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Origins of natural killer cell memory: special creation or adaptive evolution

Kayla A. Holder,
Emilie M. Comeau and
Michael D. Grant

Immunology and Infectious Diseases Programme, Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St John's, NL, Canada

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Correspondence: Dr Michael D. Grant, H1803 Immunology, Faculty of Medicine, Memorial University of Newfoundland, 300 Prince Phillip Drive, St John's, NL, Canada A1B 3V6. Email: mgrant@mun.ca
Senior author: Dr. Michael D. Grant

Summary

The few initial formative studies describing non-specific and apparently spontaneous activity of natural killer (NK) cells have since multiplied into thousands of scientific reports defining their unique capacities and means of regulation. Characterization of the array of receptors that govern NK cell education and activation revealed an unexpected relationship with the major histocompatibility molecules that NK cells originally became well known for ignoring. Proceeding true to form, NK cells continue to upend archetypal understanding of their ever-expanding capabilities. Discovery that the NK cell repertoire is extremely diverse and can be reshaped by particular viruses into unique subsets of adaptive NK cells challenges, or at least broadens, the definition of immunological memory. This review provides an overview of studies identifying adaptive NK cells, addressing the origins of NK cell memory and introducing the heretical concept of NK cells with extensive antigenic specificity. Whether these newly apparent properties reflect adaptive utilization of known NK cell attributes and receptors or a specially creative allocation from an undefined receptor array remains to be fully determined.

Keywords: Fc receptors; memory; major histocompatibility complex/histocompatibility-linked antigen; natural killer cell; virus.

Introduction

Our perception of natural killer (NK) cells has transformed dramatically since their discovery just over 40 years ago.[1–5] Once viewed as a monolithic fraction of peripheral blood lymphocytes with an innate ability to find and kill aberrant cells, NK cells have since been revealed as a highly diverse lymphocyte population within which idiosyncratic education, regulation and activation processes govern subset function and prominence. Subsets distinguished by variable constellations of regulatory receptors diverge further along functional scales delineated by the presence and affinity of polymorphic ligands. Genetic, stochastic, deterministic and epistatic factors interact to produce up to 30 000 discrete NK cell subsets.[6] We now know that the ontogenetic NK cell repertoire is dynamically modified by environmental influences (Fig. 1). As with the responses of B and T lymphocytes, NK cell engagement in an immune response reshapes the baseline repertoire, in part by introducing an adapted, preferentially selected, memory-like population with distinct phenotypic and functional features. Selective expansion, together with acquisition of novel functional and phenotypic characteristics, is common to memory B, T and NK cells alike. However, to our knowledge, only NK cells mature into immune memory cells independently of antigen recognition through somatically generated, clonotypic receptors. Therefore, the selection mechanisms underlying dynamic remodelling of NK cell repertoires and NK cell maturation into memory cells remain mostly obscure.

This review will address accumulating evidence for antigen-dependent and -independent NK cell maturation and memory formation. The response against murine cytomegalovirus (MCMV) is the first and best characterized example of antigen-driven activation and maturation of NK cells into memory cells. We will review aspects of the NK cell response to MCMV, followed by a series of as yet mechanistically unexplained examples of NK cell memory and antigen specificity in mice and primates, followed by a description of human memory-like NK cell development, primarily in response to human CMV (HCMV) infection. Of note, the process by which NK cell memory forms in the case of HCMV infection appears to

