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# Ly49H Engagement Compensates for the Absence of Type I Interferon Signaling in Stimulating NK Cell Proliferation during MCMV Infection

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## Abstract

NK cells vigorously proliferate during viral infections, resulting in an expanded pool of innate lymphocytes that are able to participate in early host defense. The relative contributions of cytokines and activation receptors in stimulating NK cell proliferation during viral infections are not well characterized. In this study, we demonstrated that signaling through the NK cell activation receptor Ly49H was able to compensate for the absence of cytokine stimulation in the preferential phase of viral-induced proliferation during MCMV infection. In the absence of type I interferon stimulation, NK cell proliferation was strongly biased toward cells expressing the Ly49H receptor, even at early time points when minimal preferential Ly49H-mediated proliferation was observed in wild-type mice. In the absence of effective Ly49H signaling or following infection with virus that did not express the ligand for Ly49H, no difference was observed in the proliferation of subsets of NK cells that either express or lack expression of Ly49H, although the overall proliferation of NK cells in IFNαβR−/− mice was substantially reduced. These results highlight the contribution of NK cell activation receptors in stimulating proliferation and subsequent expansion of NK cells that are able to recognize virally infected cells.

### Keywords

IL-15; IFNαβ; Ly49H; DAP12; MCMV; NK cells; proliferation

## Introduction

Natural killer (NK) cells are innate lymphocytes that play a pivotal role in early anti-pathogen host defense and are particularly important in resistance to viral infections. Indeed, humans with selective NK cell deficiencies uniformly have difficulty with recurrent and severe viral infections, particularly with herpesviruses (reviewed in (1)). Murine cytomegalovirus (MCMV) has proven to be an exceptionally useful model system for *in vivo* studies of NK cell responses.

NK cells are stimulated by cytokines elicited early during infections as well as by direct recognition of infected cells through activation receptors. In mice, one NK cell activation receptor that mediates resistance to MCMV is Ly49H. Mouse strains (*e.g.*, C57BL/6 or BALB/c mice reconstituted with *Ly49h* by transgenesis) that express this receptor on their NK cells

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are substantially more resistant to MCMV than mice that lack Ly49H expression (2–5). Ly49H recognition of its ligand, m157, on infected cells and subsequent signaling through its adaptor molecule DAP12 stimulates NK cell production of immunomodulatory cytokines and killing of infected cells (6–8). In addition to these effector functions, NK cells rapidly proliferate during viral infections, resulting in substantially increased numbers of peripheral NK cells (9–13). The proliferative response occurs in three distinct phases, including early nonselective NK cell proliferation, preferential proliferation of NK cells expressing activation receptors that are able to recognize the infected cells, and cessation of proliferation with contraction of the expanded NK cell population (10,11).

The regulation of these distinct phases of viral-induced NK cell proliferation is poorly characterized. In particular, little is known about the relative contributions of cytokines and NK cell activation receptors. The initial phase of nonspecific NK cell proliferation is thought to be driven by cytokines induced early during viral infections (13), in a manner similar to the nonspecific bystander proliferation observed in T cells during viral infections or following polyinosinic/polycytidylic acid treatment (14). The cytokines stimulating viral-induced NK cell proliferation are not well established, although IFNα/β up-regulation of IL-15 has been implicated (13). Other cytokines, including IL-18, may also contribute to this process (11,15, 16), although IL-18 alone is not sufficient to stimulate significant NK cell proliferation (11, 16). Type I interferons (IFN α/β) are rapidly up-regulated following viral infections (17,18), and IFN α/β R−/− mice on a 129 background (which lack expression of Ly49H) undergo less vigorous NK cell proliferation during MCMV infection than wild-type (wt) 129 mice. These observations suggest that IFN α/β participates in stimulating viral-induced NK cell proliferation (13). Although IFN α/β has an anti-proliferative effect when added to NK cells *in vitro* (13), IFN α/β up-regulates the expression of IL-15 (19–21) and thus indirectly stimulates viral-induced NK cell proliferation (13). The relative importance of IFN α/β stimulation to viral-induced NK cell proliferation has not been established in the context of specific stimulation through the NK cell activation receptor Ly49H in MCMV-resistant C57BL/6 mice.

The second, specific phase describes the preferential proliferation of NK cells that are able to recognize infected cells. An early report suggested that specific expansion of splenic Ly49H+ NK cells during MCMV infection was IL-18 (and to a lesser extent IL-12) dependent (15); however, it was subsequently demonstrated that both IL-18- and IL-18R-deficient mice (as well as IL-12-deficient mice) underwent normal preferential Ly49H+ NK cell proliferation and expansion (11). We recently demonstrated that preferential proliferation of Ly49H NK cells during MCMV infection resulted from Ly49H signaling through DAP12 augmenting pro-proliferative cytokine (*e.g.*, IL-15) stimulation and that the impact of Ly49H-mediated signaling could be masked in the presence of high concentrations of pro-proliferative cytokines (11). However, the ability of Ly49H stimulation to initiate and sustain NK cell proliferation in the context of blunted pro-proliferative cytokine stimulation secondary to the absence of type I interferon signaling has not been evaluated. Therefore, we proceeded to characterize the relative contributions of cytokines and NK cell activation receptors to NK cell proliferation during MCMV infection using IFN αβR−/− and DAP12$^{KI}$ mice on a C57BL/6 background.

## Materials and Methods

### Mice

C57BL/6 (B6) mice were obtained from NCI (Charles River, MA). 129.IFNαβR-deficient mice (29) were backcrossed onto a C57BL/6 background for 10 generations, and the completeness of the backcross was confirmed with speed congenic markers (>99% B6 with 136/137 B6 microsatellite markers) in the Rheumatic Diseases Core Center at Washington University. C57BL/6.DAP12 loss-of-function knock-in (DAP12$^{KI}$; (23)) mice were a generous gift from

Eric Vivier (CNRS-INSERM-Universite de la Mediterranee, France). C57BL/6.BXD8 (BXD8; (24)) mice that lack the Ly49H receptor were a kind gift from Wayne Yokoyama (Washington University, St. Louis, MO). Mice were maintained under specific pathogen-free conditions and used between 8 and 16 weeks of age. All experiments were conducted in accordance with institutional guidelines for animal care and use.

