Cytomegalovirus generates long-lived antigen–specific NK cells with diminished bystander activation to heterologous infection

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Natural killer (NK) cells play a key role in the host response to cytomegalovirus (CMV) and can mediate an enhanced response to secondary challenge with CMV. We assessed the ability of mouse CMV (MCMV)–induced memory Ly49H+ NK cells to respond to challenges with influenza, an acute viral infection localized to the lung, and *Listeria monocytogenes*, a systemic bacterial infection. MCMV-memory NK cells did not display enhanced activation or proliferation after infection with influenza or Listeria, as compared with naive Ly49H+ or Ly49H− NK cells. Memory NK cells also showed impaired activation compared with naive cells when challenged with a mutant MCMV lacking m157, highlighting their antigen–specific response. Ex vivo, MCMV-memory NK cells displayed reduced phosphorylation of STAT4 and STAT1 in response to stimulation by IL-12 and type I interferon (IFN), respectively, and IFN–γ production was reduced in response to IL-12 + IL-18 compared with naive NK cells. However, costimulation of MCMV-memory NK cells with IL-12 and m157 antigen rescues their impaired response compared with cytokines alone. These findings reveal that MCMV–primed memory NK cells are diminished in their response to cytokine–driven bystander responses to heterologous infections as they become specialized and antigen–specific for the control of MCMV upon rechallenge.

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Abbreviation used: BAL, bronchial alveolar lavage.

Persistent infections by viruses of the herpesvirus family, such as CMV, EBV, or HSV are extremely common in the human population, with prevalence rates of ∼60–90% (Virgin et al., 2009). Although in healthy individuals herpesvirus replication is efficiently controlled during the acute phase of infection, a clinically silent latent phase is established for the lifetime of the host and represents a carefully controlled balance of viral and host mechanisms. However, viral reactivation can result from immune suppression, such as in AIDS patients and transplant patients treated with immunosuppressive drugs, and leads to clinical pathology (Sinclair and Sissons, 2006). The impact of the latent viral pool on the immune system and subsequent response to other infections remain poorly understood (Virgin et al., 2009; Dreyfus, 2013). NK cells are important early responder cells in the immune response against infection, particularly against herpesviruses (Mossman and Ashkar, 2005); this is particularly evident in humans with primary NK cell deficiencies who often present with uncontrolled herpesvirus infections (Orange, 2002). Previous work has suggested

that latent herpesvirus infections can create an enhanced immune environment against challenge with other pathogens and may result in an increased NK cell response to heterologous infection (White et al., 2010).

Upon encountering infected cells, NK cells rapidly secrete cytokines and release cytotoxic granules; moreover, they play a key role in antibody-dependent cell-mediated cytotoxicity (Lodoen and Lanier, 2006). Studies have also identified an additional role for NK cells in the regulation of virus-specific T cell responses (Waggoner et al., 2012). NK cells are traditionally classified as innate immune lymphocytes, but recent work has revealed that these cells share more features with T and B lymphocytes than previously appreciated, including the ability to acquire immunological memory (Paust and von Andrian, 2011; Min-Oo et al., 2013). The memory-like properties of NK cells include antigen–specific recall response to haptens and

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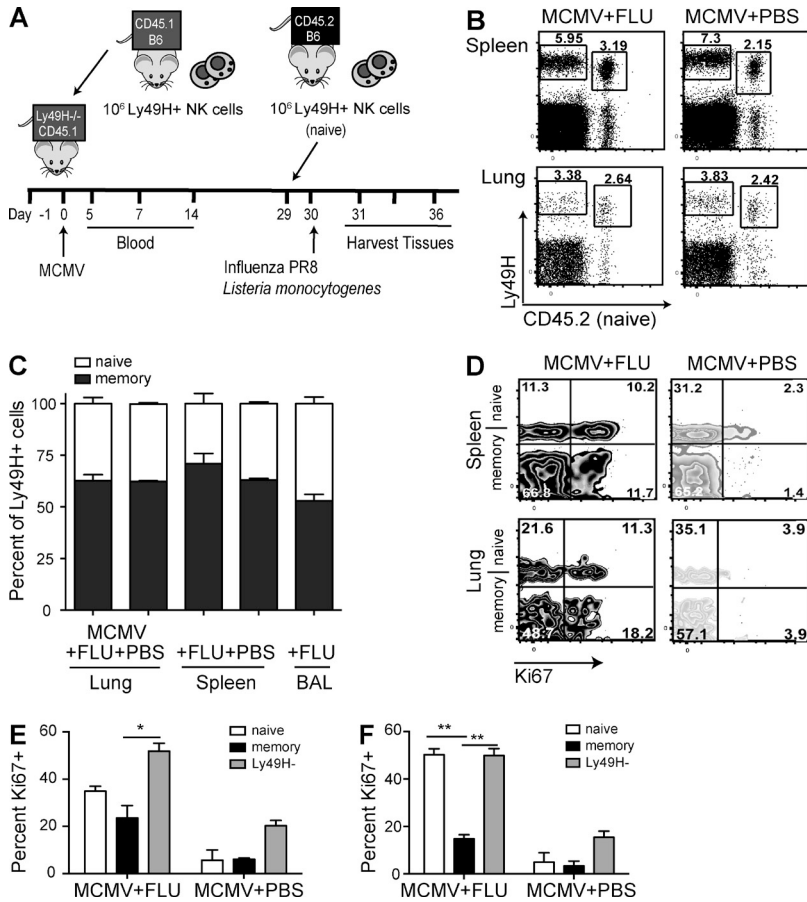www.jem.org/cgi/doi/10.1084/jem.20141172

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Figure 1. MCMV–memory NK cells are not recruited preferentially to the site of influenza infection and show reduced proliferation compared with adoptively transferred naive Ly49H⁺ NK cells. MCMV-memory NK cells were generated by adoptive transfer of 10^6 naive Ly49H⁺ NK cells into Ly49H-deficient hosts and infection with MCMV. At day 29 after infection, 10^5 CD45-congenic naive Ly49H⁺ NK cells were adoptively transferred into these Ly49H-deficient mice and then mice were infected intranasally with 50 PFU PR8 influenza virus on day 30. (A) Schematic representation of the strategy used to directly compare the response of naive and MCMV-memory Ly49H⁺ NK cells to heterologous viral or bacterial infection. (B) Transferred naive (CD45.2) and memory (CD45.1) Ly49H⁺ NK cells in the spleen and lung were analyzed by flow cytometry at day 5 after infection with influenza virus. Cells were gated on total NK cells (NK1.1⁺TCR-β⁻). (C) The ratio of naive to MCMV-memory Ly49H⁺ NK cells was calculated and is shown as a bar graph in the spleen, lung, and BAL at day 5 after infection with influenza virus. (D) Representative flow cytometry plots show Ki67 expression by naive (CD45.2⁺) and memory (CD45.1⁺) Ly49H⁺ NK cells in flu-infected and uninfected (PBS) mice. (E and F) The percentages of Ki67⁺ Ly49H⁺ and Ki67⁺ Ly49H⁻ NK cells in the lung (E) and spleen (F) on day 5 after influenza virus infection are displayed. For all panels, n = 3–4 mice, data shown are the mean ± the SEM. MCMV and influenza infection data are representative of three independent experiments. **, $P < 0.005$; *, $P < 0.05$.

virus–like particles (Paust et al., 2010), cytokine–induced memory (Cooper et al., 2009; Romee et al., 2012), and enhanced secondary response to MCMV (Sun et al., 2009). In humans, the existence of memory NK cells has been suggested by the presence of an expanded and persistent population of NK cells bearing the NKG2C receptor after CMV infection (Gumá et al., 2004; Lopez-Vergès et al., 2011).

