarily by T, NKT, and/or B cell activation (25). However, in 2006, O'Leary et al. reported that a novel subset of murine-liver-resident Thy1$^+$ (CD90$^+$) Ly49C$^+$ (in C57BL/6 mice) DX5$^+$ and NKG2D$^+$ NK cells can mediate antigen-specific long-lived immunological recall responses to haptens in a RAG-independent manner (26). These findings were highly surprising, as they demonstrated that certain subsets of NK cells are capable of adaptive CHS responses, and flow cytometric analyses and confocal microscopy further confirmed that NK cells are recruited to sites of CHS upon hapten challenge (26). Sensitization of NK cells may occur in lymph nodes, since antibody-mediated blockade of P-, E-, and L-selectin or genetic deficiency in L-selectin (27–29) blocked CHS responses in sensitized mice. Interestingly, only sensitized liver-resident Thy1$^+$ NK cells transferred CHS responses into naive lymphopenic hosts, while naive, sensitized hepatic Thy1$^-$ or splenic NK cells did not (26). In 2010, these findings were further clarified by Paust et al. in experiments demonstrating a requirement for adaptive NK cells to express CXCR6, which is expressed on about half of all murine-liver NK cells and for which the ligand, CXCL16, is constitutively expressed on liver sinusoidal

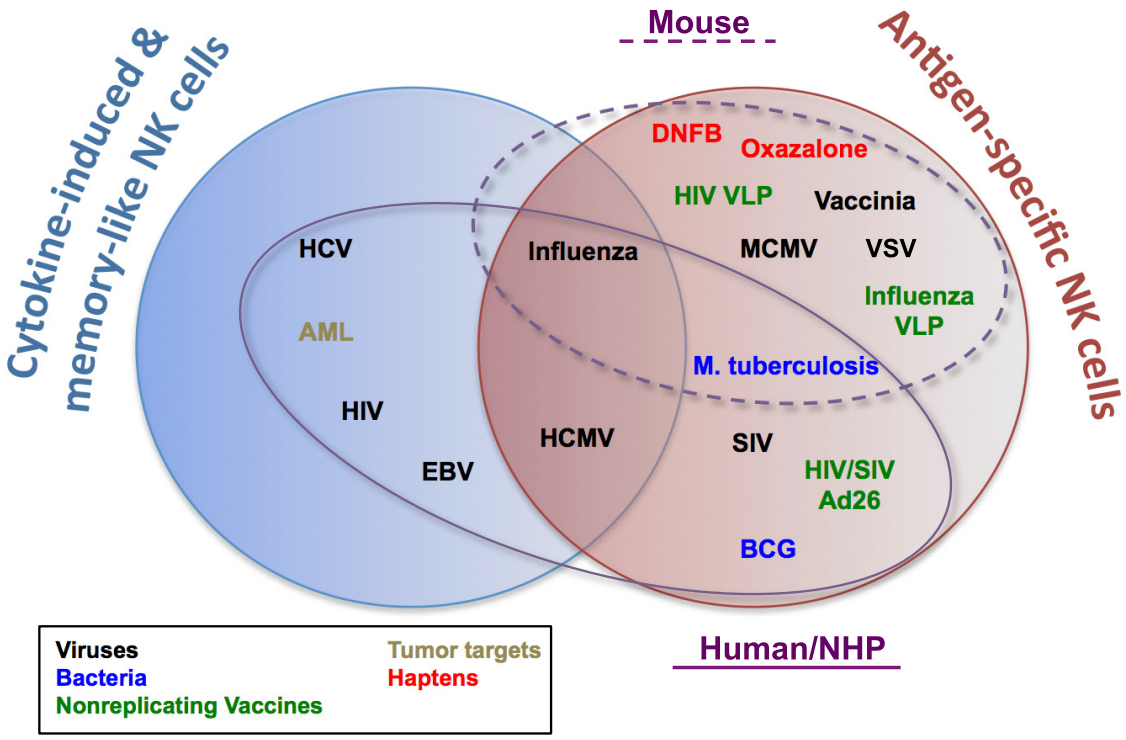

FIG 1 Examples of antigen-specific, cytokine-induced, and memory-like NK cells in mice and humans/nonhuman primates (NHP). Venn diagrams represent examples of evidence for adaptive NK cells and whether these have been demonstrated as being truly antigen specific, cytokine induced, and memory-like or a combination of these. Microbial pathogens or other agents for which memory NK cells have been demonstrated are color coded. Ellipses indicated those agents that have been demonstrated in mice (dashed line) or humans/NHP (solid line). AML, acute myeloid leukemia; VLP, virus-like particles.

