

Proinflammatory cytokine signaling required for the generation of natural killer cell memory

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Although natural killer (NK) cells are classified as innate immune cells, recent studies demonstrate that NK cells can become long-lived memory cells and contribute to secondary immune responses. The precise signals that promote generation of long-lived memory NK cells are unknown. Using cytokine receptor-deficient mice, we show that interleukin-12 (IL-12) is indispensable for mouse cytomegalovirus (MCMV)-specific NK cell expansion and generation of memory NK cells. In contrast to wild-type NK cells that proliferated robustly and resided in lymphoid and nonlymphoid tissues for months after MCMV infection, IL-12 receptor-deficient NK cells failed to expand and were unable to mediate protection after MCMV challenge. We further demonstrate that a STAT4-dependent IFN- γ -independent mechanism contributes toward the generation of memory NK cells during MCMV infection. Understanding the full contribution of inflammatory cytokine signaling to the NK cell response against viral infection will be of interest for the development of vaccines and therapeutics.

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Abbreviation used: PI, post infection.

The generation of a productive NK cell response is crucial to protect the host from viral infection. In the absence of NK cells or NK cell function, both mice and humans are susceptible to several pathogens, particularly members of the herpesvirus family (Sun and Lanier, 2010). Accumulating evidence in mice and humans suggests that like the cells of adaptive immunity, NK cells can “remember” previously encountered pathogens through the generation of long-lived memory cells after initial antigen exposure (Paust and von Andrian, 2011; Sun et al., 2011; Vivier et al., 2011). During MCMV infection, Ly49H-bearing NK cells undergo a robust clonal-like expansion (Dokun et al., 2001; Sun et al., 2009) and persist in both lymphoid and non-lymphoid organs for several months (Sun et al., 2009). During a second or third encounter with the same virus, these long-lived memory NK cells are capable of prolific recall responses, mediating greater effector function and protection than naive resting NK cells (Cooper et al., 2009; Sun et al., 2010). Similar robust NK cell clonal-like expansion and memory has been observed during acute hantavirus and HCMV infection

in humans, where recent longitudinal studies revealed virus-specific responses in the NKG2C-bearing NK cell subset (Björkström et al., 2011; Lopez-Vergès et al., 2011). The goal of immunization is to provide protection against subsequent infection, and thus it is vital to define the precise signals that promote the generation of NK cell memory.

Proinflammatory cytokines such as IL-12 are known to globally promote NK and T cell activation and cytotoxicity. Binding of IL-12 to a two-chain receptor composed of IL-12 receptor (IL-12R) β 1 and β 2 results in a signaling cascade leading to phosphorylation and dimerization of STAT4, which translocates to the nucleus and activates downstream targets and transcription of effector cytokine genes such as IFN- γ (Trinchieri, 2003). Early studies involving cytokine or neutralizing antibody treatment of mice demonstrated that IL-12 has global effects on the immune system, as many hematopoietically derived cells express the IL-12R (Trinchieri, 2003). During infection, IL-12 is primarily produced by

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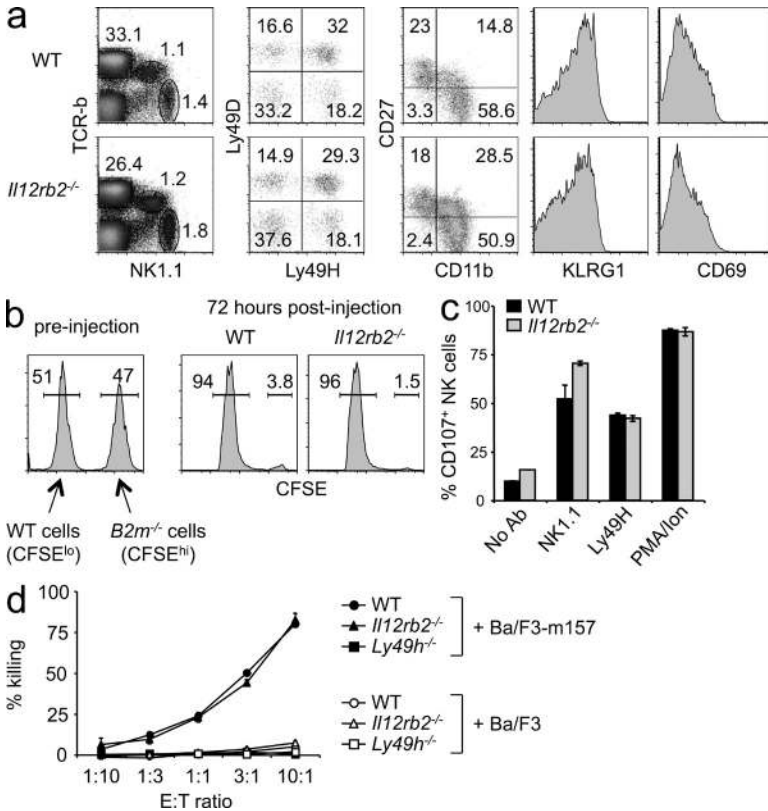


Figure 1. NK cells from IL-12R-deficient mice are phenotypically and functionally similar to NK cells from WT mice. (a) Percentages of TCR-β⁻ NK1.1⁺ NK, TCR-β⁺ NK1.1⁺ NKT, and TCR-β⁺ NK1.1⁻ T cell populations are shown for uninfected WT and *Il12rb2^{-/-}* mice (left plot). The right plots are gated on TCR-β⁻ NK1.1⁺ cells and analyzed for expression of Ly49D, Ly49H, CD27, CD11b, KLRG1, and CD69. (b) WT and *B2m^{-/-}* splenocytes were labeled with low or high concentrations of CFSE, respectively, and co-injected into WT or *Il12rb2^{-/-}* mice. Transferred cells were analyzed in spleen of recipient mice 72 h after injection. (c) NK cells from WT or *Il12rb2^{-/-}* mice were stimulated with plate-bound antibodies against NK1.1 and Ly49H, or with PMA and ionomycin. Uncoated wells (containing no Abs) served as negative control and background staining. Percentages of TCR-β⁻ NK1.1⁺ cells expressing CD107 (LAMP-1) are shown for each condition. Error bars show SEM (*n* = 2–3 for each condition). (d) Varying numbers of NK cells from WT, *Il12rb2^{-/-}*, or *Ly49h^{-/-}* mice were incubated with Ba/F3 and Ba/F3-m157 target cells labeled with ⁵¹Cr. Percentage of killing was calculated based on release of ⁵¹Cr into supernatant by lysed target cells. All data are representative of at least three independent experiments.