MCMV infection leads to the production of numerous cytokines, including type I IFNs and IL-12, which trigger cytokine-induced activation of NK cells and leads to the production of IFN-γ (Orange and Biron, 1996a). NK cells in C57BL/6 mice also possess an activating receptor, Ly49H, that specifically recognizes infected cells expressing the MCMV-encoded protein m157 (Arase et al., 2002; Smith et al., 2002) and mediates a protective response against MCMV (Brown et al., 2001; Lee et al., 2001). After early non–antigen-specific activation by cytokines, antigen-specific Ly49H⁺ NK cells undergo robust expansion after encountering m157 (Dokun et al., 2001) and generate a population of long-lived memory NK cells (Sun et al., 2009). Thus, the NK response to acute MCMV infection is governed by a combination of cytokine signals and antigen-specific activation through Ly49H. Although the mechanisms governing the expansion, contraction, and survival of MCMV-memory NK cells are still being defined, recent work has shown a role for inflammatory cytokine signaling and miRNA155 in the expansion of Ly49H⁺ NK cells and subsequent memory

formation (Sun et al., 2012; Zawislak et al., 2013). Similarly, expansion and memory formation after MCMV is dependent on signaling through the activating receptor DNAM-1 (Nabekura et al., 2014), whereas pro-apoptotic pathways regulate contraction and the size of the memory pool (Min-Oo et al., 2014). Long-lived memory NK cells show an enhanced response to rechallenge with MCMV, as demonstrated by their ability to reduce viral loads more efficiently than naive NK cells and to protect neonates from lethal MCMV infection better than naive NK cells (Sun et al., 2009; Nabekura et al., 2014) despite a similar capacity of naive Ly49H⁺ NK cells for early expansion. Furthermore, in vitro, MCMV-memory NK cells show an enhanced response to antibody stimulation through Ly49H, compared with naive Ly49H⁺ NK cells (Sun et al., 2009). The antigen specificity of memory NK cells responding to rechallenge with haptens has also been shown in a contact hypersensitivity model (Paust et al., 2010). Whether MCMV-memory NK cells are generally superior to naive Ly49H⁺ cells and, as such, offer cross-protection against heterologous pathogens or, alternatively, mediate enhanced response solely via MCMV-specific stimulation has not been addressed.

Intranasal administration of influenza to mice results in an acute infection in which viral replication is limited exclusively to the lung airways (Thangavel and Bouvier, 2014). Influenza infection in the airway induces a strong chemokine and cytokine response, including secretion of chemotactic proteins

(RANTES, MCP-1, and MIP1α) and proinflammatory factors (IL-1β, IL-6, and TNF), as well as IFN-α and IFN-β (Julkunen et al., 2001). As a result, infection by influenza virus, particularly of the subtype H1N1, causes a potent systemic immune response that can lead to an uncontrolled cytokine storm and subsequent fatal immune–mediated pathology (Oldstone et al., 2013). Limiting the intensity of the cytokine and chemokine production by the innate immune system ensures a strong antiviral cytotoxic T cell and neutralizing antibody response, while increasing survival (McGill et al., 2009). Pathology and mortality caused by H1N1 infection in humans and animals is associated with higher levels of cytokines rather than differences in viral load (Arankalle et al., 2010; Marcelin et al., 2011). The role of NK cells in response to influenza virus remains controversial; depending on the strain and dose of influenza, NK cells have been shown to have detrimental, beneficial, or no effect on the outcome (Jost and Altfeld, 2013). Intravenous infection of mice with *Listeria monocytogenes*, a Gram-positive bacterium, results in bacterial replication in the spleen and liver, which are also sites of MCMV replication during acute infection. Listeria, similarly to influenza, induces a strong systemic inflammatory response driven by the innate immune system (Pamer, 2004; Serbina et al., 2012). Here, we investigated the response of MCMV-memory NK cells when challenged with these two distinct heterologous infections, influenza virus and Listeria, as well as MCMV lacking the m157 antigen.

RESULTS

Response of MCMV–memory NK cells to influenza virus infection

Naive Ly49H^{+} NK cells were adoptively transferred into syngeneic Ly49H-deficient C57BL/6 recipient mice, and we infected mice with MCMV as previously described (Sun et al.,

2009). We tracked Ly49H^{+} NK cell expansion in peripheral blood after transfer until day 28 after infection. After expansion and contraction, transferred Ly49H^{+} NK cells displayed the phenotype associated with MCMV-memory NK cells (Sun et al., 2009; Bezman et al., 2012), with >95% expressing high levels of CD11b, KLRG1, and Ly6C and loss of CD27 (unpublished data). Intranasal infection with lethal H1N1 PR8 influenza can result in high variability between individual animals, with respect to viral load and cytokine response (unpublished data). Therefore, we devised a strategy to compare the response of MCMV-memory Ly49H^{+} cells directly to naive Ly49H^{+} cells within the same animal, persistently infected with MCMV. We transferred 10[6] Ly49H^{+} NK cells into Ly49H-deficient C57BL/6 mice and infected the mice with MCMV to generate a population of Ly49H^{+} memory NK cells (Fig. 1 A). 29 d after infection with MCMV, we transferred 10[5] CD45 congenically marked naive Ly49H^{+} NK cells into these mice containing the memory Ly49H^{+} NK cells and infected the mice intranasally with 50 PFU of the virulent H1N1 PR8 influenza virus. This allowed us to directly compare MCMV-memory NK cells that were expanded in response to MCMV infection a month earlier with naive Ly49H^{+} NK cells upon primary infection with influenza virus while controlling the viral load and cytokine environment experienced by the naive and memory NK cells. We analyzed lymphocytes from bronchial alveolar lavage (BAL), lung, and spleen at day 5 after influenza infection, when the influx of NK cells into the lung peaks (unpublished data). MCMV-memory (CD45.1) and naive (CD45.2) Ly49H^{+} NK cells were easily detected in uninfected (MCMV+PBS) and influenza-infected (MCMV+FLU) mice, with higher numbers in the spleen than lung (Fig. 1 B). The ratio of MCMV-memory to naive Ly49H^{+} NK cells did not skew significantly
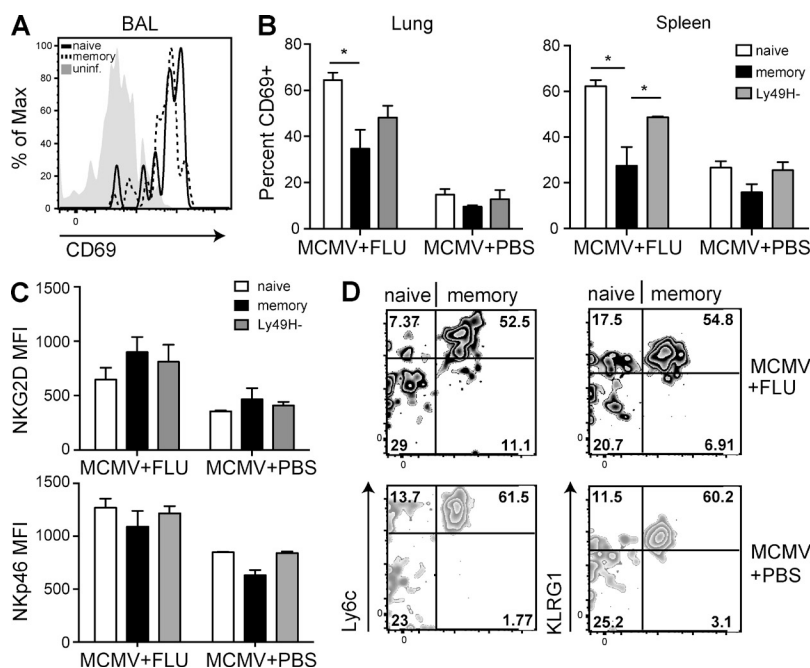