endothelial cells and upregulated at sites of inflammation (30, 31). Indeed, CXCR6-deficient mice have reduced numbers of NK cells, exhibit poor NK cell survival upon adoptive transfer, and significantly reduced memory responses. Interestingly, administration of a blocking antibody specific to NKG2D also diminishes the CHS response (26), suggesting that NKG2D may somehow be important in NK cell-adaptive responses. Further phenotyping of hapten-sensitized NK cells via adoptive transfer and CHS identified adaptive murine NK cells as liver-resident NK cells that are positive for CD45, CD90, CD11b, Ly49C/I, CXCR6, and NK1.1 (C57BL/6) or DX5 (C57BL/6 or BALB/c) but negative for CD27 (26, 31–33). Of note, while four laboratories successfully transferred NK cell memory to non-cytomegalovirus (non-CMV) antigens using $DX5^+$ as a selection marker for their antigen-sensitized liver NK cells (26, 31–33), one laboratory was unable to do so and suggested instead that CD49a-expressing NK cells mediate antigen-specific memory (34, 35).

NK CELL-MEDIATED ADAPTIVE IMMUNE RESPONSES TO NON-CMV PATHOGENS IN MICE

As discussed above, initial findings of antigen-dependent NK cell memory in mice against haptens have been described. Subsequent experiments expanded these findings of clinically relevant NK cell memory to human immunodeficiency virus (HIV) group antigen (gag)- or envelope (env)-containing virus-like particles (VLP) and those containing influenza A virus-derived matrix protein 1 (M1) (31). NK cell memory of M1 VLP was transferred to naive lymphopenic recipients of M1-sensitized lung NK cells, as demonstrated by delayed-type hypersensitivity (DTH) and prolonged survival of recipient lymphocyte-deficient mice upon challenge with influenza A PR8 virus, suggesting that liver NK cells may not be the only memory NK subset in mice. Even exposure to inactivated viruses, such as vaccination with UV-inactivated vesicular stomatitis virus (VSV), elicited adaptive immune responses in murine-liver-resident NK cells. These

TABLE 1 NK cell memory and memory-like responses[a]

Species	Type	Pathogen(s), antigen(s), or disease	Receptor(s) and/or cell phenotype	Ligand(s) or stimulus(i)	Reference(s)
Mice	Antigen specific	Haptens (DNFB, oxazalone)	NK1.1$^+$ or DX5$^+$ (CD49b) and CXCR6$^+$ NKG2D$^+$ CD90$^+$ CD11b$^+$ CD27$^-$ DX5$^-$ CD49a$^+$	?	26, 31, 32, 34
		HIV VLP (Gag, Env)	NK1.1$^+$ or DX5$^+$ and CXCR6$^+$	?	31
		Influenza VLP (M1 or HA/M1)	NK1.1$^+$ or DX5$^+$ and CXCR6$^+$	?	31
		Influenza virus	NK1.1$^+$ or DX5$^+$ and CXCR6$^+$ DX5$^-$ CD49a$^+$	?	31 35
		Vesicular stomatitis virus	NK1.1$^+$ or DX5$^+$ and CXCR6$^+$	?	31
		Vaccinia virus	NK1.1$^+$ or DX5$^+$ and CD90$^+$	?	33
		Murine cytomegalovirus	Ly49H$^+$, Ly49P$^+$	m157, m04	43–45
		BCG, *M. tuberculosis*	?	CD27, IL-21	85
	Cytokine induced		Ly49H$^+$, NK1.1$^+$	IL-12, IL-15, IL-15	87
Humans and NHP	Antigen specific	Human cytomegalovirus	NKG2C$^+$ CD57$^+$		
		SIV, SHIV	NKG2A$^+$, NKG2C$^+$	Gag, Env	78
		HIV, SIV antigens by adenovirus vectors	NKG2A$^+$, NKG2C$^+$	Gag, Env	78
		BCG	CD56$^+$ CD16^{lo}	?	86
	Cytokine induced	Acute myeloid leukemia	NKG2D$^+$ DNAM-1$^+$	IL-12, IL-15, IL-15	89
		Inactivated influenza virus	IFN-$\alpha\beta$R2$^+$	IL-2	88
	Memory-like	Human cytomegalovirus	NKG2C$^+$, γ-chain$^-$	Antibody	90, 91
		HIV	NKG2C$^+$, γ-chain$^-$	Antibody	92
		HCV	NKG2C$^+$, γ-chain$^-$	Antibody	93
		Epstein-Barr virus	CD56^{bright} NKG2A$^+$ CD56^{dim} NKG2A$^+$ NKG2A$^+$ 2B4$^+$ NKG2D$^+$	?	51, 57–60