dendritic cells and can act on many cell types including B cells, T cells, NK cells, NK T cells, and even other dendritic cells and hematopoietic progenitor cells (Trinchieri, 2003). Studies comparing the response of NK cells that can or cannot sense IL-12, in a setting where pleiotrophic effects are reduced or eliminated, have not been done. Furthermore, long-lived memory NK cell generation in the absence of IL-12 signaling was not previously investigated. Using mice deficient in the IL-12R and STAT4, and a recently developed adoptive transfer system (Sun et al., 2009), we were uniquely able to investigate the direct in vivo role of IL-12 signaling in NK cells during MCMV infection, in the absence of any indirect effects.

RESULTS AND DISCUSSION

Similar phenotype and function of WT and *Il12rb2^{-/-}* NK cells at steady state

IL-12 is not required for NK cell development or homeostasis during steady state (i.e., the absence of inflammation, infection, or lymphopenia), as normal NK cell numbers are found in IL-12⁻ and IL-12R-deficient mice (Magram et al., 1996; Wu et al., 1997, 2000; Cousens et al., 1999). In accordance with prior studies, *Il12rb2^{-/-}* mice contained similar percentages of T, B, NK, and NK T cell populations when compared with WT mice (Fig. 1 a and not depicted). Within the NK cell compartment, WT and *Il12rb2^{-/-}* mice had similar percentages of Ly49D- and Ly49H-expressing cell subsets (Fig. 1 a). Furthermore, *Il12rb2^{-/-}* NK cells exhibited a phenotype similar to WT NK cells as determined by CD27, CD11b, KLRG1, and CD69 expression (Fig. 1 a). In the absence of infection,

WT and *Il12rb2^{-/-}* mice cleared β2m-deficient target cells equally well (Fig. 1 b), suggesting that *Il12rb2^{-/-}* NK cells exhibited normal in vivo cytotoxic function. When activating NK cell receptors were triggered with plate-bound antibodies, *Il12rb2^{-/-}* NK cells were able to degranulate similar to WT NK cells (Fig. 1 c). Lastly, *Il12rb2^{-/-}* NK cells were able to kill m157-bearing target cells as well as WT NK cells ex vivo (Fig. 1 d), demonstrating that Ly49H-mediated cytotoxicity is not dependent on IL-12 signaling at steady state.

Defective expansion of *Il12rb2^{-/-}* NK cells during MCMV infection

Previous studies examined the role of IL-12 on NK cells by one of three methods: injecting the IL-12 cytokine directly, blocking IL-12 with neutralizing antibodies, or infecting cytokine-deficient mice (Magram et al., 1996; Orange and Biron, 1996; Wu et al., 1997, 2000; Cousens et al., 1999; Pien and Biron, 2000). In each of these experimental systems, uncontrolled global effects are expected as a result of the expression of the IL-12R on many immune cell populations. Infection of IL-12-deficient mice results in higher viral titers (compared with WT mice) in many instances (Cousens et al., 1999). To circumvent this problem, we generated mixed bone marrow chimeric mice, where approximately half of the hematopoietic compartment expressed the IL-12Rβ2 and the other half was deficient. Reconstitution of all immune cell populations was found to be equally distributed between WT and *Il12rb2^{-/-}*, including the NK cell compartment (Fig. 2 a and not depicted).

After MCMV infection of mixed chimeric mice, WT NK cells preferentially expanded over 7 d and became the predominant NK cell subset in the spleen (Fig. 2 a). A roughly fivefold increase in total numbers of WT compared with *Il12rb2^{-/-}* NK cells was observed at day 7 post infection (PI; Fig. 2 b).

A similar outcome was observed in nonlymphoid organs such as the liver (unpublished data). The expansion of the WT NK cell compartment was a result of the rapid proliferation of Ly49H-bearing cells (Fig. 2 c). Although the uninfected chimeric mice contained similar numbers of WT and *Il12rb2*^{-/-} Ly49H⁺ NK cells, by 7 d PI the WT Ly49H⁺ NK cells were nearly 10-fold higher in absolute number compared with *Il12rb2*^{-/-} Ly49H⁺ NK cells (Fig. 2 d). Consistent with a role for IL-12 in IFN- γ induction, fewer *Il12rb2*^{-/-} NK cells produced IFN- γ compared with WT NK cells at day 1.5 PI, and the *Il12rb2*^{-/-} NK cells made less IFN- γ per cell (as measured by mean fluorescence intensity). Interestingly, WT and *Il12rb2*^{-/-} NK cells similarly up-regulated the activation marker CD69 (Fig. 2 e). Induction of CD69 expression correlates with type I IFN-mediated activation, and our current data supports a mechanism whereby IFN- γ secretion and CD69 expression result from two segregated signaling pathways even though both are determinants of activation status. Phosphorylation of the signaling component STAT4 has been shown to be a consequence of IL-12R signaling leading to IFN- γ induction (Trinchieri, 2003); thus, we examined splenic NK cells for phosphorylation of STAT4 early after infection. Whereas robust phosphorylation of STAT4 was observed in nearly all WT NK cells on day 1.5 PI, *Il12rb2*^{-/-} NK cells showed minimal levels of phosphorylated STAT4 (Fig. 2 e). Nearly half of the *Il12rb2*^{-/-} NK cells remained unphosphorylated for STAT4, at levels comparable to uninfected WT and *Il12rb2*^{-/-} mice (Fig. 2 e). Splenic T cells from the same infected mice showed minimal amounts of STAT4 phosphorylation in both WT and *Il12rb2*^{-/-} mice (unpublished data), consistent with previous findings that NK cells are the early and major producers of IFN- γ during infection (Yokoyama et al., 2004). At day 7 PI, WT Ly49H⁺ NK cells strongly up-regulated KLRG1, Ly6C, CD90 (Thy-1), and Ki67 (a nuclear marker of cellular proliferation) and down-regulated CD27, in contrast to *Il12rb2*^{-/-} Ly49H⁺ NK cells (Fig. 2 f), suggesting that IL-12 signals during MCMV infection are crucial toward achieving a full maturation program in virus-specific NK cells.