Figure 2. Activation of MCMV–memory NK cells is reduced compared with naive Ly49H^{+} NK cells during influenza infection. MCMV-memory NK cells were generated as described in Fig. 1. At day 29 after infection, 10[5] naive Ly49H^{+} NK cells were transferred into these hosts and mice were infected with 50 PFU of PR8 influenza virus. NK cell populations were analyzed at day 5 after infection with influenza. (A) A representative histogram showing CD69 expression on memory and naive Ly49H^{+} NK cells in the BAL; uninfected splenic NK cells are shown as a control. (B) The percentages of CD69^{+} NK cells in the MCMV-memory and naive Ly49H^{+} cells and endogenous Ly49H^{−} NK cells are presented for lung and spleen. (C) The expression of NKG2D and NKp46 was assessed in MCMV-memory and naive Ly49H^{+} and endogenous Ly49H^{−} NK cells in the lung and graphed as median fluorescence intensity (MFI). (D) The expression of Ly6C and KLRG1 on memory (CD45.1[+]) and naive (CD45.1[−]) Ly49H^{+} NK cells in flu-infected and uninfected mice are shown in representative flow cytometry plots. For all panels, n = 3–4 mice and data shown are the mean ± the SEM. MCMV plus influenza infection data are representative of three independent experiments. *, P < 0.05.

after PR8 infection, in either the lung or spleen or in the BAL, the site of initial viral contact (Fig. 1 C). This demonstrates that memory NK cells did not preferentially migrate to the site of viral replication, nor were MCMV-memory NK cells expanding more rapidly than naive cells after influenza infection. To directly assess proliferation of MCMV-memory (CD45.2$^-$) versus naive (CD45.2$^+$) Ly49H$^+$ NK cells in response to influenza, we examined the frequency of NK cells expressing Ki67 at day 5 after infection. We observed increased percentages of Ki67$^+$ NK cells in PR8-infected versus uninfected mice (Fig. 1 D). Of note, the percentage of Ki67$^+$ NK cells was higher in the adoptively transferred naive Ly49H$^+$ NK cell subset in the lungs of infected mice (Fig. 1 E), and similarly significantly higher in the adoptively transferred naive Ly49H$^+$ NK cell subset in the spleen, compared with the MCMV-memory NK cell subset (Fig. 1 F). Moreover, the frequencies of both MCMV-memory and naive Ly49H$^+$ NK cells expressing Ki67 were higher or equivalent in the spleen compared with the lungs. This suggests that proliferation of the adoptively transferred naive Ly49H$^+$ NK cells is more dramatic at tissues distal to the site of infection, and that MCMV-memory NK cells are responding poorly to influenza compared with naive Ly49H$^+$ NK cells. Endogenous Ly49H$^-$ NK cells showed similar or slightly higher percentages of Ki67-expressing NK cells compared with the adoptively transferred naive Ly49H$^+$ NK cells (Fig. 1, E and F). The higher frequency of endogenous NK cells expressing Ki67 might be due to recruitment of newly generated NK cells from the bone marrow (unpublished data).

MCMV–memory NK cells are activated after influenza infection but show a reduced response to cytokines

We examined the expression of CD69 on MCMV-memory and naive Ly49H$^+$ NK cells in tissues at day 5 after infection with influenza. All of the NK cells detected in the BAL expressed CD69 (Fig. 2 A), indicating that the MCMV-memory and naive NK cells present in the airway were similarly activated. However, in the lung, a significantly higher percentage of the naive NK cell subset was CD69$^+$, compared with MCMV-memory NK cells (Fig. 2 B), after influenza infection. Surprisingly, this was also observed in the spleen, suggesting that activation was not dependent on interaction with influenza-infected cells (which are restricted to the lung) but is likely driven by systemic cytokines. Endogenous Ly49H$^-$ NK cells showed a similar level of activation as the adoptively transferred naive Ly49H$^+$ NK cells (Fig. 2 B). We examined the expression of cell surface markers associated with MCMV-induced activation and MCMV-memory, including KLRG1 and Ly6C, before and after influenza infection (Fig. 2 D), as well as activating receptors implicated in response to influenza-infected cells (Draghi et al., 2007; Mendelson et al., 2010), for example, NKG2D and NKp46 (Fig. 2 C). MCMV-memory NK cells (CD45.1$^+$) clearly showed a distinct phenotype, compared with naive cells, before influenza infection, in all tissues analyzed. As reported previously (Bezman et al., 2012), they expressed high levels of KLRG1 and Ly6C compared
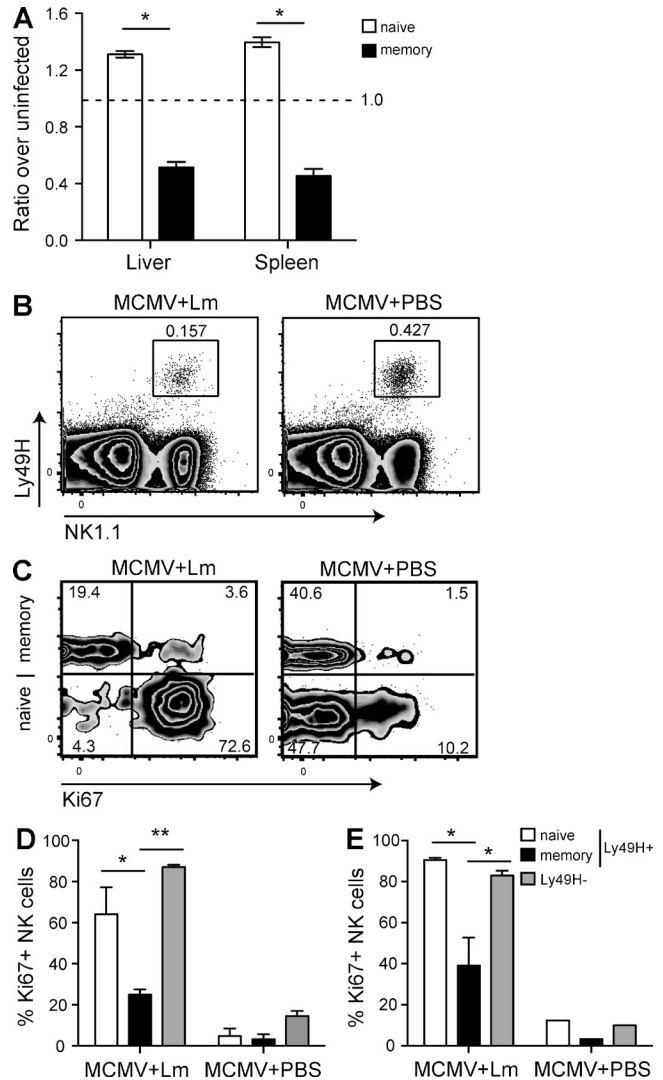

Figure 3. MCMV–memory NK cells proliferate less than naive Ly49H$^+$ NK cells after Listeria infection. MCMV-memory NK cells were generated as described in Fig. 1. 29 d after MCMV infection, 10^5 naive Ly49H$^+$ NK cells were transferred into these hosts, who were then infected with 5 × 10^3 CFU *L. monocytogenes*. Ly49H$^+$ NK cells were analyzed at day 4 after infection with Listeria. (A) The graph shows the ratio of MCMV-memory or naive Ly49H$^+$ NK cells detected in Listeria-infected over memory and naive Ly49H$^+$ cells in uninfected tissues. (B) Representative flow cytometry plots gated on total lymphocytes show the percentages of Ly49H$^+$ NK cells in the spleen after Listeria. (C) The percentages of Ki67$^+$ cells are shown in representative flow cytometry plots for naive (CD45.2$^-$) and memory (CD45.2$^+$) Ly49H$^+$ NK cells in Listeria-infected and uninfected livers. (D and E) Bar graphs show Ki67$^+$ NK cells calculated for all mice, in spleen (D) and liver (E). Data for MCMV plus Listeria infection experiments are representative of three independent experiments. n = 3–4 mice for all panels, uninfected mice were pooled from independent experiments, and data shown are the mean ± the SEM. **, P < 0.005; *, P < 0.05.