### Antibodies

Biotinylated anti-Ly49H antibody (3D10) was a gift from Wayne Yokoyama. PerCP-conjugated anti-CD3 (145-2C11), APC-conjugated anti-NK1.1 (PK136), APC-conjugated anti-Ly49G2 (4D11), APC-conjugated anti-Ly49D (4E5), and FITC-conjugated anti-BrdU antibodies as well as PE-streptavidin were all purchased from BD-Pharmingen (San Diego, CA) or eBioscience (San Diego, CA).

### MCMV and infection of mice

A salivary gland stock of Smith strain MCMV was prepared from young BALB/c mice that had been intra-peritoneally (i.p.) injected with $1\times10^6$ plaque-forming units (pfu) of tissue-culture propagated MCMV, and the titer of the stock was determined via standard plaque assay (4) using permissive NIH 3T12 fibroblasts (American Type Culture Collection (ATCC); Manassas, VA). MCMV m157-deficient isolate AT1.5 (G881A mutation resulting in a premature stop) has previously been described (22). Mice were injected i.p. with $1\times10^4$ to $5\times10^4$ pfu/mouse wt or m157-deficient MCMV salivary gland stocks. Splenic titers were determined via standard plaque assay (4).

### Splenocyte preparation, intracellular staining, and flow cytometry

To minimize the complication of bromodeoxyuridine (BrdU)+ NK cells from the bone marrow emigrating to the spleen, we utilized an acute pulse of BrdU (2 mg/mouse) injected i.p. three hours prior to euthanizing the mice at various time points after infection rather than prolonged BrdU exposure in the drinking water (11). Single-cell suspensions of splenocytes were prepared using standard techniques (10). Splenocytes were incubated in 2.4G2 (anti-Fcγ II/III receptor) supernatants (hybridoma from ATCC) prior to staining with labeled antibodies to block non-specific binding of antibodies to Fc receptors. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen), treated with DNase, and then stained with FITC-conjugated anti-BrdU antibodies. Analysis was performed with a FACScalibur flow cytometer (BD Pharmingen), and the data were analyzed with CellQuest (BD Pharmingen). A selectivity index (SI) to assess the extent of preferential Ly49H+ NK cell proliferation was used as previously defined (11).

### Quantitative RT-PCR for IL15

Total RNA of naïve or MCMV-infected spleens was isolated using an RNeasy Mini kit (Qiagen), digested with RQ1 DNase (Fisher Scientific), and reverse transcribed with Oligo dT12–18 (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on an ABI 7500 Fast Real Time system using AmpliTaq Gold polymerase (ABI) with Taqman probes and primer sets for IL-15 (previously described in (30); Integrated DNA Technologies) and β-actin (β-actin gene expression assay from ABI). After normalization with β-actin, relative expression levels of IL-15 were calculated using the ΔΔCT method.

### Statistical analysis

Unpaired, two-tailed t-test was used to determine statistically significant differences between experimental groups. Error bars in figures represent standard deviations from the mean value.

## Results

### Viral-induced NK cell proliferation during MCMV infection

Peripheral NK cells in naïve wt mice were relatively quiescent, with only ~1% of splenic NK cells dividing (*i.e.*, taking up BrdU) over a three-hour period (Fig. 1A; (10,11)). The first two phases of MCMV-induced NK cell proliferation are illustrated in the wt B6 column in Figure 1A where equivalent nonspecific NK cell proliferation was observed in both the Ly49H− and Ly49H+ subsets of splenic NK cells (17% and 22% BrdU+, respectively, with n=6) early during MCMV infection on day 1.5 post-infection (p.i.) and preferential proliferation of Ly49H+ NK cells compared with Ly49H− NK cells was evident at day 3 p.i. (22% and 39% BrdU+ in Ly49H− and Ly49H+ subsets, respectively, with n=6). Preferential NK cell proliferation during MCMV infection was restricted to the Ly49H+ subset of NK cells (Fig. S1; (10,11)). Consistent with prior studies (11), splenic NK cells from DAP12$^{KI}$ mice underwent normal early, nonspecific viral-induced NK cell proliferation during MCMV infection, but they failed to display any preferential Ly49H+ NK cell proliferation at later time points (Fig. 1A). In contrast, early proliferation was severely deficient in the IFNαβR−/− mice (Fig. 1A, day 1.5 p.i.) compared to both B6 and DAP12$^{KI}$ mice. The overall percentage of splenic NK cells that were BrdU+ in the IFNαβR−/− mice was 3.6% compared to 20% in the wt B6 mice and 15% in the DAP12$^{KI}$ mice (n=6–8). At day 3 p.i., NK cell proliferation in IFNαβR−/− mice (19% with n=15) was more vigorous but still lower than observed in wt B6 mice (32% with n=10). Interestingly, NK cell proliferation in the IFNαβR−/− mice was strongly biased toward the Ly49H+ subset of NK cells, demonstrating that these mice were able to undergo preferential Ly49H+ NK cell proliferation even in the absence of cytokine stimulation elicited by type I interferons.

### Up-regulation of IL-15 mRNA is disrupted in IFNαβ R−/− mice

The up-regulation of IL-15 transcripts observed in both wt and DAP12$^{KI}$ mice during MCMV infection was nearly abrogated in the IFN αβR−/− mice at days 1.5 and 3 p.i. (Fig. 1B), corroborating what Biron and colleagues had previously observed with semi-quantitative RT-PCR in IFNαβR−/− mice on a 129 background (13). This blunted up-regulation of IL-15 mRNA provides a plausible explanation for the near absence of non-specific, early NK cell proliferation and the minimal proliferation of Ly49H− NK cells on day 3 during MCMV infection in the IFN αβR−/− mice (Fig. 1A).

