Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells

Highlights
- Ly49H receptor density drives diversity in NK cell function during MCMV infection
- NK cells undergo avidity selection during CMV infection
- Ly49H<sup>hi</sup> NK cells possess greater potential for cytotoxic and adaptive responses
- Ly49H<sup>lo</sup> NK cells possess greater potential for IFN-γ production

Graphical Abstract

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In Brief
NK cells are innate lymphocytes capable of “adaptive” responses after infection, but whether they undergo avidity selection is unknown. Adams et al. report that diversity in antigen receptor Ly49H expression drives NK cell functional heterogeneity during MCMV infection with high-avidity NK cells being selected to dominate the adaptive response.
Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells

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SUMMARY

The process of affinity maturation, whereby T and B cells bearing antigen receptors with optimal affinity to the relevant antigen undergo preferential expansion, is a key feature of adaptive immunity. Natural killer (NK) cells are innate lymphocytes capable of “adaptive” responses after cyto-megalovirus (CMV) infection. However, whether NK cells are similarly selected on the basis of their avidity for cognate ligand is unknown. Here, we showed that NK cells with the highest avidity for the mouse CMV glycoprotein m157 were preferentially selected to expand and comprise the memory NK cell pool, whereas low-avidity NK cells possessed greater capacity for interferon-γ (IFN-γ) production. Moreover, we provide evidence for avidity selection occurring in human NK cells during human CMV infection. These results delineate how heterogeneity in NK cell avidity diversifies NK cell effector function during antiviral immunity, and how avidity selection might serve to produce the most potent memory NK cells.

INTRODUCTION

A fundamental feature of adaptive immunity is its specificity for a nearly infinite array of potential antigens (Goldrath and Bevan, 1999). During an infectious challenge, the repertoire of presented antigens and competition for those antigens shape the diversity of the effector lymphocyte population in a manner dependent on lymphocyte responsiveness to a given epitope (Johnson et al., 2016; Kedl et al., 2003; Kedl et al., 2000; Oberle et al., 2016). During a primary immune response, competition for antigen and resources results in outgrowth of CD8+ T cells bearing T cell receptors (TCRs) that recognize their cognate antigen with optimal affinity (Day et al., 2007; Trautmann et al., 2005). B cells iteratively mutate their B cell receptor (BCR) in the germinal center via somatic hypermutation until their BCR affinity is optimal for sensing antigens presented by follicular dendritic cells (FDCs), which provide high-affinity B cells with necessary survival and proliferation signals, a process known as affinity maturation (Kim et al., 1981; Küppers et al., 1993; MacLennan, 1994; Muramatsu et al., 1999). Furthermore, T cell affinity maturation occurs during a secondary or recall response, a selective process in which the diversity of the epitope-specific CD8+ T cell TCR repertoire becomes restricted (Busch and Pamer, 1999). Thus, the adaptive immune system evolves in real-time by selecting lymphocytes best able to combat the specific pathogens encountered.

Natural killer (NK) cells are innate lymphocytes that play a critical, dominant role in herpesvirus control in both mice and humans (Biron et al., 1989; Bukowski et al., 1985; Etzioni et al., 1999). Although traditionally categorized under innate immunity, NK cells can exhibit “adaptive” antiviral responses to mouse cytomegalovirus (MCMV), including robust clonal proliferation and establishment of a long-lived population of memory cells with enhanced functionality and protective capacity after MCMV reinfection (Sun et al., 2009). In C57BL/6 mice, a subset of naive NK cells bearing the activating receptor Ly49H undergoes this adaptive response after recognition of MCMV-encoded m157, a major histocompatibility complex (MHC)-class I-like glycoprotein expressed on the infected cell surface (Arase et al., 2002; Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009). In both mice and humans, the combination of stochastically expressed germline-encoded activating and inhibitory receptors generates NK cell diversity and heterogeneity at the population level (Horowitz et al., 2013; Lanier, 2005). Several groups have reported that heterogeneity within the naive Ly49H+ NK cell pool affects the antiviral NK cell response, in particular the preferential expansion of unlicensed NK cells, NK cells with a history of recombination-activating gene (RAG) expression, and NK cells that lack expression of killer cell lectin-like receptor G1 (KLRG1) or the inhibitory receptor NKR-P1B (Kamimura and Lanier, 2015; Karo et al., 2014; Orr et al., 2010; Rahim et al., 2016). However, whether Ly49H+ NK cells undergo selection during generation of the effector and memory pool on the basis of their avidity for m157 remains unresolved. Here, we interrogated the effect that heterogeneity in antigen receptor expression has on Ly49H+ NK cell functional responses and provide evidence that both...
mouse and human NK cells undergo avidity selection during CMV infection.