with naive NK cells (Fig. 2 D). After influenza infection, the cell surface density of KLRG1 and Ly6C was not remarkably changed on either the MCMV-memory or adoptively transferred naive Ly49H$^+$ NK cells (Fig. 2 D), or endogenous
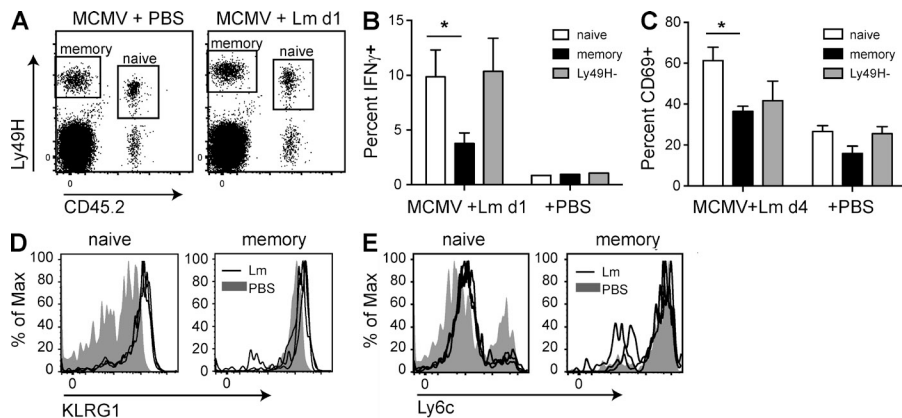
Figure 4. MCMV-memory NK cells show diminished functional responses and activation compared with naive NK cells after Listeria infection. MCMV-memory NK cells were generated as described in Fig. 1. 29 d after MCMV infection, 10^5 naive Ly49H+ NK cells were transferred into these hosts, mice were infected with 5×10^3 CFU of *L. monocytogenes*, and Ly49H+ NK cells were analyzed at days 1 and 4 after infection with Listeria. (A) Flow cytometry plots show the proportion of Ly49H+ naive and memory NK cells in the spleen 1 d after transfer and Listeria infection, gated on NK1.1+TCR-β− cells. (B) Intracellular IFN-γ was measured ex vivo by flow cytometry at day 1 after infection in spleen with Listeria in MCMV-memory and naive Ly49H+ NK cells and endogenous Ly49H− NK cells. (C) Percentages of CD69+ MCMV-memory and naive Ly49H+ NK cells or endogenous CD69+Ly49H− NK cells were assessed by flow cytometry at day 4 after infection with Listeria; results are presented in a bar graph. (D and E) Representative histogram plots show the expression of KLRG1 (D) and Ly6C (E) on MCMV-memory and naive Ly49H+ NK cells after Listeria infection (day 4) compared with uninfected mice. Data for MCMV plus Listeria experiments are representative of three independent experiments. n = 4 mice for all panels, uninfected mice were pooled from independent experiment and data shown is the mean ± the SEM. *, P < 0.05.

Ly49H− cells (our unpublished data). Expression of the activating NKG2D and NKp46 receptors was slightly increased in the lungs, but to a similar extent in naive and MCMV-memory NK cells (Fig. 2 C). These results indicate that the expression of markers associated with MCMV-memory was not impacted significantly by influenza infection.

Response of MCMV-memory NK cells to Listeria infection

As influenza viral replication is strictly limited to the lungs and airway, we examined a second model, systemic infection by *L. monocytogenes*, during which bacterial replication occurs in the spleen and liver, similar to the sites of MCMV infection. We generated MCMV-memory Ly49H+ NK cells by adoptive transfer into Ly49H-deficient recipient mice and infection with MCMV; subsequently, we transferred 10^5 naive Ly49H+ NK cells, congenically marked, into these hosts and infected the mice with 5×10^3 CFU *L. monocytogenes*, intravenously, to allow for direct comparison of the naive and memory Ly49H+ NK cells within the same animal (schematically outlined in Fig. 1 A). Memory and naive Ly49H+ NK cells were found at a 40:60 ratio before infection in spleen and liver (Fig. 3 A); however, naive Ly49H+ NK cells increased in frequency to constitute ∼80% of the transferred Ly49H+ NK cells by day 4 after infection with Listeria, such that memory NK cells in infected mice were present at only ∼50% of the level found in uninfected controls (Fig. 3 A). This suggested that naive Ly49H+ NK cells were either proliferating at an increased rate, compared with memory cells, or MCMV-memory NK cells are selectively being lost. Listeria, particularly when injected at high doses, induces apoptosis of lymphocytes (Carrero and Unanue, 2012). We observed a loss of NK cells after a high-dose infection (10^4 CFU), with a dramatic loss of MCMV-memory NK cells (our unpublished data). Therefore, we infected with a lower dose of Listeria (5×10^3 CFU) and detected increased total numbers of NK cells in target tissues, despite a decreased

percentage of Ly49H+ NK cells (Fig. 3 B). This finding suggests the possibility of a greater degree of apoptosis of MCMV-memory NK cells, compared with endogenous Ly49H− cells. Nevertheless, we assessed the proliferative response of MCMV-memory (CD45.2+, in this experiment) Ly49H+ NK cells compared with naive (CD45.2−) Ly49H+ NK cells by staining for Ki67 (Fig. 3 C). Naive NK cells proliferated to a significantly greater extent (∼70% Ki67+) compared with MCMV-memory NK cells (∼20% Ki67+) after Listeria infection, in the spleen (Fig. 3, C and D) and liver (Fig. 3 E). Adoptively transferred naive Ly49H+ NK cells showed similar levels of basal Ki67 staining in uninfected control mice, indicating that they had not undergone homeostatic proliferation as a result of adoptive transfer. In all tissues, proliferation of naive Ly49H+ NK cells after Listeria was similar to levels observed in endogenous Ly49H− NK cells (Fig. 3, D and E). These results indicate that MCMV-memory NK cells have a decreased capacity for early proliferation after cytokine stimulation induced by systemic Listeria infection, in contrast with the robust antigen-driven proliferation of MCMV-memory NK cells, when rechallenged with MCMV (Sun et al., 2009; Nabekura et al., 2014).