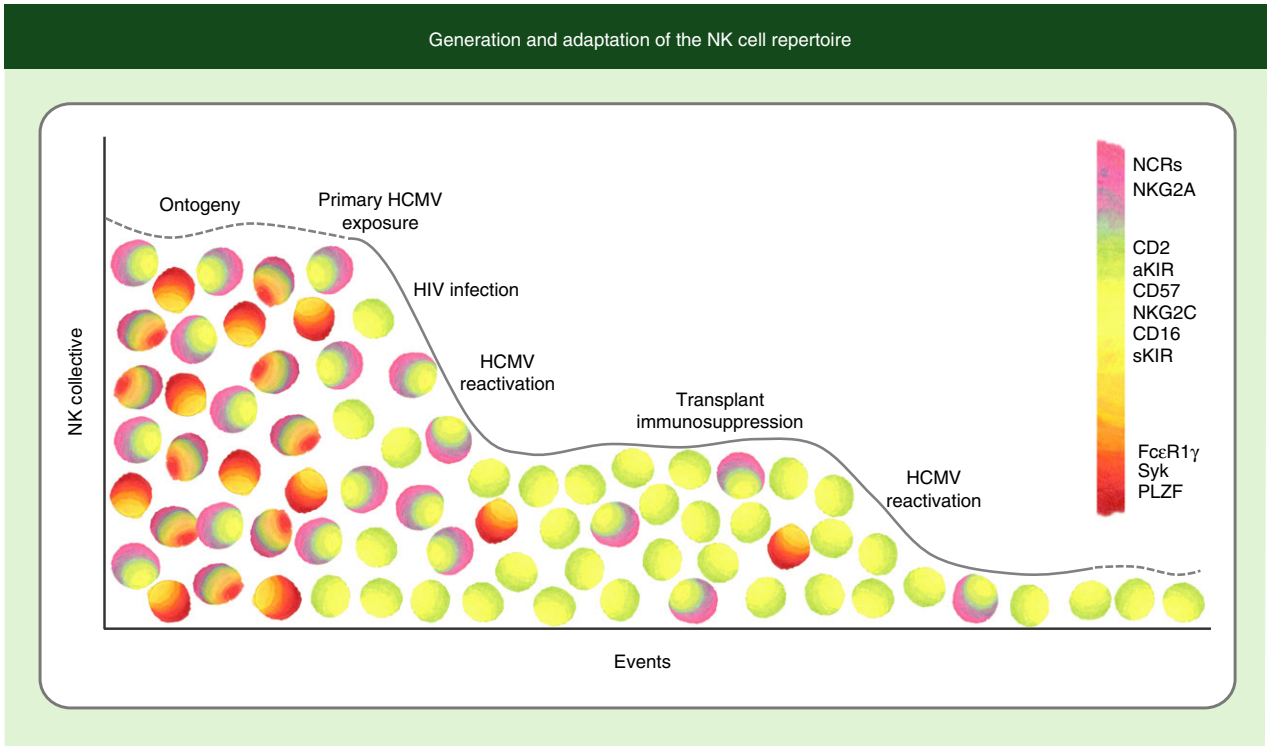

Figure 1. Generation and adaptation of the natural killer (NK) cell repertoire. A diverse NK cell repertoire is generated through variable expression of a number of germline-encoded receptors, creating a myriad collection of NK cells with different receptor constellations. Genetic and environmental influences generally lead to modest selection of NK cells with self-specific and activating killer cell immunoglobulin-like receptors (KIRs). Infection with human cytomegalovirus (HCMV) drives a more stringent selection leading to a highly expanded population of mature $CD57^{pos}$ NK cells expressing NKG2C and high levels of CD16. This selection process appears to be exaggerated in the setting of immunodeficiency or immunosuppression with increasingly narrow bottlenecks favouring selective expansion of these cells, which are specialized to mediate antibody-dependent cell-mediated cytotoxicity, into a dominant fraction of the overall repertoire. Narrowing of the repertoire with additional events on the *x*-axis represents a diminution of the frequency of certain NK cell subsets, not of total NK cell numbers.

have less in common with MCMV infection than initially expected. Finally, we will speculate on how features of NK cells, viruses and other antigens might interact to confer a semblance of antigen specificity independent of somatically rearranged clonotypic receptors.

Murine cytomegalovirus infection

The role that NK cells play against MCMV in certain strains of mice illustrates the two key aspects of immunological memory previously attributed exclusively to lymphocytes bearing clonotypic receptors. There is selective expansion of NK cells bearing antigen-specific receptors and a more efficient secondary response against the same antigen. Appreciation of the importance of murine NK cell responses to MCMV began with the observation that BALB/c mice are more susceptible to MCMV infection than C57BL/6 mice. Classical genetics mapped resistance to *Ly49H*, a gene encoding an activating receptor on murine NK cells.[7] This receptor is now known to directly interact with the MCMV-encoded m157 glycoprotein, thereby stimulating expansion of Ly49H-bearing NK cells that help to control primary infection and provide enhanced protection against subsequent challenge.[7–9] Intensive study of this system over the last two decades revealed distinct phases necessary for the propagation of memory NK cells. The first phase occurs during early infection and probably precedes physical interactions between receptor–ligand pairs. During this time, pro-inflammatory cytokines such as type-I interferon (IFN-α/β) and interleukin-12 (IL-12) provide non-specific signals to prime NK cells. These cytokines, initially produced by myeloid cells in response to MCMV infection, stimulate NK cell antiviral cytokine production, induce their activation and initiate non-specific NK cell proliferation.[10–12] Interferon-α/β-induced IL-15 is also thought to contribute to non-specific NK cell proliferation during the early stages of MCMV infection and provide stimulation pertinent to NK cell survival.[13,14] These cytokines sensitize NK cells to IL-18 and IL-33, which are required for optimal responses, but dispensable for forming memory NK cells.[15] Interactions between cytokines and their receptors

encourage NK cell adaptation to MCMV by transmitting signals through signal transducer and activator of transcription (STAT) mediators. Engaging IFN-α and IL-12 receptors stimulates STAT1 and STAT4, crucial regulators of NK cell IFN-γ expression and cytolytic activity.[16–20] Although the Yokoyama laboratory initially described the prolonged effects of IL-12 on NK cell activation, the central role for IL-12 in NK cell memory was definitively illustrated by the inability of NK cells from IL-12R$^{k/o}$ mice to expand and provide protection against MCMV challenge.[21–23] No correlations between NK cell inflammatory cytokine stimulation and antigen-specific recall responses have been noted, but IL-12 modulates NK cell adaption, enhances NK cell responsiveness and causes inheritable modifications in NK cell progeny.[23,24] Exposure to IFN-α/β and IL-12 during the later stages of NK cell adaptation also enables the proliferative burst observed after MCMV challenge, which is incited by a cytokine-specific increase in expression of zinc finger transcription factor Zbtb32.[25,26]

This early generic NK cell response to cytokines may be critical for priming NK cells to respond more effectively to virus-specific interactions during subsequent stages of MCMV infection. The initial NK cell response to cytokines is independent of activating receptor expression; however, NK cells lacking Ly49H fail to proliferate in response to MCMV during the later stages of infection.[27] The key feature in NK cell memory responses to MCMV infection is the specific interaction between the MCMV m157 glycoprotein and Ly49H activating receptors that trigger selective proliferation and differentiation into a long-lived memory cell population with enhanced protective capacity.[8,9,28,29] The interaction between Ly49H and m157 induces a two to threefold increase in NK cell numbers during the first week after MCMV infection, with this expansion remaining detectable at least 70 days after infection.[27,30] The importance of this interaction in NK cell adaptation is illustrated by adoptive transfer studies in which Ly49H^{pos} NK cells vigorously proliferate in recipients lacking Ly49H after challenge with MCMV.[30] Infection with an MCMV construct lacking m157 also fails to induce proliferation or expansion of this protective pool of NK cells, reiterating the importance of direct interaction between Ly49H and m157.[29,30]