### NK cell cytokine production is independent of type I interferonstimulation

In contrast to the deficits observed in viral-induced NK cell proliferation in IFNαβR−/− mice, no defects were noted in IFNγ production early during MCMV infection (Fig. 1C). When stained for intracellular IFNγ immediately *ex vivo* on day 1.5 p.i., 19%+/−9%, 46%+/−11%, and 25%+/−11% of the splenic NK cells from wt B6, IFNαβR−/−, and DAP12$^{KI}$ mice, respectively, were producing IFNγ (cumulative results from 4 independent experiments; n=11–15 mice/group). Consistent with a previous report in B6 mice (10), no differences in intracellular IFNγ production were observed between the Ly49H$^{+}$ and Ly49H$^{-}$ subsets of NK cells at d1.5 p.i. in wt B6, IFNαβR−/−, or DAP12$^{KI}$ mice. The increased percentage of NK cells producing IFNγ in the IFNαβR−/− mice may reflect more intense stimulation elicited in the context of less efficient early viral control. Regardless of the mechanism responsible for elevated levels of IFNγ in the IFNαβR−/− mice, these results demonstrated that IFNγ production proceeds independently of IFNαβ stimulation. These experiments using a standard inoculum dose of MCMV ($5\times10^4$ pfu/mouse) provided initial insight into the relative impact of the signaling mediated by type I interferons and Ly49H receptors on viral-induced NK cell proliferation; however, these studies were limited by the inability of the IFNαβR−/− and DAP12$^{KI}$ mice to effectively control MCMV infection at this inoculum dose. In addition to a paucity of viable splenic NK cells at later time points, we encountered considerable variability

in the proliferative response as early as day 3 p.i. For example, the percentage of splenic NK cells in the IFNαβR−/− mice that were BrdU+ at day 3 p.i. with $5\times10^4$ pfu MCMV/mouse was 19.2%+/−11.6% (n=15). $5\times10^4$ pfu MCMV/mouse is one-fifth the $LD_{50}$ in wt B6 mice and is commonly used as an inoculum dose because it provides a strong stimulus without significant morbidity in wt B6 mice (11). To facilitate a more detailed investigation of the relative influence of signaling through type I interferon and Ly49H receptors on viral-induced NK cell proliferation, we subsequently used an inoculum dose of MCMV ($1\times10^4$ pfu MCMV/mouse) that was approximately one-fifth the $LD_{50}$ in IFNαβR−/− mice. This dose allowed assessment of NK cell proliferation in IFN αβR−/− mice without causing significant morbidity, while still stimulating a robust proliferative response in wt B6 mice (Figs. 2 and 3).

## Decreased and Ly49H-biased NK cell proliferation in IFNαβ R−/− mice early during MCMV infection

The early NK cell proliferative response at day 1.5 p.i. was more robust in IFNαβR−/− mice following infection with $1\times10^4$ pfu MCMV/mouse, although it was still deficient compared to either wt B6 or $DAP12^{KI}$ mice (Figs. 2A and 2B). Moreover, the NK cell proliferation present in the IFNαβR−/− mice at day 1.5 p.i. was strongly biased to the Ly49H+ subset of NK cells (Fig. 2C) with a selectivity index (ratio of the fraction of Ly49H+ NK cells that are proliferating compared to the fraction of Ly49H− NK cells that are proliferating) of 2.1. This contrasted with equivalent levels of proliferation observed in the Ly49H+ and Ly49H− subsets of NK cells in wt and $DAP12^{KI}$ mice at this early time point. The selectivity index in wt mice was 1.2, demonstrating that little preferential proliferation was observed in these mice at day 1.5 p.i. with $1\times10^4$ pfu MCMV/mouse. These results show not only that nonselective, viral-induced NK cell proliferation was deficient in the IFNαβR−/− mice but also that even at this early time point during infection, signaling through the NK cell activation receptor was able to partially compensate for the absence of efficient cytokine stimulation in eliciting NK cell proliferation.

## Strongly Ly49H-biased NK cell proliferation in IFNαβR−/− mice at day 3 p.i. is abrogated during infection with m157-deficient MCMV

Robust NK cell proliferation was observed in the preferential phase of viral-induced NK cell proliferation in the IFNαβR−/− mice following infection with $1\times10^4$ pfu MCMV/mouse. Indeed, nearly identical overall proliferative responses (*i.e.*, the percentage of splenic NK cells that were BrdU+) were observed in the IFNαβR−/− and wt B6 mice on day 3 p.i., while lower levels of proliferation were observed in the $DAP12^{KI}$ mice (Figs. 3A and gray bars in 3B). The proliferative response in the IFNαβR−/− mice was even more strongly biased to preferential Ly49H+ NK cell proliferation than that observed in wt B6 mice (Fig. 3C with gray and black bars representing Ly49H− and Ly49H+ subsets of NK cells, respectively). This was reflected in the selectivity index of 2.3 (n=17) in the IFNαβR−/− mice and 1.5 (n=16) in the wt B6 mice at day 3 p.i. with $1\times10^4$ pfu MCMV/mouse (gray bars in Fig. 3D). In contrast, no preferential proliferation of Ly49H+ NK cells was observed in the $DAP12^{KI}$ mice (Figs. 3C and 3D). The strongly Ly49H-biased NK cell proliferation in the IFNαβR−/− mice contributed to an earlier shift in the percentage of splenic NK cells that were Ly49H+ in the IFNαβR−/− mice compared to that observed in wt B6 mice (Figs. 3E and S2).