MCMV-memory NK cells mount a diminished functional response after Listeria infection

To assess differential NK cell activation and function in naive versus MCMV-memory NK cells in response to Listeria infection, we analyzed the expression of CD69 at day 4 after infection and the in vivo production of IFN-γ at 24 h after infection. We readily detected MCMV-memory and adoptively transferred naive Ly49H+ NK cells in the spleen in both uninfected and infected mice (Fig. 4 A) at this early time point, before any significant proliferation or loss through apoptosis. The percentage of NK cells expressing IFN-γ, directly ex vivo, was significantly higher in the naive subset of Ly49H+ cells than in the MCMV-memory subset, comparable
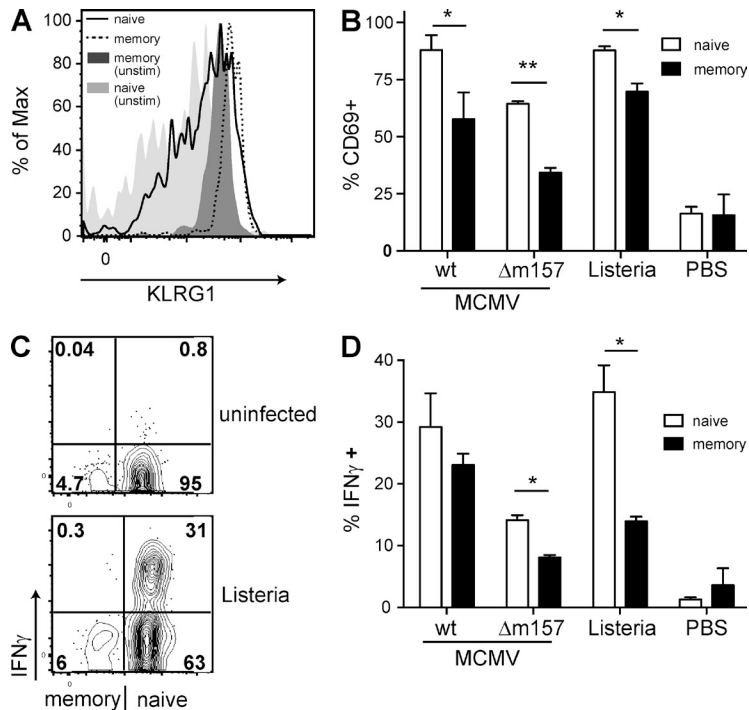
Figure 5.   MCMV–memory Ly49H+ NK cells show impaired functional response to MCMVΔm157 and Listeria in a secondary cotransfer model compared with naive cells. MCMV-memory NK cells were generated as described in Fig. 1. 28 d after MCMV infection, memory NK cells were isolated, mixed with congenically marked 10^5 naive Ly49H+ NK cells, and transferred into naive Ly49H-deficient hosts. After transfer, secondary recipient mice were infected with 10^5 PFU of WT MCMV or MCMVΔm157 or 5×10^3 CFU Listeria. (A) KLRG1 expression was assessed on splenic MCMV-memory and naive Ly49H+ NK cells after MCMVΔm157 challenge or in uninfected controls. (B) Percentages of CD69+ MCMV-memory and naive Ly49H+ NK cells were assessed by flow cytometry in the spleen at 36 h after infection with either WT MCMV or MCMVΔm157, 24 h after infection with Listeria, or in uninfected control mice (PBS). (C) Representative flow cytometry plots show percentages of IFN-γ+ MCMV-memory or naive Ly49H+ NK cells in the spleen, 24 h after infection with Listeria or in uninfected controls. (D) Percentages of IFN-γ+ Ly49H+ naive or MCMV-memory NK cells after infection with MCMV WT, MCMVΔm157, or Listeria are plotted as bar graphs. Data are representative of two independent experiments and $n = 3$ mice per group. Data shows the mean ± the SEM. **, $P < 0.005$; *, $P < 0.05$.

to endogenous naive Ly49H− NK cells in the spleen (Fig. 4 B). We assessed the activation of NK cells at day 4 after infection with Listeria to determine whether MCMV-memory NK cells were activated later than naive Ly49H+ NK cells; however, CD69 expression at day 4 was reduced in Ly49H+ memory NK cells (Fig. 4 C). This suggests that MCMV-memory NK cells are not activated by cytokines to the same extent as naive Ly49H+ NK cells after exposure to Listeria. The activation marker KLRG1 is stably expressed at high cell surface density on MCMV-memory NK cells (Sun et al., 2009; Bezman et al., 2012), and infection with Listeria also induced high amounts of KLRG1 on the naive NK cells (Fig. 4 D). However, expression of Ly6C, another marker of MCMV-induced memory NK cells, was not increased after Listeria infection in either subset (Fig. 4 E).

MCMV–memory NK cells show impaired functional response to MCMVΔm157 and Listeria in a secondary cotransfer model

Due to the possibility that persistent MCMV infection in the host animals or the differences in timing of transfer between naive and memory NK cells in our model might influence functional responses, we used a secondary cotransfer of naive and MCMV-memory NK cells into completely naive recipients. We initially generated MCMV-memory Ly49H+ NK cells by adoptive transfer into Ly49H-deficient recipient mice and infection with MCMV; subsequently, at day 28 after infection, we isolated MCMV-memory Ly49H+ NK cells from these mice and mixed them with congenically marked naive Ly49H+ NK cells. Mixed memory and naive Ly49H+ NK cells were transferred into naive Ly49H-deficient recipients and these hosts were infected with 10^5 PFU of a mutant MCMV