More recent studies show that the activating receptor Ly49P recognizes an MCMV m04-derived peptide in the context of murine H-2D class I major histocompatibility complex (MHC) molecules and acts similarly to Ly49H in conferring protection against MCMV in MA/MyJ mice.[31] With the exception of clonal selection, C57BL/6 and MA/MyJ NK cell responses against MCMV display the key defining features of adaptive immune memory displayed by B and T lymphocytes. Recognition of MCMV by NK cell activating receptors may have evolved under pressure from the numerous MCMV evasion strategies targeting NK cell inhibitory receptors. Polymorphism within MCMV m157 and m04 genes and the interacting *Ly49* alleles supports this possibility.[31–34] The evolution of both direct and, in the case of m04, MHC-restricted recognition processes illustrates multiple NK cell adaptation strategies arising against a single virus. Other similar as yet uncharted events may have advanced murine NK cell evolution and driven diversification of the analogous human killer cell immunoglobulin-like receptor (KIR) genes, however, specific recognition of MCMV by NK cells from C57BL/6 and MA/MyJ mice remain the only definitive examples.

Antigen-specific NK cell expansion initiated by m157/Ly49H interactions relies on directed signalling through adaptor proteins.[35] Although Ly49H receptors transmit signals through either DAP10 or DAP12, Orr *et al.* showed that signalling through both adaptor proteins is necessary for optimal NK cell adaptation.[35] In the absence of either DAP10 or DAP12 expression, NK cells still mediate resistance to MCMV infection; however, deleting either of these two proteins reduces surface Ly49H expression, NK cell proliferation and IFN-γ production in response to MCMV.[35] Signal transmission through either of these adaptor proteins after m157/Ly49H interactions may represent a redundancy mechanism whereby adequate memory-like NK cell responses are maintained in the absence of either DAP10 or DAP12 expression. However, if both DAP10 and DAP12 are absent, Ly49H expression is completely suppressed and mice are susceptible to MCMV challenge.[35]

Analogous to the activation of naive B and T cells, co-stimulatory receptor engagement is a necessary component of NK cell adaptation. Upon infection, MCMV induces CD155 and/or CD112 expression on monocytes or dendritic cells.[36] Engagement of NK cell-expressed DNAX accessory molecule-1 (DNAM-1) by CD112 and/or CD155 drives signal transduction through Fyn and PKCη, which promotes NK cell differentiation after initial and subsequent MCMV challenges.[37] This process generates a pool of memory NK cells with high expression levels of maturation markers (CD11b, Ly6C, and KLRG1) and of Ly49H.[37] It is unknown whether other co-stimulatory molecules are involved in the activation, proliferation or persistence of MCMV-specific memory NK cells.

During the first week of MCMV infection, NK cells are exposed to non-specific cytokines, viral proteins or virus-induced host ligands that support NK cell proliferation and expansion. Following activation and differentiation towards enhanced effector function, the next phase of memory NK cell generation begins as the NK cell pool contracts into an elite collection of highly functional mature NK cells.[38] Pro-apoptotic Bcl-2-like protein 11 (Bim) is a key regulator in this contraction phase.[39] After MCMV challenge, mice lacking Bim accumulate NK cells with a less matured phenotype (KLRG1^{lo}) that respond

poorly to secondary m157 stimulation.[39] In wild-type mice, most of these cells undergo apoptosis, but a repair process controlled by mitochondrial-associated proteins, BNIP3 and BNIP3L, spares a small proportion of NK cells by selectively removing dysfunctional mitochondria.[40] The cells that survive mitophagy persist as the long-lived memory NK cells that proliferate, expand and contract in response to repeated MCMV challenge and provide greater protection against successive MCMV infection than their naive counterparts.[30] The C57BL/6 MCMV model draws parallels with T-cell memory development, extending from the need for cytokine stimulation and co-stimulatory receptor engagement, to the critical contraction phase following antigen-driven NK cell expansion. This model has provided detailed information on the role of cytokine-priming, accessory interactions and signalling pathways underlying formation of antigen-specific NK cell memory populations, ultimately broadening our perspective on immunological memory to include cells restricted to recognition of antigens with germ-line receptors.