[a]VLP, virus-like particles; NHP, nonhuman primates.

vaccine-induced memory NK cells were pathogen specific, were developed in the absence of RAG and T and B cells, and protected T and B cell-deficient mice from lethal viral challenge (26, 31). Independent verification of NK cell-mediated innate immune memory to vaccinia virus was published by Gillard et al., who demonstrated the ability of Thy1$^+$ liver-derived NK cells to mediate adaptive immunity to this pathogen (33). In addition, Paust, et al. demonstrated clearly that NK cell memory of non-CMV viral antigens, like haptens, is confined to NK cells that express the chemokine receptor CXCR6 (31).

NK CELL-MEDIATED ADAPTIVE IMMUNE RESPONSES TO HERPESVIRUSES IN MICE AND HUMANS

Murine CMV (MCMV) is a commonly studied example of NK cell-mediated antiviral surveillance, as this virus has evolved elaborate mechanisms to evade NK cell responses and has also provided strong evidence in support of adaptive functions by NK cells (36–39). NK cells express on their cell surface activating receptors that specifically recognize MCMV-derived proteins, including Ly49H, which recognizes m157 (36–38, 40–42), and Ly49P, which recognizes m04 (43). However, this interaction is unique to B6 mice, as neither outbred mice nor inbred mice on a non-C57BL/6 background (BALB/c) express Ly49H and as such are highly susceptible to MCMV infection (44). NK cell-mediated long-term survival and memory responses to m157 MCMV antigen have recently been shown in B6 bone marrow chimera mice (45), in which Ly49H lymphopenia was induced through Dap12 deficiency. In this lymphopenic environment, Ly49H$^+$ NK cells proliferated upon MCMV infection, contracted subsequently, and persisted in lymphoid and nonlymphoid organs for several weeks (45). These self-renewing memory NK cells rapidly degranulated and produced cytokines upon reactivation, and adoptive transfer of 10-fold-fewer memory NK cells was protective upon MCMV challenge compared to what occurred with naive NK cells. Since Ly49H is expressed on splenic and hepatic NK cells, both subsets responded to m157, although liver NK cells proliferated more vigorously than those derived from spleen. In contrast, there was no correlation between Ly49-activating receptors on hepatic NK cells and CHS activity, and

splenic NK cells were unable to mediate CHS responses (26). Hence, the requirements of Ly49-activating receptors during NK cell-meditated memory responses may vary depending on the antigen and MHC haplotype, and their precise requirement to NK cell-mediated memory requires further study. Interestingly, while both splenic and hepatic NK cells respond to MCMV infection, they do so in an organ-specific manner (46, 47). In the spleen, perforin mediates viral clearance, while IFN-γ mediates protection in the liver. It would, therefore, be most informative for future studies to directly compare splenic and hepatic memory NK cell responses to MCMV challenge using adoptive transfers. While the full mechanisms mediating establishment of a long-lived memory NK cell pool of MCMV following infection are unclear, the proapoptotic factor Bim has been implicated in shaping the size and functional profile of long-lived memory NK cells in a mechanism analogous to that of memory $CD8^+$ T cells (48). Also, as with autophagy-dependent formation of $CD8^+$ T cell pools, surviving NK cells that undergo mitophagy during the contraction phase depend on BNIP3 to select survival of memory NK cells (49).