RESULTS

MCMV m157 Increases Ly49H Expression within the Antiviral NK Cell Pool

To investigate whether viral infection modulates the avidity of the virus-specific NK cell pool, we infected wild-type (WT) mice with MCMV and observed increased surface expression of Ly49H on Ly49H+ NK cells (Figures 1A and S1A). To track antigen-experienced NK cells longitudinally, we transferred Ly49H+ NK cells into Ly49H-deficient recipients (Kira8–/– mice) (Figure 1B). Because the endogenous NK cells in Kira8–/– mice cannot engage MCMV m157 (Fodil-Cornu et al., 2008), only the transferred Ly49H+ NK cells undergo MCMV-driven clonal prolifera-

tion (Sun et al., 2009). Compared with the naive Ly49H+ NK pool, effector and memory Ly49H+ NK cells displayed higher Ly49H expression (Figure 1C). In contrast, expression of NK1.1, which recognizes MCMV m12 (Aguilar et al., 2017), remained unchanged on memory NK cells (Figure 1D), indicating that the sustained change in the distribution of Ly49H expression on MCMV-driven NK cells might be unique among NK cell activating receptors. Neither infection with MCMV lacking m157 (MCMV-Jm157) nor with Listeria monocytogenes affected Ly49H expression on Ly49H+ NK cells (Figure 1E), indicating that m157 is required for this process. During MCMV infection, NK cells are exposed not only to m157 on infected cells, but also to an environment rich in pro-inflammatory cytokines (Biron and Tarrio, 2015). However, NK cells unresponsive to interleukin-12 (IL-12) (Stat4–/–), IL-18 (Il18r1–/–), and type I interferon (IFN) (Ifnar1–/– and Stat1–/–) expressed Ly49H comparably with WT

Figure 1. MCMV-Driven Changes in Ly49H Expression within the Antiviral NK Cell Pool Are m157-Dependent
(A) Histograms of Ly49H expression on splenic Ly49H+ NK cells (gating strategy in Figure S1A) from uninfected (UI) and MCMV-infected WT mice at PI day 7 (D7). Data are representative of 5–10 mice per experiment. (B and C) Experimental schematic (B). Ly49H+ NK cells were transferred into Kira8–/– mice, and their phenotype was analyzed after MCMV infection of the recipients. In (C) are histograms of Ly49H expression on Ly49H+ NK cells from blood of UI WT mice (shaded gray) versus mice at PI D7 (top left) and PI D29 (bottom left) (black lines). Quantification of Ly49H MFI on Ly49H+ NK cells at indicated PI days compared with cells of UI WT mice bled on the same day (right). Data are representative of at least 3 independent experiments with 10–25 mice per experiment. (D) As in (C), histograms (left) and MFI (right) of NK1.1 on Ly49H+ NK cells from blood of UI WT mice and at PI D28. Data are representative of at least 3 independent experiments with 10–25 mice per experiment. (E) WT mice were infected with MCMV, MCMV-Jm157, Listeria monocytogenes (L.m.), or UI. Data are represented as Ly49H MFI on Ly49H+ NK cells from blood at PI D7 for infected infections relative to UI. Data are representative of 2 independent experiments with 2–5 mice per group. (F) As in (B), except splenocytes from WT and indicated knockout (KO) mice were co-transferred. Data are represented as Ly49H MFI on KO Ly49H+ NK cells compared with WT from blood at PI D7. Data are representative of 2–4 independent experiments with 2–5 mice per group. Groups with a ratio < 1 were compared against 1 via a one sample t test.

Groups were compared using an unpaired, two-tailed Student’s t test (D) or against 1 via a one sample t test (E). In (C), each time point was compared against 1 via a one sample t test, and against each other via a paired two-tailed t test. Data are presented as the mean ± SEM. *p < 0.05; ****p < 0.0001. See also Figure S1.
Figure 2. Ly49H+ NK Cells Undergo Avidity Selection During MCMV Infection

(A) Experimental schematic (A). Equal numbers of splenic Ly49Hlo and Ly49Hhi NK cells, purified from congenically distinct WT mice, were co-transferred into Klra8−/− mice, and their responses tracked after MCMV infection of the recipients. In (B) are flow plots gated on NK cells from blood at PI D7 (left) and spleen at PI D28 (middle). Quantification of percent Ly49Hlo and Ly49Hhi NK cells within total NK cells in blood at indicated days PI (right). In (C) is the percentage of Ly49Hlo and Ly49Hhi NK cells within transferred Ly49H+ NK cells in blood at indicated PI days (left) and in indicated organs at PI D28 (right). In (D) are histograms of Ly49H expression on Ly49Hlo and Ly49Hhi NK cells from blood at PI D7. Data are representative of 2 independent experiments with 3–5 mice per experiment.

(E) WT splenocytes were labeled with CTV and transferred into congenically distinct WT mice prior to MCMV infection. Histograms of CTV in splenic Ly49Hlo and Ly49Hhi NK cells at PI D7 (left). Quantification of indicated NK cell populations that have divided at least once (right). Data are representative of 3 independent experiments with 4 mice per experiment.

(F) Percent of splenic Ly49Hlo and Ly49Hhi NK cells staining positive for FLICA at PI D4. Data are pooled from 2 independent experiments with 3–4 mice per experiment.

(G) Purified splenic Ly49Hlo and Ly49Hhi NK cells were loaded with Fura-2AM and co-cultured with Ba/F3-m157 cells. Live-cell imaging was then performed. Representative images from the time-lapse analysis (left). Scale bar, 10 μM. Quantification of the number of contacts between NK cells and target cells (middle) and contact efficiency (right), defined as the proportion of target cell contacts that resulted in an NK cell Ca2+ flux (change in NK cell color from green to yellow or red). Data are pooled from 2 experiments with 4 replicates per group per experiment.
effector NK cells after MCMV infection (Figures 1F and S1B), suggesting that these cytokines are individually dispensable for the MCMV-driven avidity changes. Thus, MCMV infection elicits an m157-dependent change in the distribution of Ly49H expression on effector and memory Ly49H+ NK cells.