We determined whether the *Il12rb2*^{-/-} NK cells were expanding as extensively as WT NK cells, or were dying faster once activated. Adoptive transfer of CFSE-labeled WT or *Il12rb2*^{-/-} NK cells into Ly49H-deficient mice confirmed that both WT and *Il12rb2*^{-/-} NK cells proliferated after infection, with the WT NK cells dividing more extensively (Fig. 2 g). By day 6 PI, the WT NK cells had fully diluted their CFSE, whereas the *Il12rb2*^{-/-} NK cells remained intermediate for CFSE staining (Fig. 2 g). These results corroborate the higher expression of Ki67 detected on WT compared with *Il12rb2*^{-/-} NK cells at day 7 PI (Fig. 2 f). At day 4 PI, WT and *Il12rb2*^{-/-} NK cells showed comparable staining for Annexin V (Fig. 2 h), suggesting that a lack of IL-12 signal does not result in greater apoptosis.

IL-12 signals required for memory NK cell generation and protection during MCMV infection

A recent study demonstrated that although defective IL-12 signaling in CD8⁺ T cells also resulted in fewer effector cells,

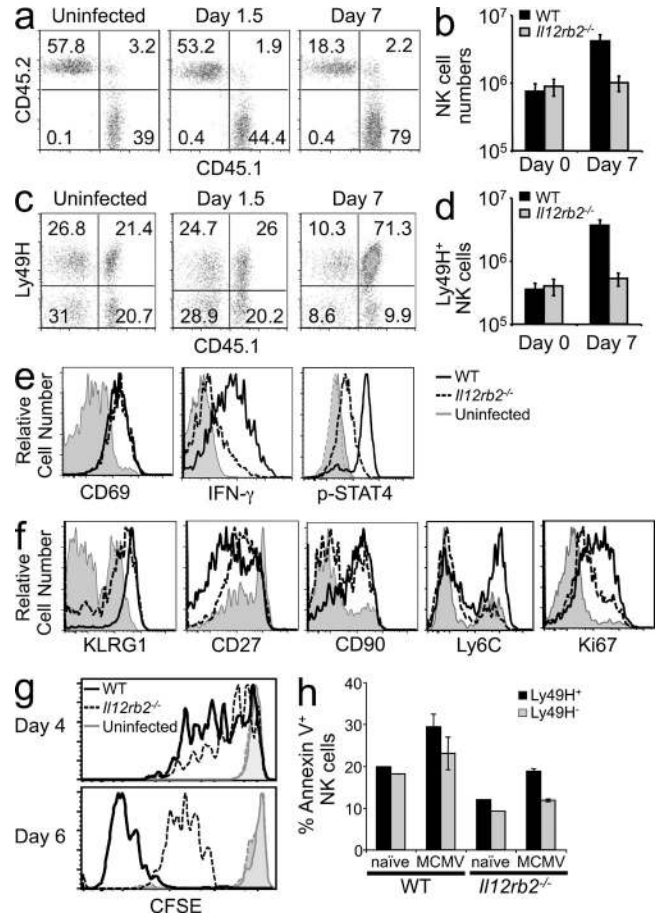


Figure 2. IL-12R-deficient NK cells exhibit defective proliferation during MCMV infection. (a) Mixed bone marrow chimeric mice were infected with MCMV and percentages of splenic WT (CD45.1⁺) and *Il12rb2*^{-/-} (CD45.2⁺) NK cells are shown (gated on CD3⁻ NK1.1⁺) in uninfected mice and various time points PI. (b) The absolute numbers of splenic WT and *Il12rb2*^{-/-} NK cells on day 0 and 7 PI are graphed. (c) Percentages of Ly49H⁺ cells within the WT and *Il12rb2*^{-/-} NK cell population (TCR- β ⁻ NK1.1⁺) are shown for uninfected mice and various time points PI. (d) The absolute numbers of WT and *Il12rb2*^{-/-} Ly49H⁺ NK cells on day 0 and 7 PI are graphed. (e) Expression of CD69, production of IFN- γ , and phosphorylation of STAT4 are shown for WT and *Il12rb2*^{-/-} NK cells (compared with uninfected mice) at day 1.5 PI. (f) Expression of KLRG1, CD27, CD90 (Thy-1), Ly6C, and Ki67 on WT and *Il12rb2*^{-/-} Ly49H⁺ NK cells (compared with uninfected mice) at day 7 PI. (g) WT or *Il12rb2*^{-/-} NK cells (CD45.2⁺) were labeled with 5 μ M CFSE and transferred into Ly49H-deficient hosts (CD45.1⁺). After MCMV infection, dividing NK cells were analyzed at days 4 and 6 PI (compared with uninfected control mice). (h) Adoptively transferred WT and *Il12rb2*^{-/-} NK cells were stained for Annexin V at days 0 and 4 PI. Percentages of Ly49H⁺ and Ly49H⁻ NK cells positive for Annexin V are shown. Error bars for all graphs show SEM ($n = 3-5$ for each time point) and all data are representative of five independent experiments.