We previously proposed that preferential viral-induced NK cell proliferation occurs as activation receptor signaling augments low levels of pro-proliferative cytokine stimulation (11). Given the blunted up-regulation of IL-15 mRNA observed during MCMV infection in the IFNαβR−/− mice (Fig. 1B), we hypothesized that the IFNαβR−/− mice would undergo minimal MCMV-induced NK cell proliferation in the absence of Ly49H stimulation. We directly tested this prediction by infecting mice with m157-deficient MCMV (22) to remove the influence of Ly49H stimulation. Following infection with $1\times10^4$ pfu m157-deficient

MCMV/mouse, we observed significantly lower levels of overall NK cell proliferation in the IFNαβR−/− mice compared to wt B6 or $DAP12^{KI}$ mice (Fig. 3A and white bars in Fig. 3B). As would be expected, no preferential Ly49H+ proliferation was observed following infection with MCMV deficient for the ligand for Ly49H (Fig. 3C). Indeed, the selectivity indexes in all three strains of mice were nearly one (Fig. 3D), and no increases were observed in the percentage of Ly49H+ NK cells (Fig. 3E). Nearly identical proliferation was observed in Ly49H+ and Ly49H− subsets of NK cells in wt B6 and $DAP12^{KI}$ mice following infection with m157-deficient MCMV (white and striped bars in Fig. 3C). Interestingly, the percentages of proliferating Ly49H+ and Ly49H− NK cells observed in these mice were very similar to the percentages of proliferating Ly49H− NK cells in B6 mice and Ly49H− and Ly49H+ NK cells in $DAP12^{KI}$ mice following infection with wt MCMV (Fig. 3C), suggesting that similar levels of MCMV-induced pro-proliferative cytokine stimulation were present in the wt B6 and $DAP12^{KI}$ mice following infection with either wt or m157-deficient MCMV. In contrast, the percentages of Ly49H+ and Ly49H− NK cells in the IFNαβR−/− mice were lower, consistent with lower levels of pro-proliferative cytokine stimulation in these mice (Fig. 3C). Overall, these results illustrated that MCMV-induced NK cell proliferation in the IFNαβR−/− mice occurred predominantly in the Ly49H+ subset of NK cells and was severely reduced following infection with MCMV deficient in the expression of the ligand for Ly49H.

One caveat to using $DAP12^{KI}$ mice as Ly49H signaling-deficient controls was that DAP12 is also expressed in other innate cells including dendritic cells and monocytes (23). In light of this potential confounding factor, we utilized B6.BXD8 mice, which lack Ly49H (4,24), as an alternative Ly49H signaling-deficient control. Although the absence of Ly49H expression on the NK cells in BXD8 mice precluded comparisons of proliferative responses in Ly49H+ and Ly49H− NK cell subsets, the overall proliferative responses observed in these mice were similar to those seen with $DAP12^{KI}$ mice following infection with either wt or m157-deficient MCMV (data not shown).

## Increased abundance of m157 may contribute to the strongly biased Ly49H+ NK cell proliferation observed in the IFNαβ R−/− mice

The absence of selective Ly49H+ NK cell proliferation following infection with m157-deficient MCMV demonstrated that Ly49H−mediated NK cell proliferation was dependent on the interaction of the receptor and its ligand. We hypothesized that the magnitude of preferential Ly49H+ NK cell proliferation would be altered by the relative abundance of m157 encountered by NK cells in the spleen, and we, therefore, investigated m157 levels following infection with MCMV in wt B6 and IFNαβR−/− mice. Unfortunately, currently available anti-m157 antibodies have not proven useful in flow cytometry studies of infected cells *ex vivo* (25). One surrogate approach to assess the relative abundance or “concentration” of m157 in the splenic environment during MCMV infection is to measure the decrement in the mean fluorescence intensity (MFI) of Ly49H expression (7,25). Interactions of Ly49H with its ligand, m157, result in decreased MFI of the receptor due to internalization of the activated receptor, although interference to binding of the anti-receptor antibody may also be contributing. Decreased Ly49H MFI can be seen *in vivo* in wt B6 mice (Fig. 1A, day 1.5 p.i. B6 mice), although it is more pronounced during *in vitro* co-culture with transfected cells expressing high levels of m157 (7,25). We consistently observed a greater decrement in Ly49H MFI on NK cells from IFNαβR−/− mice than from wt B6 mice following infection with MCMV (at inoculum doses of either $1\times10^4$ or $5\times10^4$ pfu/mouse), suggesting more frequent or persistent interactions between Ly49H and m157 in IFNαβR−/− mice (Figs. 4A and 4B). In contrast, no decrease in the MFI of Ly49H was observed in IFNαβR−/− (or wt B6) mice following infection with m157-deficient MCMV (Fig. 4B).

The apparent higher relative "concentrations" of m157 (reflected in the greater decrement in Ly49H MFI) suggested that the viral loads in the IFNαβR−/− mice would be higher than in the B6 mice. We evaluated splenic viral titers in wt B6, IFNαβR−/−, DAP12$^{KI}$, and BXD8 mice after infection with $1\times10^4$ pfu/mouse of wt or m157-deficient MCMV (Fig. 4C). Following infection with wt MCMV, we observed comparable elevated splenic titers in IFNαβR−/− and DAP12$^{KI}$ mice that were significantly higher than the viral titers seen in wt B6 mice. Type I interferons have broad anti-viral functions beyond their impact on NK cells (26), and it was not surprising to observe elevated splenic titers following infection with MCMV despite the presence of Ly49H+ NK cells in these mice. Infection with m157-deficient MCMV circumvented the impact of Ly49H+ NK cells and resulted in viral titers that were over 100 fold higher in wt B6 mice and nearly 50 fold higher in the IFNαβR−/− mice than observed following infection with wt MCMV. No difference in viral titers was observed in the DAP12$^{KI}$ mice following infection with either wt or m157-deficient MCMV, as would be expected since Ly49H signaling is deficient in these mice. BXD8 mice were included as an alternative Ly49H signaling-deficient control, and no difference in splenic titers was observed following infection with either wt or m157-deficient MCMV, although the titers were modestly higher than those observed in DAP12$^{KI}$ mice. The elevated viral titers together with the significantly decreased Ly49H MFI in the IFNαβR−/− mice provided evidence that an environment enriched in m157 (the ligand for Ly49H) may contribute to the robust Ly49H-biased NK cell proliferation observed in the IFNαβR−/− mice during wt MCMV infection.