Although adaptive NK cell responses to CMV have been delineated most clearly for MCMV, empirical evidence suggests that a similar phenomenon may occur in humans. First identified as an expansion of $NKG2C^+$ NK cells in response to human CMV (HCMV)-infected fibroblasts (50), it was later clarified that $CD57^+$ $NKG2C^{hi}$ NK cells expand early after HCMV infection *in vivo* and are highly specific to the virus (51, 52). NK cells are typically the first lymphocytes to reconstitute after hematopoietic stem cell (HSC) transplantation and during reconstitution are reciprocally modulated by reactivated CMV (52, 53). Inhibition of CMV replication is modulated, at least in part, by NKG2C, which binds to HLA-E, which in the steady state presents signal peptides derived from other MHC-I proteins (54). It is currently unknown if a CMV-encoded ligand for NKG2C exists, but 5% of humans are NKG2C null and 20% are NKG2C heterozygous, and these genetic attributes carry significant implications in transplant immunology (55). Indeed, cord blood (CB) grafts expressing an NKG2C deletion allele possessed a high risk of CMV reactivation after CB transplantation and a reduced risk with the presence of the wild-type allele (56). Further evidence that NKG2C-mediated NK cell activation has a profound effect on the NK cell repertoire and CMV-specific NK memory in humans comes from a comparison of NK cells from CMV-seronegative and CMV-seropositive recipients of CB-derived HSCs (55). While NKG2C expression remained unchanged in patients without CMV viremia, in patients who reactivated CMV, NKG2C expression increased significantly during the acute phase of CMV infection, similar to what occurred with NK cells in other patients with CMV reactivation (53). Newly formed NK cells from patients who reactivated CMV after HSC transplantation also had a more mature phenotype and produced significantly more IFN-γ both before and after detection of CMV viremia and anti-CMV therapy. Interestingly, the $NKG2C^+$ $CD57^+$ NK cells that expand in CMV infection are not responsive to Epstein-Barr virus (EBV)-infected cells, suggesting that this phenotype is not a universal response to herpesvirus infections (51). However, several independent studies have indicated that specific subsets of NK cells are also enhanced in their responses to EBV infection (57–60), including a $CD56^{bright}$ $NKG2A^+$ $CD94^+$ $CD45^+$ $CD62L^-$ population that accumulates in the tonsils of infected individuals (57). These NK cells secrete IFN-γ in response to EBV-infected cells and can restrict the transformation of EBV-infected B cells *in vitro* (57, 59). In a second study of pediatric patients, a subset of $CD56^{dim}$ $NKG2A^+$ NK cells expands for several months following acute mononucleosis (caused by EBV) and preferentially responds to EBV-expressing B cells displaying lytic antigens, suggesting that this subset may play a key role in the control of primary EBV infection (60). Finally, an $NKG2A^+$ $2B4^+$ $CD16^-$ $CD57^-$ $NKG2D^+$ NK cell subset was recently shown to mediate a specific response to lymphoblastoid cell lines latently infected with EBV (58).