surprisingly higher numbers of memory cells were generated (Pearce and Shen, 2007). Therefore, we investigated the long-term consequences of IL-12 signaling during the NK cell response against MCMV infection. Using a previously described adoptive transfer system (Sun et al., 2009), we purified NK cells from WT and *Il12rb2*^{-/-} mice and transferred an equal number

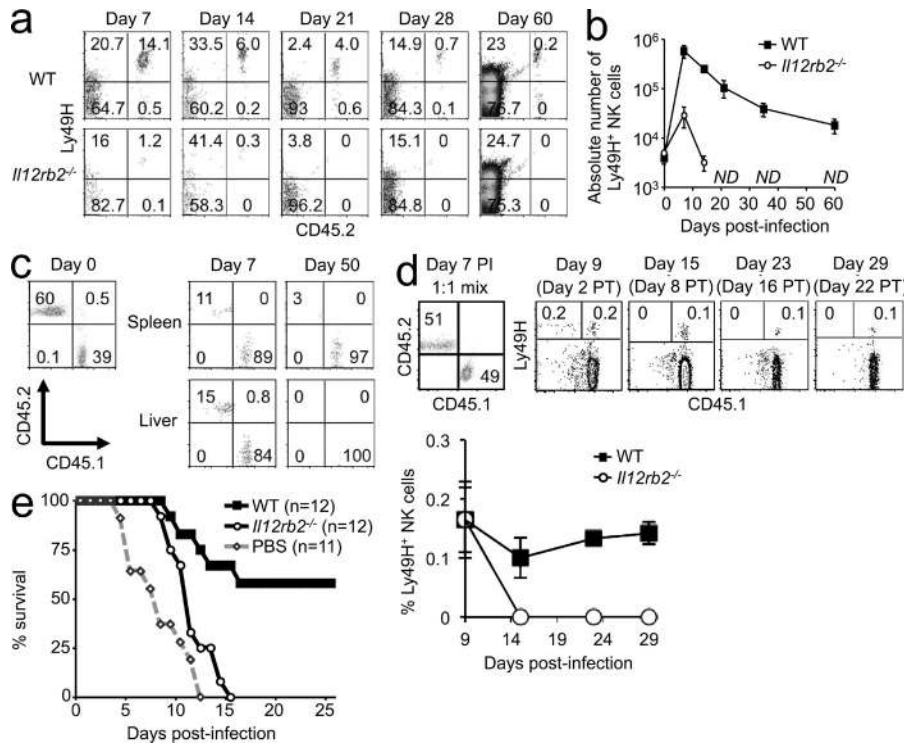


Figure 3. NK cells from *Il12rb2*^{-/-} mice fail to become long-lived memory cells and mediate protection after MCMV infection. (a and b) A total of 10⁵ WT or *Il12rb2*^{-/-} Ly49H⁺ NK cells (both CD45.2⁺) were transferred into DAP12-deficient mice (CD45.1⁺) and infected with MCMV. (a) Plots are gated on total NK cells and percentages of adoptively transferred CD45.2⁺ Ly49H⁺ NK cells are shown for each time point PI.

(b) Absolute numbers of adoptively transferred WT and *Il12rb2*^{-/-} Ly49H⁺ NK cells in the spleen of recipient mice are shown. Error bars show SEM (n = 3–5). ND, not detectable (or below the limits of detection). (c) WT (CD45.1⁺) and *Il12rb2*^{-/-} (CD45.2⁺) Ly49H⁺ NK cells were co-adoptively transferred into Ly49H-deficient mice and infected with MCMV. Plots are gated on transferred NK cells and percentages of WT and *Il12rb2*^{-/-} NK cells are shown for spleen and liver at days 0, 7, and 50 PI. All data are representative of five experiments with three to five mice per time point. (d) WT (CD45.1⁺) and *Il12rb2*^{-/-} (CD45.2⁺) mice were infected with MCMV and splenic Ly49H⁺ NK cells on day 7 PI were purified, mixed at a 1:1 ratio, and co-transferred into Ly49H-deficient mice. Plots are gated on transferred NK cells and

percentages of WT and *Il12rb2*^{-/-} NK cells are shown for at various time points PI and post transfer (PT). All data are representative of three experiments with two to four mice per time point. The graph shows the percentage of adoptively transferred WT and *Il12rb2*^{-/-} Ly49H⁺ NK cells within the total NK cell population, and error bars show SEM (n = 3–4). (e) DAP12-deficient neonatal mice received 10⁵ WT or *Il12rb2*^{-/-} NK cells (or PBS as control) followed by MCMV infection. The graph shows the percentage of surviving mice for each group, and data were pooled from three experiments.

of Ly49H⁺ NK cells from each group into separate DAP12-deficient hosts, which are deficient in Ly49H-expressing NK cells. After MCMV infection, we found that WT NK cells proliferated robustly in the new hosts, in contrast to *Il12rb2*^{-/-} NK cells (Fig. 3 a). The percentage and absolute number of WT Ly49H⁺ NK cells at day 7 PI were 15- to 20-fold higher than the *Il12rb2*^{-/-} NK cells (Fig. 3, a and b). Strikingly, although WT NK cells were easily recovered from recipient mice several weeks later, adoptively transferred *Il12rb2*^{-/-} NK cells were not detectable after week 2 PI (Fig. 3 b). Co-adoptive transfer of equal numbers of WT and *Il12rb2*^{-/-} NK cells into recipient mice yielded the same outcome after infection, with WT NK cells preferentially expanding at day 7 PI to become the only memory NK cells detected months later in both spleen and liver (Fig. 3 c). To determine whether fewer *Il12rb2*^{-/-} NK cells were recovered at later time point simply because they could not expand as well, equal numbers of WT and *Il12rb2*^{-/-} effector Ly49H⁺ NK cells isolated at day 7 PI (1:1 mix) were adoptively transferred and memory cell percentages determined at later time points (Fig. 3 d). Although the day 7 effector NK cell numbers were experimentally normalized, only WT NK cells were detected 1 mo later in recipient mice (Fig. 3 d). Thus, IL-12 signals are crucial not only for the optimal expansion of virus-specific NK cells during infection but also for the generation of a long-lived NK cell population in lymphoid and nonlymphoid tissues.