## Discussion

NK cells are stimulated to vigorously proliferate during viral infections, resulting in increased NK cell numbers early during infection before an effective adaptive immune response has been mustered (9–13). In addition, the proliferative response sculpts the make-up of the NK cell pool through the preferential proliferation of NK cells that are able to recognize infected cells (11). We have demonstrated, for the first time, the ability of NK cell activation receptors to compensate for the absence of type I interferon stimulation in mediating *in vivo* viral-induced NK cell proliferation. The results in this study strongly corroborate the hypothesis that preferential, viral-induced NK cell proliferation occurs when NK cell activation receptor signaling amplifies pro-proliferative cytokine stimulation, including low levels of cytokines that alone are insufficient to stimulate significant NK cell proliferation (11).

Biron and colleagues (13) previously demonstrated that IFNαβR−/− mice on a 129 background (which lack expression of the NK cell activation receptor Ly49H) displayed a deficit in MCMV-induced NK cell proliferation compared with wt 129 mice. Our IFNαβR−/− mice on a C57BL/6 background also had a deficit in viral-induced NK cell proliferation particularly early during MCMV infection (*i.e.*, day 1.5 p.i.) when proliferation was primarily cytokine driven. This deficiency was most evident at higher inoculum doses of MCMV (*e.g.*, $5\times10^4$ pfu/mouse) where minimal NK cell proliferation was observed in the IFNαβR−/− mice at day 1.5 p.i. However, the present studies demonstrated that signaling through the NK cell activation receptor Ly49H in IFNαβR−/− mice was able to substantially compensate for the absence of type I interferons in mediating NK cell proliferation particularly during the second, preferential phase of viral-induced NK cell proliferation (*e.g.*, day 3 p.i.). Even at earlier time points where wt B6 mice underwent minimal preferential Ly49H+ NK cell proliferation (*e.g.*, day 1.5 p.i.), NK cell proliferation in the IFNαβR−/− mice occurred predominantly in the Ly49H+ subset. The robust Ly49H+ NK cell proliferative response in the IFNαβR−/− mice was augmented by an increased abundance or elevated "concentration" of m157 in the splenic environment in these mice compared with wt B6 mice. The ability of Ly49H signaling in the IFNαβR−/− mice to compensate for the absence of type I interferon stimulation was completely abrogated during infection with MCMV that lacked expression of the ligand for the Ly49H receptor.

The strongly Ly49H-biased NK cell proliferation in the IFNαβR−/− mice resulted in an earlier shift in the percentage of splenic NK cells that were Ly49H+ in the IFNαβR−/− mice compared to that observed in wt B6 mice. A similar increase in the percentage of splenic NK cells that were Ly49H+ was observed in B6 mice at later time points (*e.g.*, 79% of B6 splenic NK cells were Ly49H+ on day 6 p.i. with $1\times10^4$ pfu MCMV/mouse; Fig. S2). The early perturbation in relative abundance of Ly49H− and Ly49H+ NK cell subsets in the spleens of IFNαβR−/− mice may reflect increased survival of Ly49H+ NK cells in addition to their preferential proliferation, and this issue is the subject of ongoing investigation.

We demonstrated that up-regulation of IL-15 mRNA observed in wt B6 mice was nearly absent during MCMV infection in the IFNαβR−/− mice. Although the regulation of IL-15 is complex and may involve a number of post-translational mechanisms, the blunted up-regulation of IL-15 transcripts provides a plausible explanation for the near absence of non-specific, early NK cell proliferation and diminished proliferation of Ly49H− NK cells on day 3 during MCMV infection in the IFNαβR−/− mice. The presence of preferential proliferation of Ly49H+ NK cells in the IFNαβR−/− mice on day 3 p.i. (as well as the Ly49H-biased although limited proliferation on day 1.5 p.i.) in the context of blunted up-regulation of IL-15 supports our hypothesis that preferential proliferation occurs when NK cell activation receptor signaling augments low levels of pro-proliferative cytokine stimulation that in isolation elicit little NK cell proliferation (11).