NK CELLS IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Multiple studies have demonstrated an association between NK cells and control of HIV replication, as well as simian immunodeficiency virus (SIV) in macaque models. NK

cells expand during primary infection (61, 62) prior to the development of $CD8^+$ T lymphocytes and have evolved multiple mechanisms to recognize, lyse, or otherwise inhibit HIV- and SIV-infected cells via virus-induced downmodulation of MHC class I molecules (63) or upregulation of NKG2D ligands on target cells (64, 65) and secretion of the infection-blocking β-chemokines CCL3, CCL4, and CCL5 (66). Although these functions are innate in nature, burgeoning evidence has suggested that the NK cell response to HIV/SIV is robust and may not be entirely nonspecific. Indeed, NK cells have been linked to controlled viremia in HIV type 1 (HIV-1) elite controllers and long-term nonprogressors and reduced acquisition in HIV-1-exposed seronegative individuals, and peptide-specific NK cell responses have been shown to block HIV mother-to-child transmission (67–73). Longitudinal studies suggest that NK cells may be associated with preventing disease progression in SIV-infected macaques (74, 75), and experimental NK cell depletion results in increased virus replication (76, 77). Importantly, Reeves et al. also recently described evidence of antigen-specific NK cell memory responses mounted against SIV/simian-human immunodeficiency virus (SHIV) infections as well as against adenovirus 26 (Ad26) vaccine antigens in rhesus macaques (78). Responses were dependent on NKG2 molecules, but delineating the full mechanisms of primate NK cell memory will require further study. Many detailed analyses of NK cells in HIV and SIV have focused on KIR interactions with cognate HLA ligands that do not represent memory *per se* but demonstrate the potential for antigen-specific modulation of the NK cell response. One example is the coexpression of the KIR3DS1 allele in conjunction with HLA class I alleles from the HLA-Bw4 family being associated with delayed AIDS progression and greater suppression of virus replication in autologous $CD4^+$ T cells (79–82). NK cells may also exert selective pressure on virus replication, as evidenced by HIV-1 polymorphisms associated with KIR2DL2 that can confer resistance to NK cells (69). Similarly, SIV peptides can modulate recognition of rhesus KIR; in one example, Mamu-KIR3DL05 is stabilized by certain peptides, but not by others, and the NK cell response can even be suppressed in this manner (83). A highly conserved HIV peptide that binds to HLA-E has also recently been shown to contribute to the sensitivity of HIV-infected cells to NKG2A-expressing NK cells (84).

NK CELL MEMORY OF *MYCOBATERIUM TUBERCULOSIS*

A nonviral form of memory NK cells has recently been described by Venkatasubramanian et al. and is present in spleens and draining lymph nodes of mice infected with *Mycobacterium tuberculosis* (85). Using a mouse model of tuberculosis (TB) infection, the authors were able to induce IFN-γ-producing $CD27^+$ memory-like NK cells upon bacillus Calmette-Guerin (BCG) vaccination (the antigen used in the tuberculin test). Memory NK cells provided protection against subsequent TB challenge but not against challenge with other bacterial pathogens. Interestingly, murine TB-specific memory NK cells are distinct from $CXCR6^+$ memory NK cells found in liver and are RAG dependent, although they do not require RAG expression but rather T cell-produced interleukin 21 (IL-21) for their induction and/or survival (85). Recently, BCG-specific memory NK cells were also identified in vaccinated humans and were found to be both long-lived and rapidly expanded upon BCG revaccination (86). Although the mechanisms and full phenotypic and functional profiles remain unclear, BCG-specific responses were found primarily among $CD56^+$ $CD16^{lo}$ NK cells. All together, these exciting findings suggest that multiple subsets of distinct memory NK cells coexist and may protect their host using distinct mechanisms of induction, maintenance, and action.

OTHER FORMS OF TRAINED NK CELL-MEDIATED IMMUNITY

Cytokine-induced memory NK cells. Another form of NK cell memory comes from initial studies by Cooper and colleagues, who demonstrated that cytokine-activated NK cells persist in naive hosts 7 to 22 days after adoptive transfer (87). Restimulation of these NK cells results in significantly elevated IFN-γ production, while granzyme B expression and killing ability are similar to those in naive NK cells. It is unlikely that this type of NK cell memory is entirely dependent on cytokine exposure after sensitization,

since cytokine-mediated NK cell activation cannot fully explain antigen-specific responses. Nonetheless, NK cells retained an intrinsic memory of prior activation, a function until now attributed only to antigen-specific adaptive immune cells. Interestingly, recent similar studies have shown that influenza vaccination can also generate cytokine-induced memory NK cells (88). Hence, NK cell-mediated effector functions during antitumor responses and allergic and infectious diseases may clinically be more important than initially appreciated, and cytokine-induced memory NK cells may be attractive therapeutic targets for disease treatment (89).