Because of the pleiotropic effects complicating the direct infection of cytokine-deficient mice, it is unknown whether defective IL-12 signaling in NK cells alone will influence the course of viral infection (without loss of the IL-12R on other cell types). Thus, we examined the contribution of IL-12R signaling specifically in NK cells toward protection against MCMV infection. We transferred an equal number of purified NK cells from WT or *Il12rb2*^{-/-} mice into neonatal DAP12-deficient mice. As a negative control, one group of neonates was given PBS without cells. All three groups were challenged with MCMV. Within the first 2 wk, all of the mice receiving PBS or purified *Il12rb2*^{-/-} NK cells had died (Fig. 3 e). During the same period, >50% of the mice given purified WT NK cells survived (Fig. 3 e). Together, the data suggest that IL-12 signaling in NK cells alone is crucial for protective immunity against viral challenge. Interestingly, the mice receiving *Il12rb2*^{-/-} NK cells died with delayed kinetics (days 9–15 PI) compared with mice receiving PBS (days 5–12 PI), perhaps because the *Il12rb2*^{-/-} NK cells could still mediate cytotoxicity against infected cells; however, their inability to undergo proliferation similar to WT NK cells might have prevented a more efficacious response.

NK cell memory depends on STAT4 signals but not IFN- γ secretion

We investigated the importance of effector molecules downstream of the IL-12R in the generation of long-lived memory

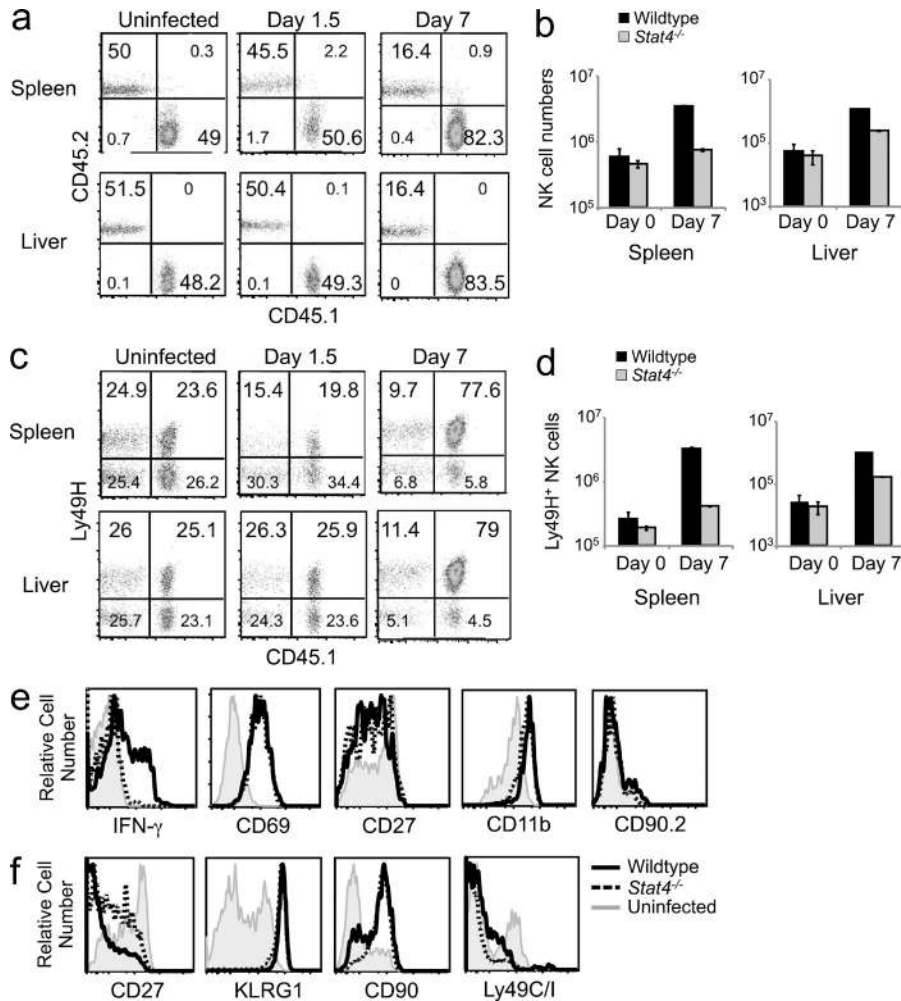


Figure 4. WT NK cells outcompete *Stat4*^{-/-} NK cells during MCMV infection. (a) Mixed bone marrow chimeric mice were infected with MCMV and percentages of WT (CD45.1⁺) and *Stat4*^{-/-} (CD45.2⁺) NK cells in spleen and liver are shown for uninfected mice and various time points PI. (b) The graph shows absolute numbers of WT and *Stat4*^{-/-} NK cells in spleen and liver on day 0 and 7 PI. Error bars show SEM ($n = 3-5$). (c) Percentages of Ly49H⁺ cells within the WT and *Stat4*^{-/-} NK cell population in spleen and liver are shown for uninfected mice and various time points PI. (d) The graph shows absolute numbers of WT and *Stat4*^{-/-} Ly49H⁺ NK cells in spleen and liver on day 0 and 7 PI. Error bars show SEM ($n = 3-5$). (e) Expression of CD69, CD27, CD11b, and CD90, and production of IFN- γ are shown for WT and *Stat4*^{-/-} NK cells (compared with uninfected mice) at day 1.5 PI. (f) Expression of KLRG1, CD90, Ly49C/I, and CD27 are shown for WT and *Stat4*^{-/-} Ly49H⁺ NK cells (compared with uninfected mice) at day 7 PI. All data are representative of three experiments with three to five mice per time point.