Although the regulation of NK cell proliferation and homeostasis remains incompletely characterized, the present study highlights the relative importance of NK cell activation receptors in mediating viral-induced proliferation even in the absence of type I interferon stimulation. Understanding the regulation of viral-induced NK cell proliferation and homeostasis is relevant to human health, as illustrated in the recent reports of a substantial NK cell expansion during a CMV infection in an infant with an unrecognized IL-7R deficiency (27), the dysregulated expansion of NK cells in patients with chronic NK cell lymphocytosis (CNKL; (28)), and the inadequate NK cell-mediated anti-viral responses in patients with functional NK cell defects (1). We propose that a more complete characterization of NK cell proliferation and homeostasis following *in vivo* stimuli, such as viral infections, will have broad translational implications and may lead to novel therapeutic interventions, either to stimulate more effective NK cell responses (*e.g.*, during intractable viral infections or solid organ malignancies) or to down-regulate over-exuberant or inappropriate NK cell responses (*e.g.*, during NK cell lymphoproliferative disorders such as CNKL or autoimmune diseases).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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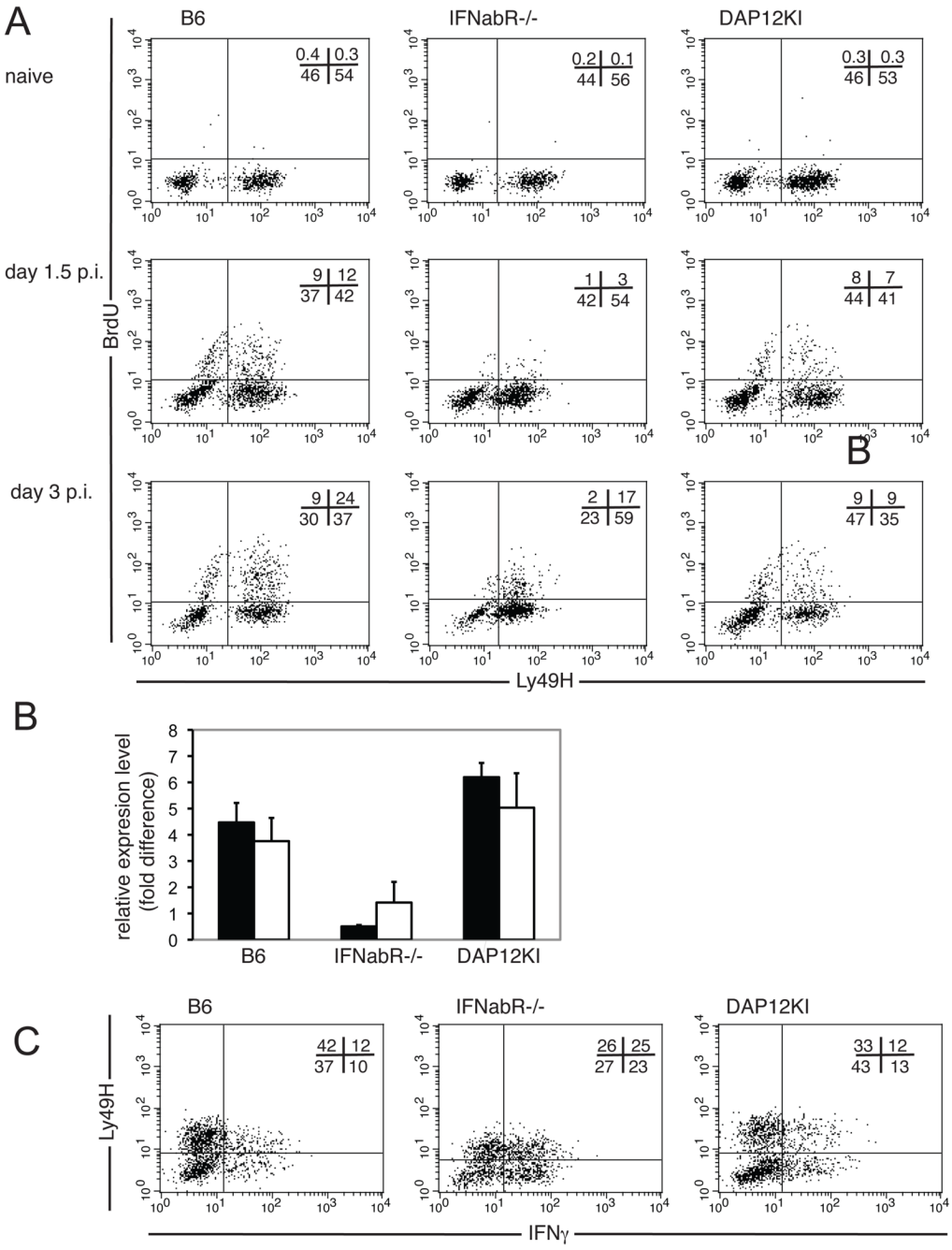


**Figure 1. Viral-induced NK cell proliferation during the course of MCMV infection**

A) Representative scatter-plots gated on splenic NK cells from wt B6, IFNαβR−/− and DAP12$^{KI}$ mice are shown with Ly49H expression on the x-axis and BrdU uptake on the y-axis. The upper row includes scatter-plots of naïve mice, while the next two rows illustrate representative scatter-plots of mice infected with $5\times10^4$ pfu MCMV at days 1.5 and 3 p.i., respectively. The results are representative of groups of 3–8 mice in four independent experiments. B) Up-regulation of IL-15 mRNA is blunted in IFNαβR−/− on days 1.5 p.i. ($1\times10^4$ pfu MCMV; black) and 3 p.i. ($5\times10^4$ pfu MCMV; white) (n= 4 mice/group). C) Intracellular staining for IFNγ in NK cells immediately *ex vivo* from mice infected with $5\times10^4$ pfu MCMV at day 1.5 p.i. (n= 6–8 mice/group from 2 independent experiments).

Representative scatter-plots gated on NK cells from wt B6, IFNαβR−/−, and DAP12$^{KI}$ mice are shown with IFNγ expression on the x-axis and Ly49H expression on the y-axis.