**Avidity Selection Shapes the Effector and Memory NK Cell Pool During MCMV Infection**

We reasoned that one explanation for a population increase in Ly49H could be preferential expansion of Ly49H+ NK cells with greater baseline Ly49H expression (i.e., avidity selection). To test this hypothesis, we purified equal numbers of Ly49H+ NK cells with different Ly49H receptor abundance (Ly49Hlo and Ly49Hhi), defined as Ly49H+ NK cells in the bottom or top ~20% of Ly49H median fluorescence intensity (MFI), respectively, from congenically distinct WT mice, co-transferred them into MCMV-infected Ly49H-deficient recipients, and longitudinally analyzed their activation and expansion (Figures 2A and S1C). Although both Ly49Hlo and Ly49Hhi NK cells clonally expanded and formed memory (Figures 2B and 2C), the magnitude of their responses differed, regardless of their congenic markers (Figure S2A). Ly49Hlo NK cells expanded more robustly and outnumbered Ly49Hhi NK cells at 7 days post-infection (PI) (the peak of the antiviral response) and preferentially contributed to the memory NK cell pool in peripheral blood and various tissues at 28 days PI (Figures 2B and 2C). The ratio of Ly49Hlo to Ly49Hhi NK cells did not diverge further during contraction (Figure 2C), suggesting the greater abundance of Ly49Hhi memory NK cells is due to their expansion advantage. Even in a non-competitive setting, when Ly49Hlo and Ly49Hhi NK cells were transferred into separate animals, Ly49Hlo NK cells produced a larger effector and memory population (Figure S2B), indicating that NK cell avidity for viral ligand regulates intrinsic aspects of the antiviral NK cell response. Despite their differential expansion, Ly49Hlo and Ly49Hhi NK cells matured similarly (Figure S2C). They also retained their relative differences in Ly49H expression after infection (Figure 2D), suggesting that Ly49H expression is clonally maintained. Compared with naive NK cells expressing either Ly49H or Ly49D alone, naive NK cells co-expressing both Ly49H and Ly49D exhibited reduced abundance of each receptor (Figure S2D). Thus, Ly49D expression might contribute to establishing heterogeneity in Ly49H expression within the naive NK cell pool.

We also evaluated whether avidity maturation (i.e., upregulation of Ly49H within an individual NK cell) and avidity selection occur concomitantly. Ly49Hlo NK cells increased their expression of Ly49H, most notably in the contraction phase, during which Ly49H expression increased 2- to 3-fold compared with that of naive Ly49Hlo NK cells (Figure S2E). In contrast, compared with naive Ly49Hlo NK cells, MCMV-driven Ly49Hhi NK cells did not considerably increase their Ly49H expression (Figure S2E), suggesting there might be an “upper limit” for Ly49H expression on NK cells.

To investigate the mechanism behind differential expansion of Ly49Hlo and Ly49Hhi NK cells, we transferred bulk splenocytes labeled with CellTrace Violet (CTV) into WT mice. After MCMV infection, Ly49Hlo NK cells divided more efficiently than did Ly49Hhi NK cells (Figure 2E). Furthermore, during MCMV-driven expansion, Ly49Hlo NK cells demonstrated lower caspase activity than did Ly49Hhi NK cells, as assessed by fluorescent-labeled inhibitors of caspases (FLICA) staining. (Figure 2F). Collectively, these data suggest that NK cell avidity regulates the proliferative capacity and survival of a given Ly49H+ NK cell during MCMV infection, which underlies the greater expansion and subsequent memory pool establishment of Ly49Hlo NK cells.

**NK Cell Avidity Regulates m157-Mediated Activation and Cytotoxicity**

These data raised the possibility that Ly49H density affects NK cell engagement with infected cells (i.e., avidity) or the magnitude of Ly49H-dependent signaling. To test this, we performed live-cell imaging of Ly49Hlo and Ly49Hhi NK cells co-cultured with Ba/F3 cells expressing m157 (Ba/F3-m157), Ly49H receptor ligation results in phosphorylation of its adaptor DAP12, which recruits and activates Syk and zeta chain of T cell receptor associated protein kinase 70 (ZAP70), initiating their signaling cascades and resulting in increased intracellular calcium (Lanier, 2008). To visualize productive target cell encounters that resulted in activation-induced Ca2+ flux, we labeled NK cells with the cell permeable Fura-2 calcium indicator. Ly49Hlo NK cells made more contacts with Ba/F3-m157 cells than did Ly49Hhi NK cells, and a greater percentage of Ly49Hlo NK cell contacts resulted in NK cell activation and Ca2+ flux (Figure 2G). These results suggest that Ly49Hlo NK cells might be preferentially recruited into the antiviral response.

Given that NK cell cytotoxicity requires activating receptor engagement, we speculated that NK cell cytotoxic function is dependent on avidity for cognate ligand. To assess this, we separately co-cultured Ly49Hlo, Ly49Hhi, and Ly49Hlo NK cells with equal numbers of target Ba/F3-m157 and control Ba/F3 cells labeled differentially with CTV. Although Ly49Hlo NK cells were capable of killing Ba/F3-m157 target cells, Ly49Hlo NK cells possessed the most potent cytotoxic function (Figures 2H, S2F, and S2G). The enhanced functionality of Ly49Hlo NK cells was strictly Ly49H-dependent, as a greater percentage of Ly49Hlo NK cells produced IFN-γ and degranulated only in response to Ly49H ligation ex vivo, but not NK1.1 ligation or phorbol 12-myristate 13-acetate (PMA) and ionomycin (Figure S2H), excluding a developmentally determined general heightened reactivity of these cells. Collectively, these data indicate that higher Ly49H expression precipitates greater Ly49H-dependent activation and effector function (i.e., functional avidity) in the presence of target cells expressing cognate ligand.