NK cells during MCMV infection. Because *Il12rb2*^{-/-} NK cells do not efficiently phosphorylate STAT4 or secrete as much IFN- γ as WT NK cells during MCMV infection (Fig. 2 e), we examined the contribution of both STAT4 and IFN- γ on the production of effector and memory Ly49H⁺ NK cells. To elucidate the importance of downstream effector molecule STAT4 on in vivo NK cell activation and response against MCMV, we generated mixed bone marrow chimeric mice. Reconstitution of all immune populations, including the NK compartment, was found to be equally distributed between WT and *Stat4*^{-/-} (Fig. 4 a and not depicted). After MCMV infection, WT NK cells preferentially expanded over 7 d to constitute the main subset in the spleen and liver, exhibiting fivefold greater numbers compared with *Stat4*^{-/-} NK cells (Fig. 4, a and b), similar to that seen for mixed WT:*Il12rb2*^{-/-} bone marrow chimeras (Fig. 2 a). In both organs, the prolific expansion of WT NK cells can be attributed to the proliferation of Ly49H-bearing cells (Fig. 4 c). Although uninfected mice had comparable numbers of WT and *Stat4*^{-/-} NK cells, by day 7 PI the WT Ly49H⁺ NK cells outnumbered their *Stat4*^{-/-} counterparts in absolute numbers by ~10-fold in spleen and liver (Fig. 4 d). Early after infection, *Stat4*^{-/-}

NK cells produced less IFN- γ than WT NK cells, even though CD69 was similarly up-regulated in both subsets at day 1.5 PI (Fig. 4 e). Although WT and *Stat4*^{-/-} NK cells similarly down-regulated CD27 and up-regulated CD11b at day 1.5 PI, phenotypic differences were evident at day 7 PI in spleen and liver, with WT NK cells strongly down-regulating CD27 and up-regulating KLRG1 (Fig. 4 e). Together, these findings suggest that IL-12-induced signaling in virus-specific NK cells primarily uses the downstream STAT4 to initiate vital signals for a complete cell maturation program during MCMV infection. Interestingly, both WT and *Stat4*^{-/-} NK cells at day 7 PI highly expressed CD90 (Thy-1; Fig. 4 f), a marker described to be present on memory NK cells (O'Leary et al., 2006; Paust et al., 2010), and were predominantly Ly49C/I⁻ (Fig. 4 f), consistent with previous data demonstrating that Ly49C/I⁻ or "unlicensed" NK cells in C57BL/6 mice dominate the NK cell response to MCMV (Orr et al., 2010).

We investigated the extent to which signaling through STAT4 influenced the generation of memory NK cells during MCMV infection. We transferred an equal number of Ly49H⁺ NK cells purified from WT (CD45.1) and *Stat4*^{-/-} (CD45.2) mice into a Ly49H-deficient host (CD45.2). Transferred WT Ly49H⁺ NK cells proliferated robustly after MCMV infection, in contrast to the minimal expansion of transferred *Stat4*^{-/-} Ly49H⁺ NK cells in the same host (Fig. 5 a). The percentage of WT NK cells at day 7 PI was ~10-fold higher than that of the co-transferred *Stat4*^{-/-} counterparts (Fig. 5 b), highlighting

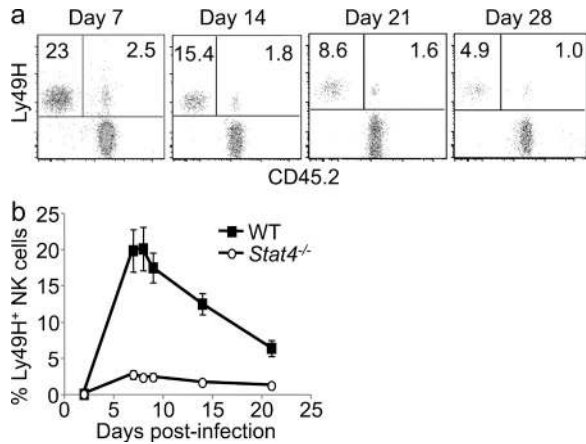


Figure 5. Defective memory NK cell generation in the absence of STAT4. (a) A total of 10⁵ WT (CD45.1⁺) and *Stat4*^{-/-} (CD45.2⁺) Ly49H⁺ NK cells were co-transferred into Ly49H-deficient mice (CD45.2⁺) and infected with MCMV. Plots are gated on total NK cells and percentages of adoptively transferred Ly49H⁺ NK cells (WT in top left quadrant and *Stat4*^{-/-} in top right quadrant) are shown for each time point PI. (b) Percentage of adoptively transferred WT and *Stat4*^{-/-} Ly49H⁺ NK cells within the total NK cell population are shown. Error bars show SEM (*n* = 3). All data are representative of three experiments with three to four mice per time point.

the importance of signaling through STAT4 in the expansion of virus-specific NK cells after MCMV infection and in the attainment of a long-lived memory NK cell population.