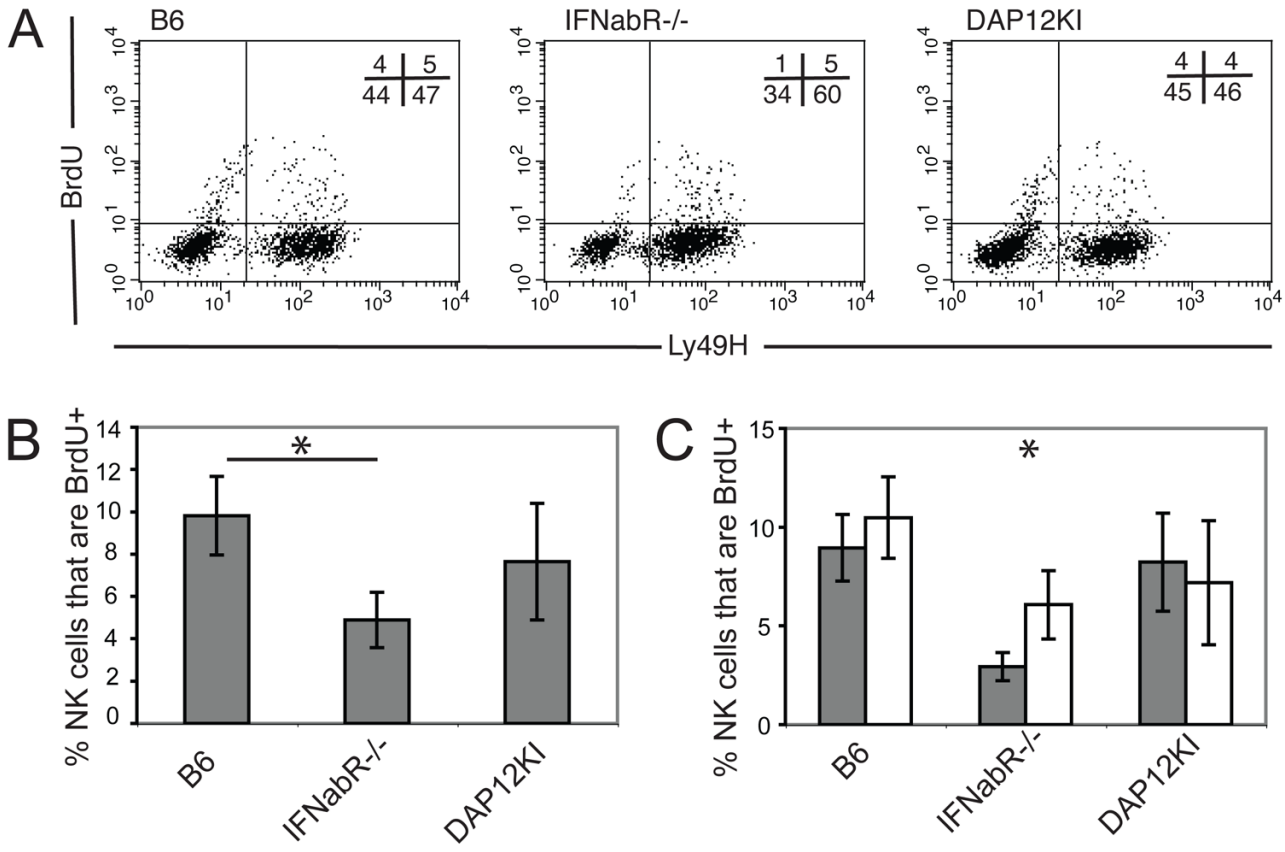


**Figure 2. Decreased but Ly49H-biased NK cell proliferation observed in IFNαβR−/− mice early during infection with $1\times10^4$ pfu MCMV**

A) Representative scatter-plots gated on splenic NK cells from wt B6, IFNαβR−/−, and $DAP12^{KI}$ mice on day 1.5 p.i. with $1\times10^4$ pfu MCMV are shown with Ly49H expression on the x-axis and BrdU uptake on the y-axis. These results are representative of two independent experiments with 6 mice per group. Cumulative results (12 mice/group) from these two experiments are quantified in figures B) the percentage of splenic NK cells that are proliferating (*i.e.*, BrdU+) and C) the percentage of Ly49H− (gray) and Ly49H+ (white) splenic NK cells that are proliferating at day 1.5 p.i. (Asterisk denotes $p<0.01$).

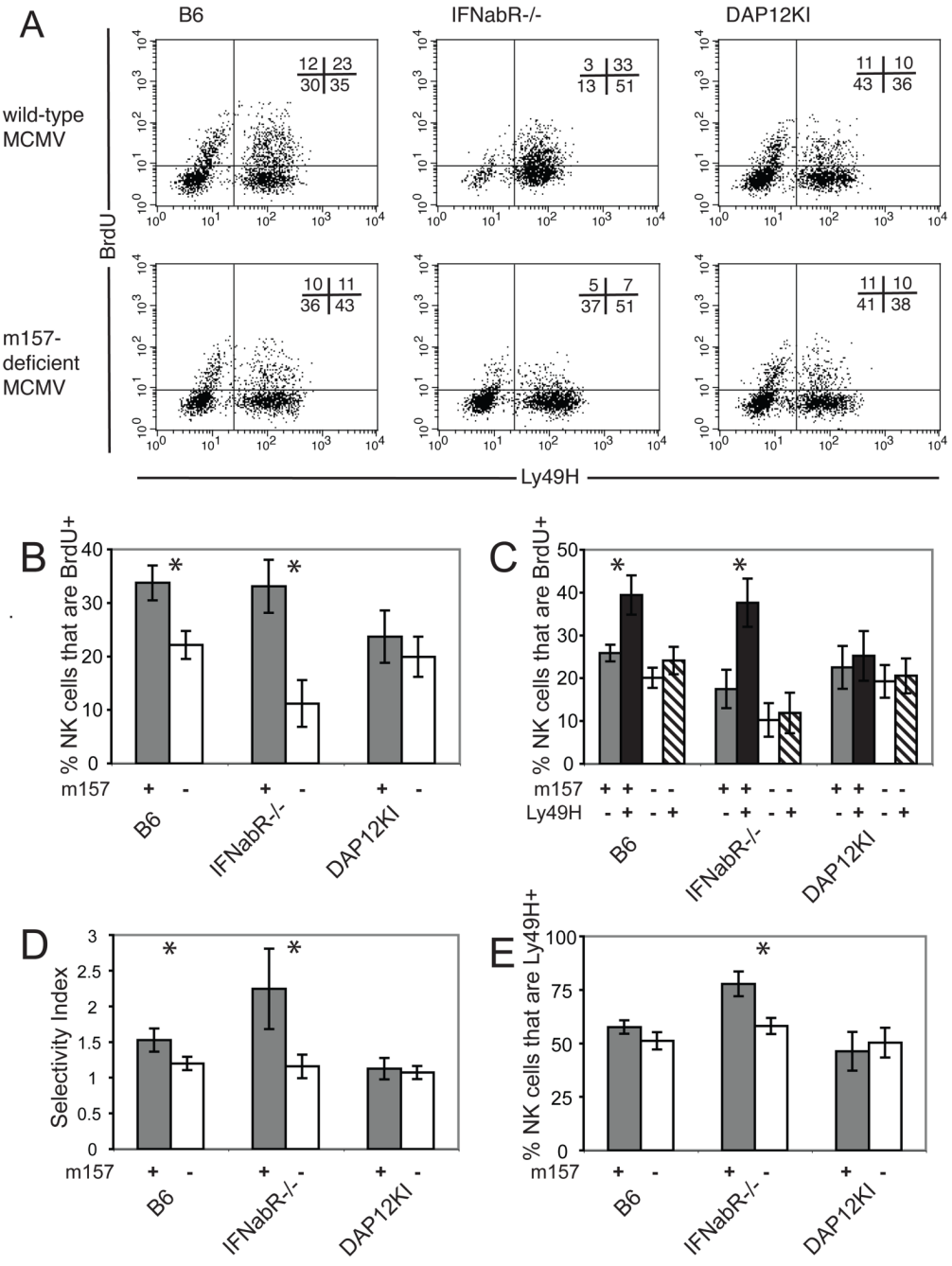


**Figure 3. Strongly Ly49H-biased NK cell proliferation in IFNαβR−/− mice at day 3 post-infection with 1×10$^4$ pfu MCMV is abrogated during infection with m157-deficient MCMV**