(H) Purified splenic Ly49Hlo, Ly49Hhi, and Ly49Hlo NK cells were incubated with Ba/F3-m157 target cells (CTV+) and Ba/F3 control cells (CTV−) at 10:1 ratio (effector:target:control) for 6 h. Quantification of target cell killing by indicated NK cell populations compared with control wells lacking NK cells. Data are representative of 3 independent experiments with 3 replicates per group per experiment. Ly49Hlo and Ly49Hhi NK cells were compared via an unpaired, two-tailed Student’s t test.

Groups were compared via a paired, two-tailed t test in (B), (E), and (F); an unpaired, two-tailed Student’s t test in (G); or against 50 via a one sample t test (C). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figures S1 and S2.
Ly49H Density Specifies Differential Effector Functions of Ly49H+ NK Cells During Early MCMV Infection

The detection of viral-ligand-bearing infected cells by Ly49H+ NK cells is an essential signal for NK cell-mediated host protection, yet MCMV also induces a highly inflammatory environment rich in IL-12, IL-18, and type I interferon (Biron and Tarrio, 2015). These cytokines can lead to non-specific activation of NK cells, promote NK cell effector function (e.g., IFN-γ production), and program their adaptive responses (Biron and Tarrio, 2015; Dokun et al., 2001; Madera et al., 2016; Madera and Sun, 2015; Sun et al., 2012). To comprehensively understand how avidity regulates the NK cell response to MCMV, we performed RNA-sequencing (RNA-seq) on splenic Ly49Hlo and Ly49Hhi NK cells during early MCMV infection (PI day 1.5). Of the 1,941 differentially expressed genes (padj < 0.05), 1,232 transcripts were upregulated and 709 were downregulated in Ly49Hlo NK cells (Table S1), the top 100 of which are shown in Figure 3A. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes revealed an enrichment of cell cycle control, DNA replication, and genome integrity maintenance genes in Ly49Hlo NK cells (Figures 3B and S3A), consistent with their proliferative advantage (Figure 2E). In contrast, genes upregulated in Ly49Hhi NK cells were primarily involved with cytokine and Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling (Figures 3C and S3B). The extent and nature of the transcriptional differences underscore the diversity within the Ly49H+ NK cell compartment and the divergence of their cellular activities within the earliest days of infection.

To validate and extend our RNA-seq results, we focused on several differentially expressed genes notable for their role in NK cells and regulation by proinflammatory cytokines. The transcript (Iil2ra) and protein (CD25) of the high-affinity α subunit of the IL-2 receptor were both more robustly upregulated in Ly49Hlo NK cells during MCMV infection (Figures S3C and S3D). Furthermore, consistent with higher quantities of Ifng (encoding IFN-γ) transcripts in Ly49Hlo NK cells (Figure 3D), a greater percentage of Ly49Hlo NK cells produced IFN-γ during MCMV infection (Figure 3E), and Ly49Hlo NK cells produced more IFN-γ than Ly49Hhi NK cells on a per-cell basis (Figure S3E). In Ifng-IRE3-YFP reporter mice (“GREAT” mice), Ly49Hlo NK cells more robustly upregulated Ifng transcript during MCMV infection (Figure 3F). However, prior to infection, there were no differences in baseline Ifng transcript (Figures 3F and S3F) or their ability to produce IFN-γ after IL-12 and IL-18 stimulation ex vivo (Figure S2H), suggesting that infection-specific differences in the regulation of the Ifng locus accounted for the observed phenotype. Although bulk Ly49H+ and Ly49H+ NK cells are thought to express similar IFN-γ at this early time point (Dokun et al., 2001), the “population average” across Ly49H+ NK cells concealed considerable heterogeneity in the Ly49H+ NK cell compartment (Figure S3G). Thus, the extent of Ly49H+ signaling in vivo during MCMV infection regulates NK cell effector properties.

Ly49Hlo and Ly49Hhi NK cells had similar maturation profiles (by CD11b and CD27 subsets) despite modest differences in KLRG1 (Figure S3H), and similar proportions of both NK cell populations expressed NKG2A (Figure S3I), one of the inhibitory receptors licensed in C57BL/6 mice. A greater proportion of Ly49Hlo NK cells expressed Ly49Cl (Figure S3I), yet consistent with an earlier report (Orr et al., 2010), Ly49Cl expression did not affect IFN-γ production in vivo during MCMV infection (Figure S3J). Thus, the functional and transcriptional differences between Ly49Hlo and Ly49Hhi NK cells during MCMV infection are likely because of infection-specific, rather than developmental differences. Furthermore, Ly49Hlo and Ly49Hhi NK cells displayed similar abundance of phosphorylated STAT4 (pSTAT4), comparable IL-18 receptor alpha (IL-18Rα), and transcripts encoding IL-12 receptor chains (Figures S3K and S3L). Thus, Ly49H-mediated regulation of proinflammatory cytokine responsiveness is likely occurring further downstream of cytokine receptor expression and STAT4 phosphorylation.

