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DAP12 Signaling Directly Augments Proproliferative Cytokine Stimulation of NK Cells during Viral Infections[1]

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NK cells vigorously proliferate during viral infections. During the course of murine CMV infection, this response becomes dominated by the preferential proliferation of NK cells that express the activation receptor Ly49H. The factors driving such selective NK cell proliferation have not been characterized. In this study, we demonstrate that preferential NK cell proliferation is dependent on DAP12-mediated signaling following the binding of Ly49H to its virally encoded ligand, m157. Ly49H signaling through DAP12 appears to directly augment NK cell sensitivity to low concentrations of proproliferative cytokines such as IL-15. The impact of Ly49H-mediated signaling on NK cell proliferation is masked in the presence of high concentrations of proproliferative cytokines that nonselectively drive all NK cells to proliferate. ***The Journal of Immunology,*** **2006, 177: 4981–4990.**

Natural killer cells play a crucial role in the initial host defense against pathogens and are particularly important in responding to viruses (reviewed in Refs. 1 and 2). In mice, resistance to a number of viruses, including murine CMV (MCMV),[3] herpes simplex type-1, and ectromelia viruses, genetically maps to the NK gene complex on mouse chromosome six (3–5). The Cmv1^r resistance locus for MCMV was determined to encode the NK cell activation receptor, Ly49H (6–8). Mice expressing this NK cell receptor, such as C57BL/6 (B6) mice or BALB/c mice reconstituted with *Ly49h* by transgenesis, are significantly more resistant to MCMV infection than mice that lack Ly49H expression (3, 6, 7, 9). Ly49H is a C-type lectin-like receptor whose expression and signaling are dependent on the ITAM-containing adaptor DAP12, also known as killer-activating receptor-associated protein or TYROBP (10, 11). Ly49H specifically recognizes the MCMV-encoded protein, m157, on infected cells (12–14). Ly49H recognition of m157 and subsequent signaling through DAP12 stimulate NK cell-mediated cytokine/chemokine production and killing of infected cells substantially, enhancing host resistance to MCMV (6, 12–17).

In addition to these effector functions, NK cells rapidly proliferate during viral infections, resulting in significantly expanded numbers of peripheral NK cells (15, 18–21). This expansion occurs in two distinct phases, including an early nonspecific phase of NK cell proliferation, followed by a phase of preferential proliferation of NK cells that are able to recognize infected cells (15). The initial stage of nonspecific NK cell proliferation is presumably driven by cytokines induced early during viral infections in a similar manner to the nonspecific bystander proliferation observed in T cells during a viral infection or following poly(I:C) treatment (22, 23). The later specific phase is illustrated by the preferential proliferation of Ly49H$^+$ NK cells during MCMV infection (15). Selective NK cell proliferation has also been observed during infection with the poxvirus vaccinia, demonstrating that this is not an MCMV- or even herpesvirus-restricted phenomenon (15). After resolution of the infection, the NK cell population contracts back to steady-state levels (20, 24).

The cytokines stimulating in vivo NK cell proliferation during infections are not well characterized, although there is evidence implicating the up-regulation of IL-15 by IFN-$\alpha\beta$ in stimulating early, nonselective NK cell proliferation (19, 22, 25). However, efforts to directly delineate the contribution of IL-15 to viral-induced NK cell proliferation have been limited by the paucity of available reagents and the absence of NK cells in IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ mice (26, 27). In addition, the complex interactions between IL-15 and its trimeric receptor have also hampered the investigation of the role of IL-15 in stimulating NK cell proliferation in vivo (28). For example, the coordinate expression of IL-15 and IL-15Rα and subsequent *trans* presentation to cells expressing the dimeric IL-2/15Rβ common γ-chain receptor complex allow IL-15 stimulation to be tightly controlled and localized (29), but make quantification of IL-15 levels very difficult.

The factors driving the specific proliferation and expansion of the subset of NK cells that recognize infected cells during viral infections are even less well characterized and may involve cytokine

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[3] Abbreviations used in this paper: MCMV, murine CMV; DAP12, DNAX-activation protein 12; p.i., postinfection; SI, selectivity index; wt, wild type.