A) Representative scatter-plots gated on splenic NK cells from wt B6, IFNαβR−/−, and DAP12$^{KI}$ mice on day 3 p.i. with 1×10$^4$ pfu wt MCMV (top row) or m157-deficient MCMV (bottom row) are shown with Ly49H expression on the x-axis and BrdU uptake on the y-axis. These results are representative of three independent experiments with 4–6 mice per group. Cumulative results (12–17 mice/group) from these experiments are quantified in the remaining figures with gray (and black) bars indicating infection with wt MCMV and white (and striped) bars indicating infection with m157-deficient MCMV: B) the percentage of splenic NK cells that are proliferating, C) the percentage of Ly49H− (gray or white) and Ly49H+ (black or

striped) splenic NK cells that are proliferating where gray and black bars represent infections with wt MCMV while white and striped bars represent infections with m157-deficient MCMV, D) selectivity index, and E) the fraction of splenic NK cells that were Ly49H+ at day 3 p.i. (Asterisk denotes $p<0.01$).

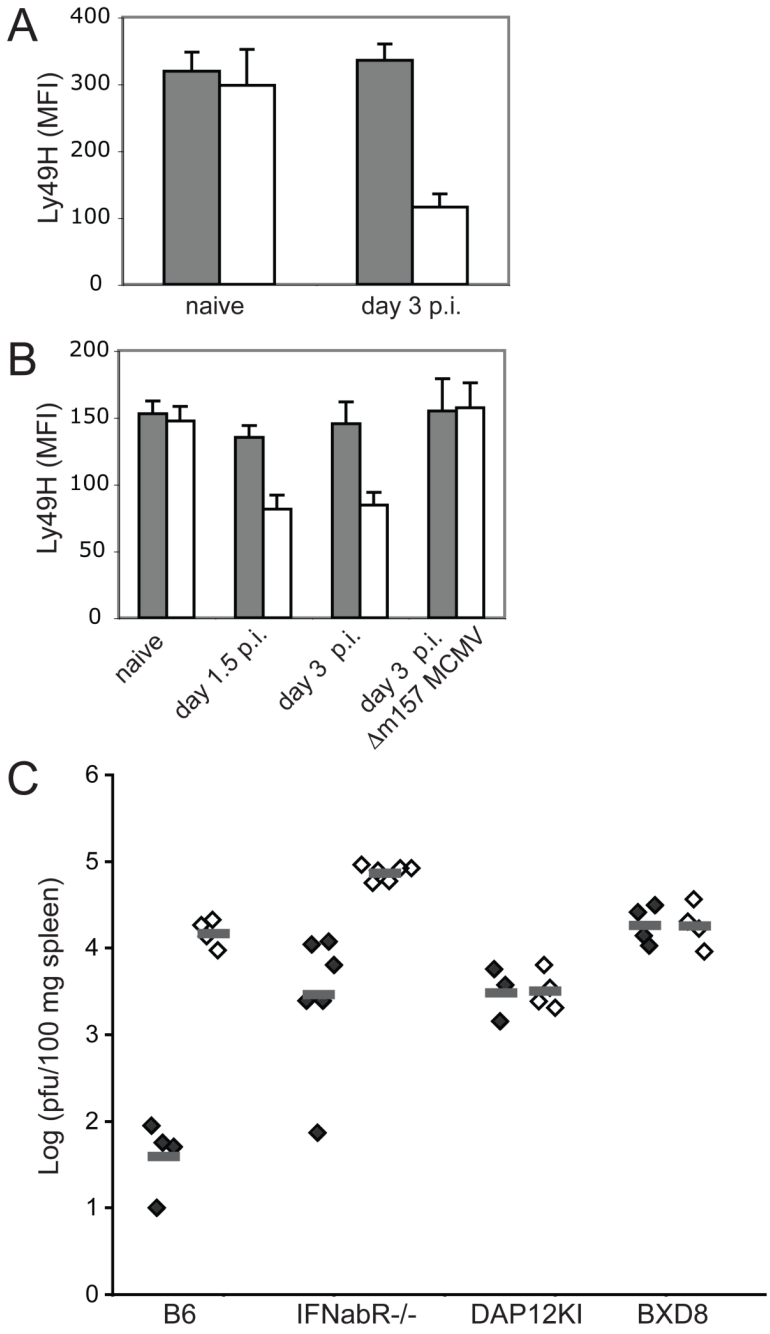


**Figure 4. Increased m157 in spleens of IFNαβR−/− mice as assessed by decreased expression levels of Ly49H on NK cells and higher viral titers**

A) Variation in expression levels of Ly49H (assessed by mean fluorescent intensity, MFI) on splenic Ly49H+ NK cells from wt B6 (gray bars) and IFNαβR−/− (white bars) mice on day 3 p.i. with $5\times10^4$ pfu MCMV (cumulative results of two independent experiments with n=4 naïve mice/group and n=8–13 infected mice/group). B) Variation in expression levels of Ly49H (*i.e.*, MFI) on splenic NK cells (post-fixation and permeabilization with 4% paraformaldehyde and saponin in preparation for BrdU staining) on days 1.5 and 3 post-infection with $1\times10^4$ pfu wt or m157-deficient MCMV (cumulative results of three independent experiments n=12–15 infected mice/group). C) Splenic viral titers at day 3 post-infection with $1\times10^4$ pfu wt (filled

symbols) or m157-deficient (open symbols) MCMV (3–6 mice/group). Mean values per group are shown as bars. Representative of 2 independent experiments.