Memory-like NK cells. In addition to describing true antigen-specific NK cells, a recent study has identified a subpopulation of "memory-like" NK cells, which are exquisite effector cells when granted specificity through antibody binding. These cells, described in humans in 2013 by Zhang et al. (90), express high levels of FcγR (including CD16) but lack the intracellular γ-signaling chain. So-called (g–) or FcγRΔg NK cells are found at low frequencies in all individuals but expand in HCMV-seropositive persons. Following initial antibody binding, these cells become epigenetically modified and long-lived and are capable of significantly enhanced antibody-dependent functions and numerical expansion upon new antibody binding (91). FcγRΔg NK cells, partially identifiable by NKG2C and NKp30 expression, have been shown to exhibit potent antiviral functions against HCMV, herpes simplex virus (HSV), and influenza virus in the presence of their respective antiviral antibodies, regardless of previous antigen exposure. Recently, FcγRΔg NK cells with enhanced antibody-dependent cellular cytotoxicity (ADCC) have been shown to be increased 7-fold in HIV-infected persons and are also associated with protection from progressive liver disease in hepatitis C virus (HCV) infection (92, 93). Thus, this memory-like NK cell subpopulation has become an attractive target for antibody-based vaccines and immunotherapeutics.

NK CELL DIVERSITY IN VIRAL INFECTIONS AS A MECHANISM FOR MEMORY NK CELL GENERATION

It is unclear how NK cell memory of viral pathogens affects the diversity of the human NK cell repertoire. In the adaptive immune system, immune memory decreases repertoire diversity by increasing the frequency of cells expressing a single receptor specific for pathogens that have been encountered before. Among NK cells, this relationship is less clear, because with a few exceptions (such as the Ly49H-mediated recognition of m157 of murine CMV [45]), a specific receptor that mediates NK cell recognition and memory has not been identified. Instead, there are numerous associations between certain NK cell receptors and different viral infections in human, yet there are only a few situations in which there is a mechanistic understanding of how these receptors contribute to viral recognition (reviewed in reference 94). It is possible that distinct receptor combinations are required to respond to different viruses, making the elucidation of the requirements for a specific virus more challenging in light of the diversity receptor expression profiles within the NK cell repertoire. NK cell diversity may be best defined based on combinatorial expression patterns of activating and inhibitory receptors, whose signals are integrated to control NK cell function (95). Recent work has revealed that these receptors assort on the cell surface to generate a vast diversity of distinct phenotypic subsets, with 6,000 to 30,000 unique NK cell subsets per individual (95). This raises the possibility that specific subsets might be enhanced in their ability to recognize distinct pathogens, and a memory response may result in a shift in the NK cell repertoire to increase the frequency of these subsets. However, to date, only human CMV infection is associated with dramatic imprinting on the NK cell repertoire (96).

Interestingly, *in vitro* experiments suggest that short-term exposure to virus-infected cells actually increases human NK cell diversity (95). This shift in diversity may represent a short-term accommodation to "tune" the NK cell response to detect a specific pathogen. Consistently with this idea, a human immune repertoire with increased expression of maturity markers, such as CD57, is associated with shifts in the expression patterns of activating and inhibitory receptors *in vivo* (97). Such shifts, which generally favor greater expression of activating receptors, might decrease the threshold for NK

cell activation upon a secondary exposure. Consistently with this idea, more mature CD57$^+$ NK cells display enhanced cytokine secretion but diminished cytotoxic activity in response to autologous HIV-infected T cells *in vitro* (98), suggesting that the viral exposure modulates the quality of the NK cell response to subsequent infections. Consistently with this idea, exposure to many pathogens is associated with shifts in the expression patterns of a variety of natural killer cell receptors (94, 99).