strain lacking m157 (MCMVΔm157) or WT MCMV, or 5×10^3 CFU *L. monocytogenes*. At 36 h after MCMV infection or 24 h after Listeria infection, when the NK cell response is induced primarily by cytokines, we analyzed the transferred Ly49H+ cells by flow cytometry. We initially assessed KLRG1 expression, a marker of activation and memory on NK cells, in uninfected mice and confirmed high expression of KLRG1 on the transferred memory NK cells but not naive NK cells. KLRG1 expression was further unregulated after MCMV Δm157 challenge (Fig. 5 A) or Listeria infection (not depicted). This suggests that KLRG1hi expression is not dependent on persistent MCMV infection or reactivation in the host. MCMV-memory NK cells showed significantly reduced CD69 expression compared with naive NK cells in the same animal after challenge with MCMVΔm157 or Listeria (Fig. 5 B). A similar, but less significant, difference was seen 36 h after WT MCMV challenge, which also primarily activates NK cells via cytokines at this time point. Importantly, we assessed functional response by measuring IFN-γ production in vivo after MCMV or Listeria infection (Fig. 5, C and D). MCMVΔm157 induced a greater functional response in naive NK cells than MCMV-memory NK cells, whereas WT MCMV infection induced a response in both memory and naive NK cells (Fig. 5 D). A more striking impairment of function was seen in MCMV-memory NK cells after infection with Listeria, where IFN-γ production by MCMV-memory Ly49H+ NK cells was significantly reduced compared with the naive Ly49H+ NK cell subset (Fig. 5, C and D). These data validate our initial findings in the persistently infected mice (Figs. 3 and 4) and suggest that the impaired response of MCMV-memory NK cells to cytokines produced in vivo early during infection
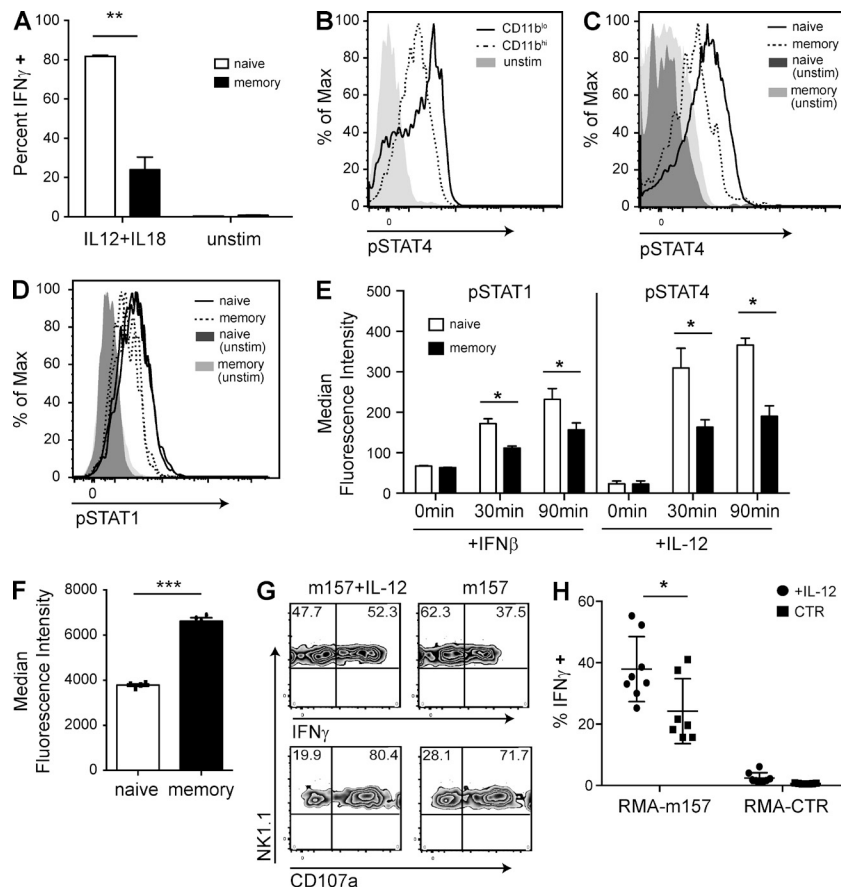
Figure 6.   MCMV–memory NK cells respond poorly to cytokine stimulation alone in vitro but show an enhanced response after costimulation by m157. MCMV-memory NK cells were generated as described in Fig. 1. (A) MCMV-memory and naive Ly49H+ NK cells were cultured ex vivo with IL-12 + IL-18 or without cytokines for 6 h and the percentages of IFN-γ+ Ly49H+ NK cells were assessed by flow cytometry. The results are presented as a bar graph. (B) A histogram plot shows levels of pSTAT4 in CD11bhi and CD11blo naive NK cells compared with unstimulated naive NK cells after 10 min stimulation with IL-12. (C) MCMV-memory and naive Ly49H+ NK cells were co-cultured ex vivo with IL-12 for 15 min and levels of pSTAT4 were assessed by flow cytometry and plotted as histograms; unstimulated MCMV-memory and naive Ly49H+ NK cells are shown as controls. (D) MCMV-memory and naive Ly49H+ NK cells were co-cultured ex vivo with IFN-β for 90 min and pSTAT1 levels were assessed by flow cytometry and plotted as histograms; unstimulated Ly49H+ cells are shown as controls. (E) The median fluorescence intensity (MFI) of pSTAT1 and pSTAT4 was assessed in MCMV-memory and naive NK cells after 30 or 90 min of stimulation by IFN-β or IL-12, respectively, and is summarized as a bar graph. (F) The MFI of Ly49H expressed on cotransferred MCMV-memory cells (dashed line) and naive Ly49H+ cells (solid line), in vivo, is shown as a representative histogram plot and summarized in a bar graph. MCMV-memory NK cells harvested at day 28 after infection were co-cultured with RMA cells expressing m157 or control RMA cells for 6 h, with or without 100 ng/ml of IL-12; IFN-γ and CD107a were assessed by flow cytometry. (G) Representative flow cytometry plots showing the proportion of IFN-γ+ and CD107a+ Ly49H+ memory NK cells after stimulation with m157-expressing RMA cells, with or without IL-12. (H) The percentages of IFNγ+Ly49H+ NK cells for each condition are shown in a bar graph. Data are representative of 2 independent experiments and n = 3–4 mice per group. Statistical comparisons were performed using a paired Student's t test, or Mann-Whitney testing. Data shows the mean ± the SEM. **, P < 0.005; *, P < 0.05.

is neither dependent on persistent MCMV in the host nor due to adoptive transfer of the NK cells. Furthermore, these data indicate the diminished response of MCMV-memory NK cells during infection with MCMV Δm157 is caused by the lack of recognition of m157, whereas challenge with WT MCMV induced a similar early functional response of memory and naive NK cells (being driven by both cytokines and antigen). The enhanced function of memory NK cells after WT MCMV challenge prevails later, during the m157–driven proliferative phase of infection when cytokine levels have declined, and results in host protection and survival (Sun et al., 2009; Nabekura et al., 2014).

MCMV–memory NK cells show diminished signaling downstream of cytokine receptors after stimulation ex vivo

Intranasal influenza infection and intravenous infection with Listeria, as well as MCMV infection, induce strong early systemic cytokine responses, including type I IFNs that induce the expression of IL-12 and -18 (Orange and Biron, 1996b; Mocci et al., 1997; Julkunen et al., 2001), which stimulate NK cells to produce IFN-γ. Expression data taken from the

ImmGen consortium revealed a reduction in the level of various cytokine receptor transcripts (including Ifnar1, Ifnar2, and IL12rb1) in sorted MCMV-memory compared with naive Ly49H+ cells (unpublished data). Therefore, we examined the functional response of memory and naive Ly49H+ NK cells to IL-12 + IL-18 stimulation in vitro. MCMV–memory NK cells produced significantly less IFN-γ than naive Ly49H+ NK cells after 6 h of stimulation (Fig. 6 A). As IL-12 receptor expression at the cell surface is difficult to examine by flow cytometry, we assessed downstream signaling of the IL-12 receptor by measuring intracellular levels of phosphorylated STAT4 (pSTAT4) in memory and naive NK cells. We initially determined an optimal dose and length of stimulation with IL-12 in vitro to assay pSTAT4 in naive splenic NK cells (our unpublished data). We observed significant induction of pSTAT4 within 10 min of stimulation with 20 ng/ml of IL-12. Interestingly, we noted that among the naive population, mature CD11bhi naive NK cells had decreased levels of pSTAT4 compared with more immature CD11blow NK cells (Fig. 6 B). Naive Ly49H+ CD45.1+ NK cells were mixed in the same well with MCMV-memory Ly49H+ CD45.2+ NK cells and stimulated with

20 ng/ml IL-12. After 15 min of stimulation, naive Ly49H^{+} NK cells showed higher levels of pSTAT4 than MCMV-memory Ly49H^{+} NK cells (Fig. 6 C). We similarly assessed the MCMV-memory cell response to type I IFN, IFN-β, by measuring phosphorylated STAT1 (pSTAT1) downstream of the type I IFN receptor (IFNAR) after 30 and 90 min of stimulation in vitro. Our results showed similar basal levels of pSTAT1 in MCMV-memory NK cells compared directly within a well with naive Ly49H^{+} cells, but importantly, MCMV-memory NK cells did not up-regulate pSTAT1 levels to the same extent as naive cells after 30 min (not depicted) or 90 min of IFN-β stimulation (Fig. 6 D). The decreased level of pSTAT1 and pSTAT4 in MCMV-memory NK cells compared with naive cells was significant across multiple biological replicates (P < 0.05, paired Student's *t* test; Fig. 6 E). We have not established whether the diminished cytokine-induced activation of MCMV-memory NK cells results from a reduction in cytokine receptor expression or potential changes in total levels of downstream signaling molecules, such as STAT4 and STAT1, as has been previously shown to be critical in NK cell response regulation (Miyagi et al., 2007). Nonetheless, these findings clearly validate our results showing reduced response of MCMV-memory NK cells after influenza or Listeria infection in vivo and suggest that this is due to diminished signaling downstream of the IL-12 and type I IFN receptors.