To understand whether the functional differences between these two NK cell subsets have a physiological role in host defense, we compared protection of susceptible Rag2−/−Il2rg−/− mice by Ly49Hlo or Ly49Hhi NK cells. Although Ly49Hlo NK cells provided some protection against MCMV (compared with mice that did not receive NK cells), Ly49Hhi NK cells significantly extended the survival of susceptible hosts beyond that of Ly49Hlo NK cells (Figure 3G). Collectively, these results reveal that the avidity-dependent functional differences between Ly49Hlo and Ly49Hhi NK cells regulate the protective antiviral responses mounted by NK cells.

Human NK Cells Show Evidence for Avidity Selection During HCMV Infection

Analogous to the Ly49H+ NK cell response against MCMV in mice, CD94/NKG2C+ NK cells are expanded in the peripheral blood of human cytomegalovirus (HCMV)-seropositive healthy individuals (Gumà et al., 2004; Lopez-Vergés et al., 2011), as well as in recipients of solid organ or hematopoietic stem cell (HSC) transplants who reactivate HCMV (Della Chiesa et al., 2012; Foley et al., 2012; Horowitz et al., 2015; Lopez-Vergés et al., 2011; Muccio et al., 2016). HCMV-encoded UL40 peptides loaded onto HLA class I histocompatibility antigen, alpha chain E (HLA-E) were recently shown to activate human NKG2C+ NK cells in a peptide-specific manner (Hammer et al., 2018), comparable to the interaction between MCMV m157 and Ly49H in mice. To determine whether HCMV infection similarly modulates the avidity of human NKG2C+ NK cells, we analyzed NK cells from peripheral blood of T-cell-depleted HSCT recipients. In the transplant setting, the timing of HCMV infection (or reactivation in patients previously reported to be HCMV-seropositive) can be estimated, which enabled longitudinal analysis of NK cells in the same patient. Consistent with previous reports (Foley et al., 2012; Horowitz et al., 2015; Muccio et al., 2016), NKG2C+ NK cells were expanded in HSCT patients after HCMV detection (Figure 4A). Phenotypic analysis revealed higher NKG2C expression on the expanded NKG2C+ NK cell population from HCMV-reactivating patients compared with the population prior to expansion (Figure 4B). In contrast, the unexpanded population of NKG2C+ NK cells from patients who remained HCMV-seronegative after transplant maintained stable NKG2C expression during the post-transplant period analyzed (Figure 4B). This evidence supports the notion that NK cell avidity selection during CMV infection might be conserved between mouse and human.

In a larger healthy human cohort, HCMV-seronegative donors had minimal variation in NKG2C expression and NKG2C+ NK cell percentage, whereas HCMV-seropositive donors displayed a
positive linear relationship between NKG2C expression and percentage of NKG2C+ NK cells (Figure 4C). Similarly, during the mouse Ly49H+ NK cell response to MCMV, Ly49H expression strongly correlated with expansion of the Ly49H+ NK cell population at the peak of the antiviral response (Figure 4D). These data support the idea that the degree of virus-driven NK cell...
expansion and the extent of NK cell avidity selection are closely linked processes in both mice and humans.

**DISCUSSION**

Our study provides a mechanistic understanding of how avidity for viral ligand regulates the functional contribution of an NK cell during MCMV infection. Although NK cells of varying avidities were recruited during early MCMV infection, similar to CD8+ T cells with a range of TCR affinities (Zehn et al., 2009), high avidity NK cells preferentially expanded. Concurrent with our studies using NK cell populations sorted on the basis of Ly49H surface density, Grassmann et al., 2019 used retrogenic color barcoding to track single NK-cell-derived responses. In accordance with our findings, they also observed that Ly49H expression in individual NK cell clones correlated with the degree of their clonal expansion. Thus, the major conclusions from both of these complementary studies are in agreement. Furthermore, it appeared that effector and memory Ly49H+ NK cells expressed more Ly49H than they do as naive NK cells, suggesting that avidity maturation (i.e., upregulation of Ly49H within an individual NK cell) and avidity selection might be occurring concomitantly. Together these processes might contribute to the generation of a memory NK cell pool with heightened specificity for MCMV re-encounter (Sun et al., 2009).

Previous in vivo imaging studies have described that TCR transgenic CD8+ T cell priming by cognate-peptide-loaded DCs occurs in three phases: (1) transient serial encounters with DCs, (2) stable contacts with DCs, and (3) CD8+ T cell motility and proliferation (Mempel et al., 2004). Stable contacts between CD8+ T cells and DCs are required for the full differentiation program. These stronger and longer interactions program CD8+ T cell clonal expansion and are dependent on the absolute number of TCR-pMHC interactions (Henrickson et al., 2008). Furthermore, high-affinity TCR ligands and prolonged antigen exposure dictate the duration and magnitude, respectively, of the CD8+ T cell expansion (Prlic et al., 2006; Zehn et al., 2009). Our data reveal that many requirements for naive CD8+ T cell
and NK cell priming and clonal expansion are conserved. Although MCMV infection activated both Ly49Hlo and Ly49Hhi NK cells, these cells were transcriptionally distinct during the earliest days of infection. We speculate that the number of activating receptor-cognate ligand interactions regulates the strength or duration of contact between an NK cell and an infected cell, which plays a fundamental role in programming the response of that cell.

Despite the greater potential of Ly49Hhi NK cells for expansion and cytotoxicity, Ly49Hlo NK cells were the primary IFN-γ-producing NK cells during the early effector response, suggesting a division of labor between these two cell populations. One possibility is that an NK cell must allocate cellular and metabolic resources for the competing demands of IFN-γ secretion and expansion or that strong Ly49H receptor ligation might antagonize NK cell responsiveness to proinflammatory cytokines. Indeed, MCMV-driven memory NK cells, which have greater Ly49H surface density, have diminished bystander responses (Min-Oo and Lanier, 2014). Thus, strong Ly49H activation precipitates both immediate and heritable changes in IFN-γ production.