Lastly, we examined a possible autocrine role for IFN- γ produced by NK cells during infection. Because WT NK cells produced more IFN- γ than *Il12rb2*^{-/-} or *Stat4*^{-/-} NK cells early during MCMV infection, could this IFN- γ feed back on the NK cells themselves and drive stronger proliferation and generation of memory cells? We first generated mixed bone marrow chimeric mice consisting of a 1:1 ratio of WT:*Ifngr*^{-/-} hematopoietic cells. After MCMV infection of chimeric mice, WT and *Ifngr*^{-/-} NK cells proliferated similarly over the first 7 d (Fig. 6 a), with nearly equal ratio of WT to *Ifngr*^{-/-} NK cells. On day 7 PI, similar percentages of Ly49H-bearing cells existed in total WT and *Ifngr*^{-/-} NK cell populations (Fig. 6 a), suggesting that IFN- γ does not influence the ability of NK cells themselves to undergo expansion. On day 1.5 PI, expression of CD69 and secretion of IFN- γ were also independent of IFN- γ receptor expression on the NK cell (Fig. 6 b). Similarly, no phenotypic differences were observed between WT and *Ifngr*^{-/-} NK cells at day 7 PI (unpublished data). When we co-transferred equal numbers of enriched WT and *Ifngr*^{-/-} NK cells into recipient mice followed by MCMV infection, we observed a similar expansion at day 7 PI and equal percentages of memory NK cells at day 50 PI (Fig. 6 c). Altogether, these data suggest that the robust generation of NK cell memory during MCMV infection requires STAT4-dependent but IFN- γ independent signals.

The recent discovery that primed NK cells can become long-lived memory cells during infection begs the question of what the mechanisms are behind such a phenomena. Past studies using neutralizing antibodies and cytokine-deficient mice did not specifically address whether there was an in vivo

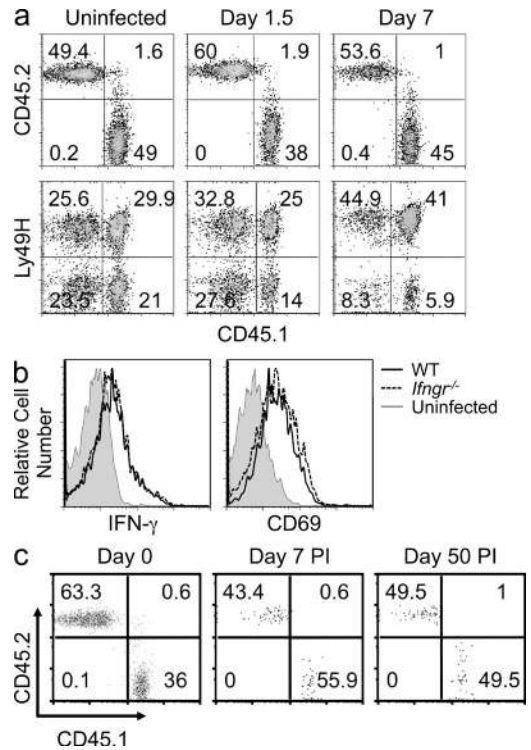


Figure 6. WT and *Ifngr*^{-/-} NK cells expand equally and generate memory cells after MCMV infection. (a) Mixed bone marrow chimeric mice consisting of a 1:1 mixture of WT (CD45.1⁺) and *Ifngr*^{-/-} (CD45.2⁺) hematopoietic cells were infected with MCMV. Top row: percentages of total WT and *Ifngr*^{-/-} NK cells (gated on CD3⁻ NK1.1⁺) are shown for uninfected mice and at day 1.5 and 7 PI. Bottom row: percentages of Ly49H⁺ cells within the WT and *Ifngr*^{-/-} NK cell populations are shown. (b) Production of IFN- γ and expression of CD69 are shown for WT and *Ifngr*^{-/-} NK cells in chimeric mice (compared with uninfected chimeras) at day 1.5 PI. (c) WT (CD45.1⁺) and *Ifngr*^{-/-} (CD45.2⁺) Ly49H⁺ NK cells were co-adoptively transferred into Ly49H-deficient mice and infected with MCMV. Plots are gated on transferred NK cells and percentages of WT and *Ifngr*^{-/-} NK cells are shown for day 0, 7, and 50 PI. All data are representative of three experiments with three to five mice per time point.

role for IL-12 on NK cells independent of global effects on other cell types (Magrath et al., 1996; Orange and Biron, 1996; Wu et al., 1997, 2000; Cousens et al., 1999; Pien and Biron, 2000). Although these previous studies found a role for IL-12 in activating NK cells, our current study demonstrates the absolute requirement for both IL-12 and STAT4 in the clonal expansion of antigen-specific NK cells and the generation of memory NK cells during MCMV infection.

The amount of inflammation in the host environment during CD8⁺ T cell priming has been suggested to dictate the production of effector and memory cells. In recent studies where the degree of inflammation was varied while antigen concentration was kept constant, memory CD8⁺ T cell potential was determined by a gradient of T-bet expression in which moderate inflammation correlated with robust memory cells, whereas high inflammation correlated with short-lived effector cells and reduced memory potential (Joshi et al., 2007;

Pipkin et al., 2010). Consistent with this study, another group showed that IL-12-deficient mice produced a diminished effector CD8⁺ T cell response during *Listeria monocytogenes* infection but generated higher numbers of memory CD8⁺ T cells and greater protection against reinfection (Pearce and Shen, 2007). However, when mice receiving WT or IL-12R-deficient TCR transgenic CD8⁺ T cells were infected with viral pathogens, the conclusions were less clear. One study observed normal clonal expansion but diminished memory responses in CD8⁺ T cells that could not sense IL-12 during vaccinia virus infection (Xiao et al., 2009), whereas another study showed normal effector and memory responses during infection with vaccinia virus, LCMV, and VSV (Keppler et al., 2009). These discrepancies may be a result of different TCR transgenic mice and recombinant pathogens used in the studies, but they highlight the need to resolve these inconsistencies.