Infection by MCMV causes a potent cytokine response, including the secretion of type I IFNs, IL-12, and IL-18. In vivo, MCMV-memory NK cells challenged with WT MCMV responded similarly to naive NK cells at 36 h, likely due to the predominance of high levels of cytokines at that time point (Fig. 5). Thus, we assessed if MCMV-memory NK cells stimulated through Ly49H in vitro integrate simultaneous cytokine signals to increase their functional responses, as would be the case in vivo. Importantly, the cell surface density of Ly49H on MCMV-memory NK cells was significantly and consistently increased compared with naive NK cells when analyzed from the same mouse (cotransfer, Fig. 6 F). This suggests that persistent MCMV infection and a latent viral reservoir were not sufficient to induce the high level of expression of Ly49H on the adoptively transferred naive NK cells but likely resulted from the preferential expansion and/or survival during the differentiation of NK cells into the memory compartment. To assess whether MCMV-memory NK cells show enhanced responses to simultaneous cytokine and antigen signaling, we stimulated memory Ly49H^{+} cells in vitro with low levels of IL-12 and RMA target cells expressing m157, or control RMA cells lacking m157. IL-12 in combination with control RMA cells did not induce IFN-γ production above baseline (Fig. 6 H). In contrast, incubation with m157-expressing target cells alone was sufficient to induce IFN-γ in ~30% of MCMV-memory cells (Fig. 6, G and H) and cause strong degranulation (Fig. 6 G). Of note, when MCMV-memory NK cells were stimulated simultaneously with m157 and IL-12, they produced significantly more IFN-γ (P = 0.05, nonparametric Mann-Whitney test; Fig. 6, G and H) and were able to degranulate to a slightly greater extent than when stimulated

with m157 alone. Stimulation of the MCMV-memory NK cells with IL-12 in the absence of m157 failed to induce any IFN-γ production (Fig. 6 H). Collectively, our findings indicate that MCMV-memory NK cells are more responsive to an integration of signals from cytokines in the presence of m157 antigen while showing a dampened response to cytokines alone, as in the case of challenge with heterologous pathogens or MCMV lacking m157.

DISCUSSION

CMV establishes persistent infection in mice and humans; therefore, NK cells might have evolved specific receptors for CMV and the capacity to generate memory NK cells to work cooperatively with T and B cells to control CMV replication for the lifetime of the host. Because MCMV-memory NK cells demonstrate an enhanced effector response to rechallenge with MCMV, we addressed whether these MCMV-memory NK cells respond more robustly to heterologous infections, or alternatively become preferentially focused on the control of MCMV. Our findings reveal that long-lived MCMV-memory NK cells are in fact less responsive to heterologous infection with other viruses and bacteria than naive NK cells within the same host.

The antigen specificity of T cells is conferred by TCRs that are assembled by the somatic recombination of their receptor-encoding genes performed by the RAGs (Schatz, 2004). Upon encounter with antigen, T cells undergo clonal expansion and produce long-lived memory cells (Mueller et al., 2013). NK cell recognition and memory formation does not require RAG. In C57BL/6 mice, the NK cell response to MCMV is mediated by the germline-encoded Ly49H receptor, which is exquisitely specific for the MCMV-encoded m157 glycoprotein that is displayed on the surface of infected cells. Both the primary MCMV-specific NK cell response and recall response of MCMV-memory NK cells is strictly dependent on recognition of m157, a viral protein which is not shared by any other known pathogen (Arase et al., 2002; Smith et al., 2002). Recent studies by Paust et al. (2010) have described NK cells with enhanced recall responses to a variety of haptens, as well as virus-like particles carrying influenza and HIV antigens; these recall responses were also highly specific to the sensitizing antigen, but only a certain subset of CXCR6^{+} NK cells located in the liver possess the capacity to mediate these antigen-specific recall responses. As yet, the NK receptors responsible for these responses have not been identified, but there is no requirement for RAG, and without the identification of a specific receptor, it has not been possible to determine the behavior of these NK cells to heterologous infection.

In humans, NK cells expressing the activating CD94-NKG2C receptor are expanded in CMV-seropositive individuals (Gumá et al., 2004; Lopez-Vergès et al., 2011) and preferentially expand during acute CMV infection in hematopoietic stem cell transplant and immunosuppressed solid organ transplant patients (Lopez-Vergès et al., 2011; Foley et al., 2012). CMV-seronegative individuals possess a low frequency of NK cells expressing CD94-NKG2C at low cell

surface density; during acute CMV infection there is an expansion of NK cells expressing high cell surface density CD94-NKG2C and acquisition of the CD57 marker that is associated with NK cell maturation (Lopez-Vergès et al., 2011; Foley et al., 2012). We have recently examined the response of these NKG2C⁺ NK cells in CMV-seronegative and –seropositive university students during acute infectious mononucleosis caused by Epstein-Barr virus. There was no evidence for expansion of NKG2C⁺ NK cells in these individuals during acute EBV infection, although other alterations in the NK cell population were observed during and after EBV infection (Hendricks et al., 2014). An expansion of NKG2C⁺ NK cells has been noted in individuals during acute infection with Hantavirus infection (Björkström et al., 2011); however, this only occurred in CMV-seropositive patients, suggesting that the expansion might be due to subclinical CMV reactivation. Thus, similar to the behavior of MCMV-memory NK cells in mice, the NKG2C⁺ NK cells in humans appear specific for CMV and unresponsive to an acute heterologous herpesvirus infection.

Francois et al. (2013) examined the impact of MCMV persistence on retroviral infection, focusing on the role of NK cells. They demonstrated that MCMV infection enhanced NK cell responses to Friend virus infection and implicated NK cells in improving the antiviral T cell responses. They concluded that this enhanced response was attributable to the Ly49H⁺ NK cell subset (Francois et al., 2013) but did not discriminate between the role of MCMV-memory and naive Ly49H⁺ NK cells per se. Similarly, in a mouse model of latent MuHV4 infection, White et al. (2010) reported enhanced NK cell responses with respect to granzyme B levels, IFN-γ production, and cytotoxicity against tumor target cells. These findings are reminiscent of the cytokine-induced memory-like NK cell described by Cooper et al. (2009). NK cells that were cultured in vitro with IL-15, IL-12, and IL-18 and then adoptively transferred into naive mice demonstrated enhanced cytokine production, but not cytolytic activity, when restimulated ex vivo a few weeks later. Similarly, Chaix et al. (2008) have demonstrated that for optimal production of IFN-γ, NK cells need to be primed by IL-18. For many years, it has been common practice to inject naive mice with poly I:C (which induces type I IFNs and IL-15) before harvesting NK cells for in vitro functional studies to obtain robust responses. Thus, it appears that there is a fundamental difference in NK cells that have been primed by the cytokine environment and those that have undergone expansion induced by cognate recognition of ligand by an ITAM-based activating NK cell receptor such as Ly49H. The m157-driven proliferative response has recently been shown to be dependent on the Zbtb32 transcription factor in MCMV-specific NK cells (Beaulieu et al., 2014).

The enhanced response of MCMV-memory NK cells is exquisitely antigen-dependent in that these MCMV-memory NK cells fail to mount an elevated response when challenged with MCMV lacking the m157 antigen. Thus, although cytokines, including IL-12, are clearly required for the m157-induced expansion of Ly49H⁺ NK cells and the generation of long-lived memory NK cells (Sun et al., 2012), the enhanced

functional response of MCMV-memory NK cells becomes more stringently regulated and antigen-specific, as the MCMV-memory NK cells become limited in their responsiveness to cytokines alone.

Previous studies have focused on the impact of MCMV persistence on the overall response to a secondary superinfection. Persistent infection by either MCMV or gamma-herpesvirus 68 was shown to protect the host against subsequent lethal infection by bacteria; this was linked to increased IFN-γ secretion and activation of macrophages (Barton et al., 2007). In contrast, MCMV persistence did not confer protection against West Nile virus, LCMV, or influenza virus (Cicin-Sain et al., 2012; Mekker et al., 2012) but altered the CD8⁺ T cell responses to the heterologous pathogen. Here, although we focused on the specific response of MCMV-memory NK cells in direct comparison with naive Ly49H⁺ NK cells, we also observed an enhanced clearance of Listeria bacterial load in mice that were persistently infected with MCMV (unpublished data), whereas overall the response to influenza virus was slightly impaired (unpublished data). However, MCMV-memory NK cells were not responsible for these differences in the overall host responses.