Ly49Hlo and Ly49Hhi NK cells maintained their relative differences in Ly49H density after MCMV-driven proliferation, suggesting clonal maintenance of Ly49H expression. Given that Kira8 (encoding Ly49H) was the most differentially expressed gene ranked by p value between Ly49Hlo and Ly49Hhi NK cells at PI day 1.5 (Table S1), we hypothesize that Ly49H expression is transcriptionally maintained during infection. Because the effector function profile of an NK cell during MCMV infection hinges on its Ly49H expression, further investigation into the full complement of factors (of which, Ly49D might be one such variable) that regulate and maintain Ly49H expression during development, homeostasis, and infection is warranted.

We demonstrated that the NKG2C+ NK cell pool that arose in HSCT recipients after HCMV infection or reactivation expressed more NKG2C, which was analogous to avidity selection of mouse NK cells during MCMV infection. NKG2C zygosity has been reported to influence surface receptor density and NKG2C+ NK cell numbers in HCMV+ subjects (Muntasell et al., 2013), and could contribute to the range in these parameters observed in our transplant patient cohort that reactivated HCMV. Future work is required to determine the molecular mechanisms behind avidity selection of human NK cells, and how avidity affects human NK cell repertoire diversity on the basis of prior pathogen experience (Strauss-Albee et al., 2015). Nevertheless, our findings collectively indicate that avidity selection might be a general mechanism in NK cell biology conserved between mouse and human, likely resulting from the extensive co-evolution of NK cells with CMV, and can inform vaccination strategies to elicit memory NK cells with the greatest avidity for viral ligands.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2019.04.009.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

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<td><strong>Bacterial and Virus Strains</strong></td>
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<td>J. Sun (PI)</td>
<td>Smith Strain</td>
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<td>U. Koszinowski (PI) (Subić et al., 2004)</td>
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<tr>
<td>Listeria monocytogenes</td>
<td>J. Sun (PI)</td>
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<td><strong>Biological Samples</strong></td>
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<td>Healthy human donor buffy coats</td>
<td>New York Blood Center</td>
<td><a href="http://nybloodcenter.org/">http://nybloodcenter.org/</a></td>
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<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
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<td>Recombinant Mouse IL-18</td>
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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joseph Sun (sunj@mskcc.org).

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
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<tbody>
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<td>Phorbol 12-myristate 13-acetate (PMA)</td>
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<td>Ionomycin calcium salt from <em>Streptomyces conglobatus</em> (Ionomycin)</td>
<td>Sigma-Aldrich</td>
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Critical Commercial Assays

| TRIZOL™ Reagent | Thermo Fisher Scientific | Cat#15596026 |
| eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat#00-5523-00 |
| BioMag Goat Anti-Rat IgG (NK cell enrichment) | QIAGEN | Cat#310107 |
| CellTrace™ Violet Cell Proliferation Kit | Thermo Fisher Scientific | Cat#C34557 |
| FAM FLICA™ Poly Caspase Kit | Bio-Rad | Cat#ICT092 |
| Fura-2, AM, cell permeant | Thermo Fisher Scientific | Cat#F1201 |
| eBioscience™ Fixable Viability Dye eFluor™ 506 | eBioscience | Cat#65-0866-14 |
| LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation | Thermo Fisher Scientific | Cat#L34966 |

Raw Data Files for RNA Sequencing (Ly49H
|-- versus Ly49H
|--)
| NCBI Gene Expression Omnibus | GEO: GSE129490 |

Experimental Models: Cell Lines

| Mouse: Ba/F3 cells | L. Lanier (PI) | N/A |
| Mouse: Ba/F3-m157 cells | L. Lanier (PI) | Derived from parental Ba/F3 cells |

Experimental Models: Organisms/Strains

| Mouse: WT or CD45.2: C57BL/6J | The Jackson Laboratory | Stock#000644; RRID: IMSR_JAX:000664 |
| Mouse: WT or CD45.1: B6.SJL-Ptprca Pepcb/J | The Jackson Laboratory | Stock#002014; RRID: IMSR_JAX:002014 |
| Mouse: Kira8\^/- or Ly49H-deficient | S. Vidal (PI) | N/A |
| Mouse: Stat4\^/-, Ifnar1\^/-, Stat1\^/-, Il18r1\^/- | J. Sun (PI) | N/A |
| Mouse: Ifng-ires-YFP or GREAT: B6.129S4-Ifng\^tm3.1Lky/J | The Jackson Laboratory | Stock#017581; RRID: IMSR_JAX:017581 |
| Mouse: Rag2\^/-, I2rg\^/-: C;129S4-Rag2\^tm1.1Ry \ I2rg\^tm1.1Ry/J | The Jackson Laboratory | Stock#014593; RRID: IMSR_JAX:014593 |

Software and Algorithms

| Trimmomatic (v.0.36) | Bolger et al., 2014 | http://www.usadellab.org/cms/?page=trimmomatic |
| Salmon (v0.10.2) | Patro et al., 2017 | https://combine-lab.github.io/salmon |
| goseq (v1.33.0) | Young et al., 2010 | https://bioconductor.org/packages/release/bioc/html/goseq.html |
| KEGG.db (v3.2.3) | | https://bioconductor.org/packages/release/data/annotation/html/KEGG.db.html |
| R (v.3.5.1) | | https://www.r-project.org/ |
| SlideBook software (v6.0.15) | Intelligent Imaging Innovations | https://www.intelligent-imaging.com/slidebook |