In contrast to the inconclusive role of IL-12 on T cells, the NK cell response in the absence of IL-12 signaling resulted in a severe detriment in both effector and memory NK cell numbers. During MCMV infection, the IL-12R-deficient Ly49H⁺ NK cells showed a remarkable 10- to 20-fold reduction in absolute number of effector cells in spleen and liver compared with WT NK cells, and a complete deficiency in memory cells. Even when effector NK cell numbers (isolated at day 7 PI) were normalized, only WT NK cells could be detected at later time points after adoptive transfer (Fig. 3 d), suggesting that IL-12 signaling programs NK cells during priming to demonstrate longevity after activation and expansion. Unlike CD8⁺ T cells that expanded less in the absence of IL-12 but generated higher memory cell numbers (Pearce and Shen, 2007), our results suggest an absolute requirement for IL-12 signals in the generation of memory NK cells. These findings are strengthened by the severe defect in clonal expansion and memory cell formation in STAT4-deficient NK cells. Interestingly, we do observe some low level phosphorylation of STAT4 in the *Il12rb2*^{-/-} NK cells compared with WT NK cells (Fig. 2 e), suggesting that other cytokines produced during MCMV infection may share the STAT4 signaling pathway. IFN- α/β has been shown to activate STAT4 (in addition to STAT1) in T cells (Nguyen et al., 2002); however, findings in IFN- α receptor-deficient mice that STAT4 is normally phosphorylated during MCMV infection suggest that IL-12 signaling is the predominant pathway for STAT4 phosphorylation leading to IFN- γ production in NK cells (Nguyen et al., 2002; unpublished data). Developing a system for NK cells where we can vary the inflammation while keeping the antigen dose constant will allow us to determine whether, like CD8⁺ T cells, there exist optimal and suboptimal degrees of inflammation that drive generation of effector and memory NK cells. In addition, the molecular mechanisms behind NK cell programming by IL-12 during MCMV infection remain to be elucidated. Future studies will determine how IL-12 signaling influences histone acetylation and chromatin remodeling at promoter sites of genes important for the generation of NK cell memory.

Like NK cells, memory CD8⁺ T cells surprisingly possess the innate-like ability to produce IFN- γ and proliferate after

exposure to proinflammatory cytokines alone, without TCR and co-stimulatory receptor ligation (Berg and Forman, 2006). Given their relatedness during ontogeny (both are derived from a common lymphoid progenitor) and possible shared evolutionary ancestry, perhaps it is not surprising that parallel mechanisms exist in NK cells and T cells for their activation, proliferation, and generation of memory during infection. The importance of environmental cues, in addition to antigen receptor triggering, is becoming more evident, and a greater understanding of these mechanisms will allow for development of therapeutics and vaccines against infectious disease.

MATERIALS AND METHODS

Mice and infections. C57BL/6 and congenic CD45.1⁺ mice were purchased from the National Cancer Institute. IL-12R β 2 chain-deficient (Wu et al., 2000; provided by J. DeRisi, University of California, San Francisco [UCSF], San Francisco, CA), STAT4-deficient (Kaplan et al., 1996), IFN- γ receptor-deficient, β 2m-deficient, Ly49H-deficient (Fodil-Cornu et al., 2008; provided by S. Vidal, McGill University, Montreal, Canada), and DAP12-deficient (Bakker et al., 2000) mice on a C57BL/6 background were bred at the UCSF and Memorial Sloan Kettering Cancer Center and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Mixed bone marrow chimeric mice were generated as described previously (Sun et al., 2009). Mice were infected by intraperitoneal injections of Smith strain MCMV (5×10^4 PFU). Newborn mice were infected with 2×10^3 PFU of MCMV.

Flow cytometry. Fc receptors were blocked with 2.4G2 mAb before surface or intracellular staining with indicated antibodies or isotype-matched control immunoglobulin (BD, eBioscience, or BioLegend). For measuring apoptosis, freshly isolated splenocytes were first stained with antibodies against NK1.1, CD3, and Ly49H, and a Live/Dead fixable near-IR stain (Invitrogen), washed, and then stained with PE-conjugated Annexin V (BD), according to the manufacturer's protocol. Samples were acquired on an LSRII (BD) and analyzed with FlowJo software (Tree Star).

NK cell enrichment, CFSE labeling, and adoptive transfer. NK cells were enriched with an NK Cell Isolation kit (Miltenyi Biotec) or a method where spleen cells were incubated with purified rat antibodies against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119, followed by anti-rat IgG antibodies conjugated to magnetic beads (QIAGEN). Purified NK cells were injected intravenously into adult recipients or intraperitoneally into neonatal recipients 1 d before viral infection. In some experiments, NK cells or unfractionated splenocytes were labeled with varying concentrations of CFSE before intravenous injection. Labeling of cells with CFSE was performed in accordance with the manufacturer's instructions (Invitrogen).

Ex vivo stimulation of NK cells and cytotoxicity assay. Tissue culture plates treated with *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium methylsulphate (Sigma-Aldrich) were coated with 10 μ g/ml anti-NK1.1 or anti-Ly49H antibodies (provided by W. Yokoyama, Washington University, St. Louis, MO), and enriched NK cells or whole splenocytes were incubated for 5 h at 37°C in the presence of GolgiPlug (BD), followed by staining for intracellular cytokines. Uncoated or PBS-coated wells served as negative controls, and addition of 50 ng/ml PMA and 1 μ g/ml ionomycin during incubation served as a positive control.

Enriched NK cells were used as effector cells *ex vivo* in a 6-h ⁵¹Cr release assay against Ba/F3 and m157-transfected Ba/F3 target cells. Percentage of Ly49H⁺ NK cells in each group was determined by flow cytometry and absolute numbers were normalized before incubation with targets. Large numbers of NK cells from Ly49H-deficient mice were included as a negative control to demonstrate specificity of receptor-ligand interactions.

Statistical methods. The Mann–Whitney nonparametric *U* test was used to compare survival between groups of mice. A value of 25 d was assigned to survivors living >25 d after MCMV infection. Student's *t* test was used to compare groups in ex vivo stimulation experiments.

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