Antigen-specific NK cells in mice

Other adaptive properties that were previously thought to be restricted to lymphocytes bearing somatically rearranged antigen-specific receptors were demonstrated for NK cells by O'Leary *et al.*[41] In classic crossover control style experiments, they showed that *rag*$^{k/o}$ mice lacking B and T cells can be sensitized against a particular hapten and mount hapten-specific contact hypersensitivity reactions for at least 4 weeks afterwards.[41] This phenomenon reflects selective expansion and differentiation of hapten-specific cells during sensitization, persistence of some fraction of the sensitized cells as memory cells, recruitment of memory cells to the site of secondary challenge and their *in situ* antigen-specific reactivation. Adoptive transfer of NK cells from sensitized mice enabled naive mice to mount contact hypersensitivity responses specific for the sensitizing hapten, recapitulating a phenomenon previously associated exclusively with adoptive transfer of antigen-specific T cells.[41] The haptens used to induce contact hypersensitivity (dinitrofluorobenzene and oxalazone) fall within a special category of antigens that trigger immune responses by modifying self-proteins, but investigators have since demonstrated similar NK cell-mediated responses following immunization with viral proteins. Human immunodeficiency virus (HIV) gag or env proteins, or influenza M1 matrix protein delivered in virus-like particles or immunization with ultraviolet-light-killed vesicular stomatitis virus also elicited NK-dependent immune memory.[42] Although it lacks mechanistic explanation, this form of apparent antigen-specific NK cell reactivity has now been generalized across a range of antigens. Characterization of the murine NK cell subset responsible for antigen specificity indicates CXCR6-dependent liver residency, expression of Ly49C, DX5 (in most cases) and NKG2D with dependence on IL-12, IFN-α and IFN-γ priming for their initial development.[43,44] A notable feature of the contact hypersensitivity mediated by sensitized NK cells is the speed with which the reaction occurs following secondary exposure to the sensitizing antigen. Unlike T-cell-dependent contact hypersensitivity, which takes 24–48 hr to develop, NK cell-dependent responses are detectable within 30 min.[43] Presumably, this reflects the lack of any need for antigen processing and presentation and possibly faster recruitment or enhanced potency on a per cell basis. If so, this might either be an intrinsic property of the NK cells active in this context or related to the absence of (regulatory) T cells in the *rag*$^{k/o}$ models. Further studies have extended the phenomenon of antigen-specific NK cell memory to recognition of vaccinia virus and *Mycobacterium tuberculosis*.[45,46] In contrast to the case of influenza M1 matrix protein, where the memory NK cells induced selectively reside in the lungs, respiratory influenza virus infection induces memory-like NK cells resident in the liver.[42,47] Although *M. tuberculosis*-induced memory NK cells can develop in *rag*$^{k/o}$ mice, they require T-cell-derived IL-21 for induction.[46]

These studies in mice have been rigorously conducted with a general consensus emerging from multiple laboratories as to the phenotype, effector functions and homing behaviour of the antigen-specific NK cells detected. There is preliminary evidence of a genetic basis for the murine NK cell specificity, but the nature of any receptor genes responsible remains mysterious.[48] Adaptation to ancient, chronic pathogens like MCMV is a logical explanation for pressure to evolve activating receptors that counter viral decoy proteins, but it is difficult to envision something similar for adaptation to the synthetic haptens that specifically sensitize NK cells. More research is clearly required to discern the full antigenic range of this phenomenon and whether specific recognition of synthetic haptens and microbial products reflects their inclusion in some form of pre-existing or inducible antigen receptor repertoire unique to NK cells.

Antigen-specific NK cells in primates

Demonstration of antigen-specific memory NK cells in mice raises the question of whether similar phenomena occur in primates. Subsets of human NK cells respond selectively to certain viruses, bacteria and fungi with proliferation and augmented effector functions.[49–55] Expanded numbers of these cells can persist after initial exposure and mount memory-like reactions upon secondary exposure. A number of associations between protection against viral infection or disease progression and co-ordinate inheritance of NK cell receptor/ligand

gene pairings indicate that particular subsets of NK cells have pathogen-selective, if not pathogen-specific, activity in humans and other primates. One of the more prominent associations with enhanced NK cell function, protection from viral infection or protection from disease progression, involves inheritance of *KIR3DS1* together with or without class I human histocompatibility-linked antigen (HLA) molecules bearing the HLA-Bw4*80I motif.[53,54,56–60] The human *KIR* locus, analogous to murine *Ly49*, encodes inhibitory and activating receptors that interact selectively with class I HLA molecules. In HIV infection, NK cells bearing particular KIR molecules increase in prominence, suggesting that they might play a role in pathogen recognition.[54,60] Interaction between class I HLA molecules and KIR proteins can be modulated by the nature of the HLA-bound peptide, as can the interaction between C-type lectin-like receptors and HLA-E.[61–67] It is possible, therefore, that viral infection alters the peptide composition of expressed class I HLA molecules in ways that tilt the balance between NK cell inhibition and activation. Selective expansion of NK cells bearing KIR3DS1 occurs during acute HIV infection, and with co-ordinate expression of HLA-Bw4*80I, NK cells bearing KIR3DS1 more effectively inhibit HIV replication *in vitro*.[53,54] There is epidemiological evidence of a co-ordinate role for KIR3DS1 expression together with HLA genotypes expressing the HLA-Bw4*80I epitope in protection from HIV disease progression as well as some indirect evidence for enhanced NK cell function with this combination, but the nature of this effect remains controversial. No functional interaction between KIR3DS1 and HLA molecules bearing the Bw4*80I epitope was ever demonstrated and in fact, it was recently shown that KIR3DS1 binds open conformers of the relatively non-polymorphic HLA-F molecule.[68,69] In light of this finding, the data indicating epistatic interaction between KIR3DS1 and HLA-B molecules bears re-assessment or re-analysis. A study of inhibitory and activating KIR expression on NK cells from monozygotic twins in comparison with unrelated individuals suggested that expression of inhibitory receptors is highly influenced by genetics, but that environmental factors have more influence on expression of activating KIR than does co-ordinate expression of their cognate ligands.[6] As none of the subjects in this study were infected with HIV, this implies that a number of other infections might select for NK cells expressing activating KIR. Conversely, there is also evidence for HIV escape mutations selective for individuals expressing KIR2DL2 that work by enhancing its inhibitory function.[70] While fine antigenic specificity is not implied by any of these examples, expression of various KIR molecules with or without their ligands clearly affects, and is affected by, the interface between NK cells and multiple pathogens.