*Immunity* 50, 1381–1390.e1–e5, June 18, 2019
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All mice used in this study were housed and bred under specific pathogen-free conditions at Memorial Sloan Kettering Cancer Center (MSKCC), and handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The following mouse strains were used in this study: C57BL/6 (CD45.2), B6.SJL (CD45.1), Klra8−/− (Ly49H-deficient) (Fodil-Cornu et al., 2008), Stat4−/−, Il18r1−/−, Ifnar1−/−, Ifng-IRES-YFP (GREAT), and Rag2−/− Il2rg−/− mice. Experiments were conducted using age- and gender-matched mice in accordance with approved institutional protocols. Mice used in this study were of both genders and were 6-8 weeks of age at the time of experimentation.

Primary Human Cells

Peripheral blood samples were collected from allogeneic bone marrow transplantation patients and healthy human donors following approval from the MSKCC Institutional Review Board, and donors provided informed, written consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll centrifugation. Additional PBMCs were isolated from buffy coats obtained from healthy volunteer donors via the New York Blood Center (NYBC, http://nybloodcenter.org/). The MSKCC IRB waived the need for additional research consent for anonymous NYBC samples. Donors were of both genders and ranged in age between 12 and 76 years old. PBMCs were cryopreserved in fetal bovine serum with 10% DMSO. HCMV serostatus was provided by NYBC.

Virus

MCMV (Smith strain) was serially passaged through BALB/c hosts three times, and then salivary gland viral stocks were prepared with a dounce homogenizer for dissociating the salivary glands of infected mice 3 weeks after infection.

Bacteria

Frozen stocks of Listeria monocytogenes were grown in brain-heart infusion (BHI) broth. Bacteria culture samples were grown to mid-log phase, measured by optical density (A600), and diluted in PBS for injection.

METHOD DETAILS

In Vivo Virus Infection

Adoptive co-transfer studies were performed by transferring 10⁵ Ly49Hlo NK cells and/or 10⁵ Ly49Hhi NK cells, purified from spleens of congenically distinct WT mice (CD45.1 or CD45.2), into Klra8−/− mice 1 day prior to MCMV infection. Recipient mice in adoptive transfer studies (both competitive and non-competitive) were infected with MCMV by intraperitoneal (i.p.) injection of 7.5 × 10² plaque-forming units (PFU) in 0.5 mL.

Survival studies were performed by transferring either no cells, 5x10⁴ purified splenic Ly49Hlo NK cells, or 5x10⁴ purified splenic Ly49Hhi NK cells into Rag2−/− Il2rg−/− mice 2 days prior to MCMV infection. Recipient mice in survival studies were infected with 7.5 × 10³ PFU MCMV in 0.5 mL by i.p. injection.

In several experiments, WT mice were directly infected with MCMV (7.5 × 10³ PFU) or MCMV-Dm157 (10⁵ PFU) (Bubić et al., 2004) in 0.5 mL by i.p. injection.

In Vivo Bacterial Infection

Mice were infected with priming doses equivalent to 2000-5000 colony forming units (CFU) by tail vein injection.

Lymphocyte Isolation

Spleens were dissociated using glass slides and filtered through a 100-μm strainer. To isolate lymphocytes from liver, the tissue was physically dissociated using a glass tissue homogenizer and purified using a discontinuous gradient of 40% over 60% Percoll. Red blood cells in blood, spleen, and liver were lysed using ACK lysis buffer.

Flow Cytometry and Cell Sorting

Cell surface staining of single-cell suspensions from various organs was performed using fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo Biosciences, Beckman Coulter, Miltenyi). Intracellular staining was performed by fixing and permeabilizing with the eBioscience Foxp3 Transcription Factor Staining Set (Thermo Fisher) for staining intranuclear proteins and cytokines, or with formaldehyde and methanol for staining phosphorylated STAT proteins.

Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. Data were analyzed with FlowJo software (Tree Star). Flow cytometry of mouse lymphocytes was performed using the following fluorophore-conjugated antibodies: CD3ε (17A2), TCRβ (H57-597), CD19 (6D5), NK1.1 (PK136), Ly49H (3D10), CD45.1 (A20), CD45.2 (104), CD11b (M1/70), CD27 (LG.7F9), KLRL1 (2F1), Ly49D (4E5), Ly49C/I (5E6), NK2CA/C/E (20D5), CD25 (PC61), IFN-γ (XMG1.2), CD107a (1D4B), STAT4 pY693 (38/p-Stat4), IL-18Ra (P3TUNYA), and Fixable Viability Dye. Staining of human PBMCs was performed using: CD3 (UCHT1), CD6 (N901), NKG2C (REA205), and LIVE/DEAD as viability marker.
Apoposis was evaluated by caspase activity staining using the carboxyfluorescein FLICA poly caspase assay kit (Bio-Rad). NK cell proliferation was analyzed by labeling cells with 5 μM CellTrace Violet (CTV, Thermo Fisher) prior to transfer, and CTV labeling was performed according to manufacturer protocol.