Encounter with heterologous infectious agents by the host has been shown to induce contraction of preexisting memory T cell populations (Selin et al., 1996). Moreover, recently it has been shown that LPS administration during latent infection can also induce CMV-specific T cell contraction (Campbell et al., 2012), thus contributing to potential reactivation of MCMV. Our findings suggest that acute challenge with Listeria also reduces the number of MCMV-memory NK cells, although the mechanism for this is unclear and may be an indirect consequence of the overall lymphocyte apoptosis induced by Listeria (Merrick et al., 1997). Stelekati et al. (2014) have recently shown that the development of CD8⁺ T cell memory to secondary infections is impacted by preexisting chronic infections with various pathogens. Furthermore, T cell function during chronic viral infections can be influenced by continued exposure to antigen and persistent inflammation. If antigen amounts are relatively low, as is the case in CMV, virus-specific T cells maintain a high state of activity (Virgin et al., 2009). However, when antigen load remains high or increases, as is the case in chronic HIV, HBV, or HCV infection, T cells display increased and sustained expression of inhibitory receptors such as PD-1 and become less functional. Studies using transgenic mice constitutively expressing m157 demonstrated impaired response of the Ly49H⁺ NK cells to MCMV infection (Tripathy et al., 2008). In the case of MCMV infection in WT mice, viral load during latent infection is undetectable in most tissues apart from the salivary gland, the site of persistent infection and virus dissemination. Presumably, during persistent infection MCMV-memory NK cells would not be exposed to high levels of viral ligands in the spleen or liver, which might adversely affect their MCMV-specific responsiveness. Accordingly, our results indicate that naive Ly49H⁺ NK cells transferred into a host infected a month earlier with MCMV do not acquire markers of MCMV-induced activation

(as marked by up-regulation of Ly49H or KLRG1), suggesting that they are not exposed to latent MCMV in the host within 1–4 d after transfer.

Overall, our findings reveal that the long-lived memory NK cells generated by Ly49H-dependent activation and expansion become highly specialized for responsiveness to MCMV infection and exquisitely antigen (m157)-specific. This renders MCMV-memory NK cells hypo-responsive during challenge with heterologous bacterial or viral infections, including infection with MCMV that lacks a ligand for Ly49H. Our findings also suggest that the mechanism underlying the specialization of MCMV-memory NK cells might involve increasing the cell surface density of the antigen-specific Ly49H receptor and decreasing responsiveness to cytokines alone. However, cytokine stimulation that occurs concurrently with antigen stimulation can augment the functional response of MCMV-memory NK cells. Becoming more antigen-specific and less responsive to bystander cytokine activation may serve to focus the immune response of these NK cells on the specific virus responsible for inducing their memory state. Thus, although cytokine-primed NK cells might contribute as bystanders to infection with many different pathogens, the ligand-specific expansion and differentiation induced through Ly49H might serve to generate a pool of NK cells specifically to control persistent infection with MCMV.

MATERIALS AND METHODS

Mice. Female C57BL/6 (CD45.2) and congenic C57BL/6-Ly5.2 (CD45.1) mice were purchased from National Cancer Institute and all animal protocols were approved by the UCSF IACUC. Ly49H-deficient (*Klra8*$^{-/-}$) C57BL/6 mice were a gift from S. Vidal (McGill University, Montreal, Quebec, Canada).

MCMV, influenza, and Listeria infections. MCMV (Smith strain) was passaged in BALB/c mice; a salivary gland stock was prepared as previously described (Bubić et al., 2004). Δm157 MCMV (Bubić et al., 2004; provided by U. Koszinowski, Max von Pettenkofer-Institut, München, Germany) and MCMV (Smith strain) were prepared by using C57BL/6 3T3 cells as described previously (Bubić et al., 2004). Stocks were titered on mouse embryonic fibroblasts by using a standard plaque assay. Mice were infected intraperitoneally with 10^4 PFU of the salivary gland stock, or 10^5 PFU of the stocks propagated in 3T3 cells. PR8 influenza virus stocks were propagated in Madin-Darby canine kidney (MDCK) cells; supernatant was harvested, snap frozen, and titer was determined on MDCK cells by using a standard plaque assay (Balish et al., 2013). *L. monocytogenes* stock was grown in brain heart infusion (BHI) broth to an OD_{600} of 0.2, and mice were infected intravenously with 5×10^3 CFU. Dose was determined by CFU assays for each infection.

Preparation of single cell suspensions. Leukocytes were isolated from spleen, lung, liver, and BAL. In brief, single cell suspensions were prepared from lung tissue after tissue perfusion with 10 ml of PBS and digestion for 20 min in 3.2 mg/ml type IV collagenase (Worthington Biochemical Corporation) and 0.1 mg/ml DNase I (Roche). Livers were subjected to Dounce homogenization and lymphocytes were prepared using centrifugation on a 40/60% Percoll gradient (GE Healthcare). BAL was performed by washing the bronchial space with 1 ml PBS through a cannula; lymphocytes were isolated from the lavage fluid. NK cells were enriched by staining splenocytes with rat mAbs against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119, followed by depletion with anti–rat IgG antibodies conjugated to magnetic beads (QIAGEN). 500,000 to 1 million Ly49H$^+$ NK cells were injected intravenously into Ly49H-deficient recipient mice on the day before MCMV

infection. Similarly, 100,000–500,000 naive Ly49H$^+$ NK cells were injected intravenously into MCMV-infected mice at days 29–30 after infection on the day before infection with either influenza or Listeria. For cotransfer into naive Ly49H-deficient recipients, $1–5 \times 10^4$ memory Ly49H$^+$ and 5×10^5 naive Ly49H$^+$ NK cells were injected intravenously on the day before challenge with WT MCMV, MCMVΔm157, or Listeria.

Flow cytometry and antibodies. Fc receptors were blocked with anti-CD16 + CD32 mAb (clone 2.4G2) before staining with the indicated mAbs (BD, eBioscience, or BioLegend). We assessed proliferation by intracellular staining of fixed and permeabilized cells for Ki67, a marker of recent cell division. Samples were analyzed on a LSRII (BD) using FlowJo software (Tree Star).

In vitro cytokine stimulation and phospho-STAT assay. Enriched NK cells (naive or memory) were incubated with 20 ng/ml mouse IL-12 (R&D Systems) + 10 ng/ml IL-18 (R&D Systems) for 6 h at 37°C in the presence of PE-conjugated anti-CD107a mAb and GolgiStop (BD), followed by staining for surface molecules and intracellular cytokines. For the phospho-STAT4 assay, enriched NK cells (naive plus memory, CD45 congenically marked) were cultured with 2, 5, 10, 20, or 50 ng/ml of IL-12 for 5, 10, 30, or 90 min at 37°C. Cells were fixed (1.6% paraformaldehyde in phosphate-buffered saline), permeabilized with 90% methanol, and stained with an antibody against pSTAT4 (Alexa Fluor 647–conjugated anti–STAT4-pY693; BD). For the phospho-STAT1 assay, enriched NK cells (naive plus memory, CD45 congenically marked) were cultured with 5,000 U/ml IFN-β (PBL Assay Science) for 30 or 90 min at 37°C; cells were fixed as for pSTAT4 and stained with pSTAT1 (PE-conjugated anti–STAT1-pY701; BD).

Statistical analysis. Groups were compared using standard Student's *t* tests when normally distributed and otherwise subject to nonparametric analysis (Mann-Whitney). Paired samples that were directly cotransferred were analyzed using paired Student's *t* tests.

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