Two published studies describe antigen-specific NK cell behaviour in primates. Splenic NK cells from rhesus macaques, either infected with simian immunodeficiency virus (SIV) constructs or vaccinated with recombinant adenoviruses expressing SIV antigens, selectively killed dendritic cells pulsed with relevant retroviral Env or Gag antigens.[71] Specialized longer-term killing assays were used in these cases, suggesting either a low frequency or low cytotoxic potency of the relevant NK cell population. However, SIV-specific NK cell activity detected by this assay persisted up to 5 years after vaccination with the recombinant adenoviruses, indicating a robust and durable response.[71] Blocking either NKG2C or NKG2A reduced killing, but it is unclear if or how either receptor is directly involved. In similar assays, circulating human NK cells were reported to selectively kill autologous B cells pulsed with viral peptides, most notably those pulsed with HCMV pp65 peptides.[72] Significant NK cell killing was reported with cells from 9 of 35 donors tested, but the representative data shown in the publication indicated weak killing barely above background and these findings have not since been corroborated by other published research.

Cytokine-dependent NK cell memory

Another interesting feature of NK cells is their ability to manifest a form of immunological memory simply from exposure to pro-inflammatory cytokines in the absence of virus, hapten or other specific receptor/ligand engagement. The first indication that cytokines invoke NK cell adaptation followed an adoptive transfer study of murine splenic NK cells that were briefly activated *in vitro* with IL-12, IL-15 and IL-18 before administration to naive recipients.[23] Cytokine-primed NK cells proliferated in their new host and generated a stable population with augmented IFN-γ responses upon re-exposure to IL-12, IL-15 and IL-18, or upon activating receptor engagement.[23] Heightened IFN-γ responses were maintained for up to 12 weeks, and cytokine-induced NK cell memory was demonstrable as a heritable property.[23,24] Similar effects were noted with human NK cells, where *ex vivo* IL-12, IL-15 and IL-18 re-stimulation induced robust IFN-γ production in NK cells previously exposed to the same cytokine milieu *in vitro*.[73] In contrast with the phenotype associated with HCMV-induced NK cell adaptation, namely acquisition of the maturation marker CD57 and loss of NKG2A and natural cytotoxicity receptors, the cytokine-induced IFN-γ-producing adaptive NK cell population lacks CD57, while retaining NKp46 and NKG2A.[73] It remains to be established whether epigenetic modification of the *IFNG* locus occurs, and if the cytokine-induced alterations to NK cell functions are as durable as those seen in HCMV-driven NK cell adaptation. Another intriguing question is whether immunomodulatory

cytokines produced during viral infections contribute to *in vivo* cytokine-induced NK cell adaptation. Commonly produced cytokines, such as IFN-α/β, or in the case of HCMV, a viral homologue to IL-10, could act alone or in concert with signalling through various NK cell receptors to promote adaptation.

The NK cell response to HCMV

There is now clear appreciation that diversity within the NK cell repertoire at the population and individual levels affects interactions with a multitude of pathogens and that in turn, these pathogens effect enrichment of certain NK subsets. Diversity within NK cells is thought to have arisen in part under pressure from what has been described as an evolving 'arms race' between pathogens and the host immune system.[74] If longevity and antagonistic recidivism in this arms race are important factors, then the influence of human herpesviruses on the NK cell repertoire should be prominently reflected. Despite evidence of marked susceptibility to multiple herpesvirus infections in NK-deficient humans, only CMV infection is known to have a dramatic effect on the composition of the NK cell repertoire.[75] This is true in the case of both MCMV and HCMV, but despite superficial similarities, the operative mechanism for NK cell adaptation to murine and human CMV appears mostly unrelated.

In 2004, Guma *et al.*[76] first reported that infection with HCMV leaves a durable imprint on the human NK cell repertoire. This imprint is reflected in an increased frequency of NK cells expressing CD57 and C-type lectin-like activating receptor NKG2C, together with high levels of the IgG Fc receptor CD16 (Fig. 2). Expression of activating and self-specific KIRs is higher on these NK cells, whereas levels of natural cytotoxicity receptors and NKG2A are reduced.[76–83] Functionally, the cells are especially efficient at mediating antibody-dependent cell-mediated cytotoxicity, perhaps in adaptation to down-modulated natural cytotoxicity receptor signalling. Recently, data from two independent laboratories demonstrated substantial overlap of the population of NK cells co-expressing CD57 and NKG2C with NK cells that have reduced expression of the FcεR1γ signalling protein, Syk kinase, promyelocytic leukaemia zinc finger protein (PLZF) transcription factor and Ewing's sarcoma's/FLI-1 activated transcript-2 (EAT-2).[84,85] Although accumulation of these cells was initially associated with a number of other viral infections, it later became clear that HCMV infection is the one constant required for marked expansion of CD57^{pos} NK cells expressing NKG2C. This association between selective expansion of NK cells bearing the activating receptor NKG2C and HCMV infection inspired consideration that NKG2C might be analogous to Ly49H with its specific recognition of an HCMV protein or peptide driving expansion of NKG2C^{pos} NK cells. Lending credence to this idea, in some *in vitro* HCMV infection/NK cell co-culture systems, CD57^{pos} NKG2C^{pos} NK cell expansion is blocked by antibodies against either HLA-E or NKG2C.[86] Both NKG2C and its inhibitory counterpart NKG2A bind to HLA-E, which in turn is modulated by HCMV infection.[64] Expression of HLA-E is generally stabilized by peptides derived from class I molecules and its function as an inhibitory or activating ligand for NKG2A or NKG2C receptors, respectively, is critically dependent on the peptide presented.[61–63,87] Although peptides derived from HCMV UL40 protein (i.e. VMAPRTLIL) bind and stabilize HLA-E, expression of HCMV UL16, UL18 and UL40 is dispensable for NKG2C^{pos} NK cell expansion *in vitro*.[77,88] In contrast, the US2–US11 genes crucially contribute to *in vitro* NKG2C-driven NK cell expansion, indicating a role for class I HLA molecules.[77,89]