Collectively, these findings raise the question of what effects chronic exposure to different viruses has on the NK cell repertoire. On one hand, exposure to viruses might give rise to adaptive and pathogen-specific cells, but on the other hand, it might lead to a more mature repertoire that favors cytokine secretion over direct killing. In a small study of women at risk of HIV infection, higher NK cell diversity was associated with increased HIV acquisition risk (98). Together, these data support a model in which exposure to a given pathogen might give rise to rare populations of memory cells that are difficult to detect among the overall increase in diversity. However, the cells that have diversified in response to one pathogen may be less "flexible" in their ability to respond to a *de novo* pathogen.

SPECULATION ON EVOLUTIONARILY CONSERVED ADAPTIVE IMMUNE MECHANISMS

The phenomenon of antigen-specific NK memory is entirely unprecedented and suggests an alternative pathway to generate immunological memory that is fundamentally distinct from all known cellular and molecular mechanisms of adaptive immunity. Based on the findings presented herein, we hypothesize that vertebrates have evolutionarily conserved the ability to generate a diverse antigen receptor family in a RAG-independent manner, resulting in NK cell-mediated adaptive immunity. Interestingly, evidence for a RAG-independent generation of a clonal repertoire of lymphocytes has been described in the only two surviving jawless vertebrates, lampreys and hagfish, which use recombinatorial assembly of leucine-rich-repeat genetic segments to generate diversified variable lymphocyte receptors (VLRs) (22, 100). Lamprey-expressed VLRs allow adaptive, clonal immune responses to a variety of antigens, rejection of secondary-skin allographs, and DTH not unlike DTH responses mediated by murine NK cell subsets. While lampreys express several genes or gene homologues that are important for adaptive immune responses (100–102), the numbers of immune gene homologs are comparatively low relative to that of jawed vertebrates. That said, lampreys are considered the most phylogenetically primitive species that may have an adaptive immune system. We are tempted to speculate that their ability to develop an adaptive immune system may have been key in their evolutionary survival. Further evidence that NK cell memory may not be restricted to higher-order vertebrates and may be highly evolutionarily conserved can be found in a recent report from Garcia-Valtanen et al. (103), who demonstrated that RAG-deficient zebrafish are also capable of antiviral innate immune memory. The responsible cell type could not be identified in this species, as regents to distinguish between innate immune cells of zebrafish are currently lacking; however, gene expression profiling did uncover an enhanced cytotoxic response. Whether mouse (or human) NK cells utilize similar or distinct mechanisms to generate a diverse antigen-receptor repertoire in a RAG-independent manner is under intense investigation. Indeed, data presented by Paust et al. outlined the development of a sorting strategy to identify and isolate 2,4-dinitrofluorobenzene (DNFB)-specific NK cells from livers of DNFB-sensitized RAG-knocked-out mice, whereby the nuclei were then transplanted into enucleated oocytes for the generation of embryonic stem cell lines that were used to clone mice (104). NK cells from cloned animals and about 50% of the F2 offspring instantly responded to DNFB without requiring prior sensitization but could not be sensitized to other haptens. These data strongly suggest that the nuclear information for hapten specificity of memory NK cells persists even in a donor nucleus whose epigenetic state was reset by nuclear reprogramming and subsequent breeding. This apparent genetic fixation would not be explained by epigenetic regulation of conventional "hard-wired" NK

receptors but is expected if antigen receptor specificity is encoded at the level of genomic DNA and may suggest an entirely novel mechanism to generate receptor diversity (104).

CONCLUDING REMARKS

All together, the findings discussed herein challenge the notions that innate cells are incapable of innate immune memory or that adaptive immune memory is somehow strictly RAG dependent. Significant data also suggest that it is unique populations of NK cells or receptors that mediate adaptive immunity and that these functions might be conserved among species. Mechanistic evaluations remain ongoing, but the concept of NK cell memory has now evolved from immunologic heresy to a broad field of study, and it will be exciting to see how NK cell memory responses can be harnessed for improved vaccines and novel immunotherapies.

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