**NK Cell Enrichment and Purification**

NK cells (TCRβ−CD3ε−CD19−F4/80−CD45−NK1.1−) were enriched from spleens of pooled C57BL/6 mice by negative selection over BioMag goat anti-rat IgG beads (QIAGEN) coated with rat anti-mouse CD8α, CD4, CD19, and Ter-119 antibodies (Bio X Cell, clones 2.43, GK1.5, 1D3, and TER-119 respectively) before being sorted to high purity on an Aria II cytometer (BD Biosciences).

**Ex Vivo Stimulation of Lymphocytes**

10⁶ purified splenic Ly49Hlo or Ly49Hhi NK cells were stimulated for 4 hours in RPMI containing 10% fetal bovine serum with 20 ng/mL recombinant mouse IL-12 (R&D Systems) plus 10 ng/mL IL-18 (MBL), 10 ng/mL PMA (Sigma) plus 1 μg/mL Ionomycin (Sigma), or 25 μg/mL of plate-bound anti-mouse NK1.1 (PK136, BioLegend) or anti-mouse Ly49H (3D10, BioLegend). Cells were cultured in media alone as a negative control.

**Ca²⁺ Imaging of NK Cells**

Purified splenic NK cells with different Ly49H expression were loaded with 5 μg/mL Fura-2AM for 30 min and transferred into colorless RPMI (no phenol red) containing 5% FCS. 8 × 10⁴ NK cells were mixed 1:4 with Ba/F3-m157 cells and imaged in 8-well chamber slides (Thermo Fisher Scientific) using an inverted fluorescence video microscope (IX-81; Olympus) fitted with a 20 ×, 0.75 NA objective lens (Olympus). A Xen lamp (DG-4; Sutter Instrument) was used for fluorophore excitation, and data were collected on an electron-multiplying charge-coupled device camera (ImageEM; Hamamatsu Photonics). Time-lapse recordings were made using SlideBook software (Intelligent Imaging Innovations). One brightfield image and one Fura-2 image were taken every 20 s for 20 min.

**Ex Vivo Killing Assay**

Ba/F3 control cells and Ba/F3-m157 target cells (Arase et al., 2002) were labeled differentially with CTV (Ba/F3, CTVlo; Ba/F3-m157, CTVhi). 5 × 10⁴ of each cell line were mixed with 5 × 10⁴ purified splenic NK cells with different Ly49H expression, or without NK cells (control condition). Effector and target cells were co-cultured for 6 hours at 37°C in RPMI-1640 containing 10% FBS. After 6 hours, cells were stained with propodium iodide prior to flow cytometry. The percentages of target cell killing were determined using the following formula, adapted from (Viant et al., 2017): 100 − [((% Ba/F3-m157 cells/% Ba/F3 cells)/(% Ba/F3-m157 cells/% Ba/F3 cells) control) × 100]. For this formula, only CTV+ cells within live cells were considered. The percentage of propodium iodide-staining target cells was determined from total CTV+ cells.

**RNA Sequencing**

Ly49Hlo and Ly49Hhi NK cells were sorted from the spleens of WT mice 1.5 days post MCMV infection. Spleens from three infected mice were pooled to achieve the necessary cell numbers. RNA from cells suspended in Trizol was extracted with chloroform. Isopropanol and linear acrylamide were added, and the RNA was precipitated with 75% ethanol. Samples were resuspended in RNase-free water. After Ribogreen quantification and quality control by Agilent BioAnalyzer, 2ng total RNA with RNA integrity numbers ranging from 8.1 to 10 underwent amplification using the SMART-seq v4 Ultra Low Input RNA Kit (Clontech catalog # 63488), with 12 cycles of PCR. Subsequently, 10ng of amplified cDNA were used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) using 8 cycles of PCR. Samples were barcoded and run on a HiSeq 4000 in a 50bp/50bp paired end run, using the HiSeq 3000/4000 SBS Kit (Illumina). An average of 38 million paired reads were generated per sample and the percent of mRNA bases per sample ranged from 79% to 81%.

**Quantification and Statistical Analysis**

**RNA Sequencing Analysis**

Paired-end reads were trimmed for adapters and removal of low-quality reads using Trimmomatic (v0.36) (Bolger et al., 2014). Trimmed reads were mapped to the Mus musculus genome (mm10 assembly) and counted at transcript-level using quasi-mapping approach by Salmon (v0.10.2) (Patro et al., 2017). These transcript-level estimates were then summarized at gene-level using tximport (v1.9.12) (Soneson et al., 2015). Differential expression (DE) analysis was executed with DESeq2 (v1.12.17) (Love et al., 2014) using UCSC knownGene model as a reference annotation. Genes were considered to be differentially expressed between two groups (Ly49Hlo versus Ly49Hhi) if their adjusted p values were less than 0.05. Gene Ontology (GO) analysis was performed using goseq (v1.33.0) (Young et al., 2010) on KEGG pathway database (KEGG.db v3.2.3) with a cutoff of log₂ fold change > 0 or < 0 for DE upregulated or downregulated genes, respectively.

**Statistical Analysis**

For graphs, data are shown as mean ± SEM, and unless otherwise indicated, statistical differences were evaluated using an unpaired, two-tailed Student’s t test, assuming equal sample variance. For experiments in which Ly49Hlo and Ly49Hhi NK cells were compared within the same mouse, a paired, two-tailed t test was used. Statistical differences in survival were determined by the Log-rank test.
(Mantel-Cox) test. \( p < 0.05 \) was considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the RNA-seq data reported in this paper is GEO: GSE129490.