The largely NKG2C^{pos} phenotype of adapted NK cells in HCMV infection supports the possibility that NKG2C plays a direct role. However, in co-culture systems without exogenous cytokines, NK cells from HCMV-infected individuals selectively proliferate and release IFN-γ in response to HCMV-infected fibroblasts with little apparent role for NKG2C.[90] In addition, NK cells from individuals lacking the NKG2C gene respond similarly to HCMV infection with accelerated maturation, and co-express activating KIRs (KIR2DS1, KIR2DS2, KIR2DS4 and KIR3DS1) to a similar extent to NK cells from NKG2C^{pos} donors.[91] Therefore, at the very least, NKG2C-mediated interactions are not the only driving force behind HCMV's impact on the NK cell repertoire.[92] An extensive study of adaptive NK cell responses in NKG2C-bearing and NKG2C^{null} individuals suggests that CD2 co-stimulation may be a critical component in effector potency, irrespective of NKG2C expression.[92] Paralleling the 'second signal' or DNAM-1 co-activation of adaptive murine NK cells, human CD2 on NK cells interacts with CD58 on target cells.[93] Increased CD2 expression on *in vitro*-expanded adaptive NK cells favours increased IFN-γ and TNF-α production, and is critical for robust antibody-dependent responses.[92,93] However, the extent to which CD2 engagement contributes to adaptive NK cell formation *in vivo*, or whether other ligands for CD2 provoke the same response, remains unresolved.

Elucidation of intracellular mechanisms accompanying NK cell adaptation to HCMV infection was bolstered by identification of NK cells deficient for FcεR1γ, as emergence of adaptive NK cells in HCMV-infected individuals is closely associated with loss of this transmembrane signalling adaptor protein.[94,95] Although CD57 and NKG2C are mostly co-expressed on adapted NK cells, some FcεR1γ^{neg} NK cells lack NKG2C and retain low levels of NKG2A.[85,95] The FcεR1γ adaptor protein associates with NKp30, NKp46 and/or CD16 receptors as a homodimer or heterodimer with CD3ζ to signal through immunoreceptor

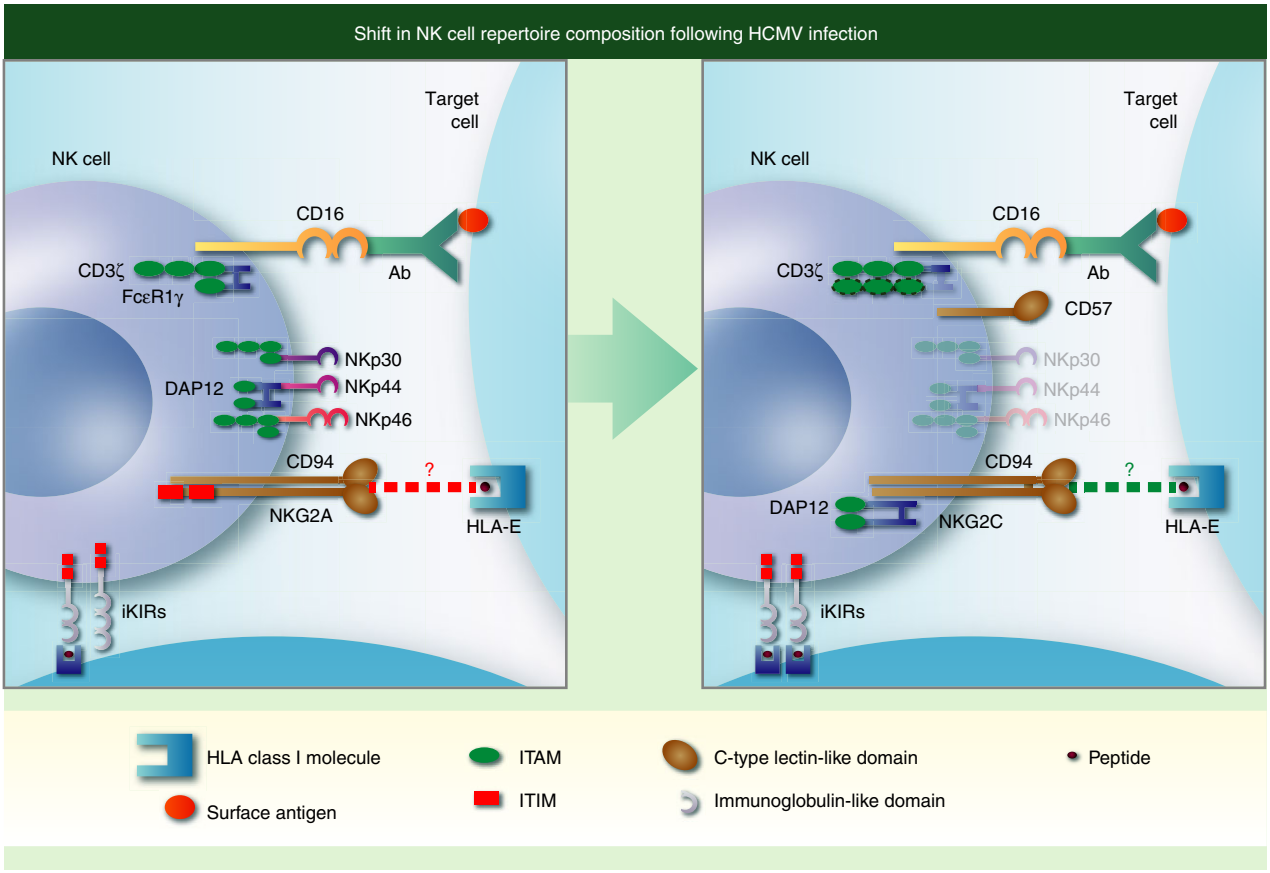

Figure 2. Adaptation of natural killer (NK) cells in response to human cytomegalovirus (HCMV) infection. Individuals infected with HCMV (right hand panel) have an increased fraction of circulating NK cells that express CD57 and the activating C-type lectin-like receptor NKG2C with reduced levels of natural cytotoxicity receptors NKp30, NKp44 and NKp46. These NK cells are skewed towards expression of activating KIR and self-specific inhibitory killer cell immunoglobulin-like receptors (iKIR) with cognate HLA-I ligands present in the host, express higher levels of CD16 and are more effective at mediating antibody-dependent cell-mediated cytotoxicity. Enhanced triggering through CD16 is associated with down-regulation of the FcεR1γ adaptor protein [one immunoreceptor tyrosine-based activation motif (ITAM) each] and potential replacement with the CD3ζ adaptor protein (three ITAMS each). Additional changes not shown include epigenetic modifications (demethylation) activating the interferon-γ locus and down-regulation of Syk, Ewing's sarcoma's/FLI-1 activated transcript (EAT) and move as indicated to end of sentence promyelocytic leukaemia zinc finger protein (PLZF) transcription factor. Although NK cells expressing NKG2C and CD57 have undergone differentiation and are considered adaptive NK in the setting of HCMV infection, specificity for HCMV has not been demonstrated. Both NKG2A and NKG2C interact with HLA-E and CMV infection can increase expression of HLA-E, but the role that either interaction plays in shaping the NK cell repertoire in CMV infection is unknown, hence a '?' punctuates the dotted lines denoting receptor–ligand interactions.

tyrosine-based activation motifs.[96,97] Although CD3ζ expression remains unchanged, loss of NK cell FcεRIγ expression in response to HCMV parallels the development of adaptive NK cells with low levels of NKp30 and NKp46 and impaired cytokine and cytotoxic responses against classical targets.[94,95] In contrast to the reduced functional and phenotypic natural cytotoxicity receptor expression, loss of FcεR1γ has limited impact on CD16 expression and is associated with broadly enhanced antibody-dependent cytokine production and cytotoxic potential.[95] Further, HCMV-dependent loss of NK cell FcεR1γ relates to markedly decreased expression of the PLZF transcription factor and DAB2.[84,85] Loss of PLZF, in concert with epigenetic hypermethylation of the FcεR1γ, Syk and EAT-2 promoter regions to which PLZF binds, reduces FcεR1γ, and in some instances Syk and EAT-2 transcription, producing a pattern that reliably identifies adaptive NK cells.[84,85] The differential methylation pattern observed in adaptive NK cells represents a shift from the epigenetic profile of conventional NK cells towards that of $CD8^{pos}$ cytotoxic T cells.[84]

Ancillary to the epigenetic modifications that create a population of NK cells highly specialized for antibody-dependent cell-mediated cytotoxicity, partial demethylation of the *IFNG* locus gives rise to daughter cells with heritable DNA modifications and an enhanced capacity

Table 1. Human cytomegalovirus-encoded immune evasion genes

HCMV immune evasion gene	Description of interaction	References
UL16	Blocks surface expression of NKG2D ligands MICB and ULBP1/2	106–108
UL18	(i) Class I HLA homologue (ii) Binds NK cell receptor CD85j (LIR-1) (iii) Weakly binds NK cell activating receptor NKG2C	109–111
UL40	(i) Class I HLA homologue (ii) Leader sequence peptide (VMAPRTLIL) binds and stabilizes both UL18 (see above) and HLA-E (NKG2A ligand)	88,112–114
UL83 (pp65)	Interacts with NKp30 to dissociate CD3ζ	115
UL111a	hIL-10 homologue with immunoregulatory properties	116–119
miR-UL112-1	Down-modulates NKG2D ligand, MICB	120
UL135	Remodels actin cytoskeleton to disrupt immune synapse formation	121
UL141	(i) TRAIL ligand (ii) Sequesters CD155 (PVR) in the ER (iii) Decreases CD112 expression in concert with US2 to reduce NK cell co-stimulation through DNAM-1	122–125
UL142	Class I HLA homologue that retains NKG2D ligands, MICA and ULBP3, in Golgi	126–128
UL146	Chemoattractant virokine (vCXCL1) that binds NK cell and neutrophil IL-8R	129,130
gp34 & gp68	HCMV-encoded FcγR that impairs CD16-mediated NK cell activation	131–134
US2	Prevents HLA-A2 and B27 expression by translocating HLA complexes from ER lumen to cytosol	135–138
US3	Blocks tapasin and retains class I HLA in ER	137–139
miR-US4-1	Inhibits ERAP1, prevents peptide loading into class I HLA complexes	140
US6	Inhibits TAP, prevents peptide loading into class I HLA complexes	138,141,142
US9	Targets MICA*008 for proteasomal degradation	143,144
US10	Induces degradation of HLA-G; classical HLA complexes resist degradation	145,146
US11	Degrades HLA-A2 by transporting to cytoplasmic proteases; HLA-C and HLA-G are resistant	135,138,147,148
US18 & US20	(i) Targets MICA for lysosomal degradation (ii) Down-modulates B7-H6 expression	149–151

Abbreviations: ER, endoplasmic reticulum; HCMV, human cytomegalovirus; HLA, human histocompatibility-linked antigen; NK, natural killer.

for IFN-γ production.[98,99] Epigenetic imprinting of both the *IFNG* promoter and conserved non-coding sequence 1 (CNS-1), located 4 kbp upstream of the human *IFNG* promoter, fixes NKG2C^{hi} NK cells with strong and stable IFN-γ responses.[98–101] During NK cell differentiation, hypomethylated CNS-1 encourages binding of T-bet, STAT4, nuclear factor-κB and nuclear factor of activated T cells to enhance downstream *IFNG* transcription after stimulation through activating NK cell receptors, particularly NKG2C.[99–102] The epigenetic imprint that HCMV leaves on the NK cell repertoire contributes to a highly specialized collective readily able to produce IFN-γ in response to appropriate stimuli.

In the absence of demonstrable specific interactions between activating NK cell receptors and HCMV proteins, the question remains as to why HCMV in particular has such a dramatic effect on the NK cell repertoire. Latency and reactivation are common features of all herpesviruses, but the apparently special relationship between HCMV and the immune system is also reflected in conventional adaptive immunity, wherein phenotypically and functionally altered T cells with clonotypic receptors specific for HCMV expand into an unusually large fraction of the T-cell repertoire.[103] This process of T-cell memory inflation parallels phenotypic adaptation of the NK cell repertoire, suggesting that similar stressors impact on both responses.[104] Like other herpesviruses, a long period of co-existence with the human immune system infers an extended arms race influencing both host and virus evolution. Accordingly, a large number of HCMV genes encode proteins that function to promote evasion of T or NK cells (Table 1). Through the nature of its latent reservoir and mode of reactivation, HCMV may disseminate more broadly than other herpesviruses and adopt a wider cellular host range. Hence, the adapted lymphocyte population emerging following primary infection or persisting following viral reactivation should be selectively configured to resist HCMV immune evasion mechanisms more effectively than the naive population. Possibly a number of as yet undefined features of the NK cells that expand following HCMV infection serve to counter one or more of the multiple NK cell evasion

mechanisms enacted by HCMV. Although NKG2C is an effective surrogate marker for NK cell adaptation to HCMV infection, its role in either *in vivo* selection or protection against HCMV has not been confirmed. In fact, there is only one reported example of a T-cell-deficient child where HCMV replication was reduced *in vivo* coincident with emergence of a predominantly NKG2C^{pos} NK cell subset with high CD16 expression.[105] The most notable functional feature attributed to the NK cells responding to HCMV infection is an enhanced capacity for antibody-dependent activation.[95] Whether CD16, NKG2C, activating KIR, self-specific KIR, the absence of inhibitory receptors such as NKG2A, epigenetic remodelling, or all of the above endow NK cells with a selective advantage for expansion and persistence in HCMV-infected individuals remains to be clarified. More effective suppression of HCMV by the adapted NK cell population through antibody-dependent or other mechanisms has also not been proven, but the consistency and robustness of the response suggests either a strong adaptive advantage to the host or that HCMV has evolved to shape the NK cell repertoire to its own advantage. Many individuals infected with HCMV do not have large CD57^{pos} NKG2C^{pos} NK cell populations and low numbers of these cells are not associated with reduced containment, at least not symptomatically. Conversely, the NK cell response to HCMV infection is markedly accentuated in conditions of immune deficiency, immune suppression or ongoing immune reconstitution, such as organ transplantation, bone marrow transplantation and antiretroviral suppression of HIV infection.[81,83,104,105] Whatever selection criteria are generally active appear to operate with greater stringency in these settings, creating a narrow bottleneck strongly favouring eminence of NK cells phenotypically and functionally adapted in response to CMV infection (Fig. 1). Either the frequency or magnitude of viral reactivation in these instances may be an important factor underlying the more pronounced NK cell adaptive responses that occur. The impact of viral variation or superinfection has not been addressed.

Concluding remarks

Ample evidence has accumulated that NK cells proliferate, mature and differentiate in response to various environmental cues. These responses transform the naive NK cell repertoire, which already varies with host genetic background, into a mature repertoire adapted in terms of activating and self-specific KIR expression, C-type lectin-like receptor expression, CD16 expression, maturation markers, intracellular signalling molecules and transcription factors. Although this constitutes a memory population of immune effector cells, only in the case of MCMV is there definitive evidence of a mechanism for selective expansion of NK cells with specificity for a particular antigen. A limited number of examples in mice and macaques hint at the possibility of antigen specificity involving an NK cell activating receptor repertoire beyond that currently known, but evidence for the nature of a receptor repertoire specially created for NK cell adaptive responses is lacking. Hence, some ambivalence still exists as to the perceived nature of NK cell memory. In the clearest examples, there is monochromatic recognition of select foreign proteins and non-specific cytokines that enhance secondary effector function. Still to be fully explained is the NK cell antigen specificity similar to that bestowed by clonotypic receptors reported in mice and peptide or other modification of NK cell receptor ligands that tilt the balance towards activation of NK cells with particular receptor constellations. In the latter case, memory for any particular pathogen would reflect collective recognition of multiple ligands with signal integration triggering a select set of NK cells to proliferate and differentiate in a manner that lowered the threshold for activation upon secondary exposure to the same set of environmental cues. This possibility requires only subtle extension of what is already accepted about integration of positive and negative signals to control NK cell activation. A more speculative possibility would be collective memory of specific environmental cues distributed among a network of NK cells, rather than attributable to single antigen-specific cells. In this scenario, NK cells would have evolved to provide context-dependent help through intercellular communications in a manner similar to the establishment of memory with neuronal networks. Much remains to be explained regarding the unanticipated behaviour of NK cells revealed over the last two decades. The massive implications for basic and translational science will continue to drive research on NK cell memory that promises more surprises.

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