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stimulation and/or specific NK cell activation receptor-dependent signaling. For example, a recent report suggested that the specific expansion of splenic Ly49H$^+$ NK cells during MCMV infection was IL-18 dependent (30). Previous work in our laboratory indicated that the selective proliferation of Ly49H$^+$ NK cells during MCMV infection was dependent on Ly49H recognition of infected cells (15). Pretreatment of B6 mice with F(ab′)$_2$ of anti-Ly49H mAbs before MCMV infection resulted in significant reductions in overall NK cell proliferation and absolute NK cell numbers late during MCMV infection. Although this observation implicated Ly49H in contributing to selective NK cell proliferation, the anti-Ly49H treatment precluded the direct assessment of proliferation in the Ly49H$^+$ and Ly49H$^-$ NK cell subsets due to residual bound F(ab′)$_2$. We have also recently reported that infection of B6 mice with m157-defective MCMV resulted in no accumulation of splenic Ly49H$^+$ NK cells at day 4 or 5 postinfection (p.i.), providing indirect evidence that preferential proliferation is dependent on the interaction of Ly49H with m157 (31). However, neither of these studies conclusively demonstrated that the interaction between Ly49H and m157 was required for selective NK cell proliferation nor provided insight into how Ly49H-mediated recognition of infected cells resulted in preferential NK cell proliferation.

We hypothesized that Ly49H recognition of m157 on infected cells would cause differential responsiveness to cytokines other than those involved in stimulating early, nonspecific NK cell proliferation. This differential cytokine responsiveness could result from up-regulation of cytokine receptors on Ly49H$^+$ NK cells that were absent on Ly49H$^-$ NK cells in an analogous manner to the up-regulation of IL-2Rs on T cells following engagement of the TCR (32). Alternatively, Ly49H activation could alter the response threshold for proproliferative cytokines, such as IL-15. We tested these hypotheses and found that Ly49H signaling mediated through DAP12 directly augments NK sensitivity to low concentrations of proproliferative cytokines, such as IL-15, resulting in preferential Ly49H$^+$ NK cell proliferation.

Materials and Methods

Mice

B6, C57BL/6.RAG1$^{-/-}$ (RAG$^{-/-}$), C57BL/6.SCID (SCID), C57BL/6.IL-2$^{-/-}$ (IL-2$^{-/-}$), C57BL/6.IL-4$^{-/-}$ (IL-4$^{-/-}$), C57BL/6.IL-10$^{-/-}$ (IL-10$^{-/-}$), C57BL/6.IL-12$^{-/-}$ (IL-12$^{-/-}$), C57BL/6.IL-18$^{-/-}$ (IL-18$^{-/-}$), and C57BL/6.IL-18R$\alpha^{-/-}$ (IL-18R$^{-/-}$) mice were obtained from The Jackson Laboratory. B6 mice from National Cancer Institute (Charles River Laboratories) were also used. C57BL6.IL-15R$\alpha^{-/-}$ (IL-15R$\alpha^{-/-}$) (26) mice were generated in the Ma laboratory (University of California). C57BL/6.DAP12 loss-of-function knock-in (DAP12^{KI}) (33) mice were generated in the Vivier laboratory (Centre National de la Recherche Scientifique-Institut National de la Sante et de la Recherche Medicale-Universite de la Mediterranee). C57BL/6.IL-21R$^{-/-}$ (IL-21R$^{-/-}$) (34) mice were provided by Wyeth-Ayerst Pharmaceuticals. Mice were maintained under specific pathogen-free conditions and used between 8 and 16 wk of age. All experiments were conducted in accordance with institutional guidelines for animal care and use.

IL-15Rα-deficient chimeric mice

IL-15Rα-deficient chimeric mice were generated, as previously described (35). Briefly, bone marrow was harvested from IL-15R$\alpha^{-/-}$ mice that had been backcrossed to B6 mice for at least eight generations or from wild-type (wt) B6 mice. Recipient B6 mice were irradiated with 950 rad total body irradiation, and then reconstituted with 3–6 $\times$ 10^6 bone marrow cells i.v. injected within 1 h of irradiation. NK cell chimerism was complete as determined by Ly-5.1 or IL-15Rα expression. Eight or more weeks after bone marrow transplant, the IL-15Rα-deficient chimeric mice as well as control chimeric mice reconstituted with wt bone marrow were infected with MCMV.

Abs and cell lines

The 3D10 Ab (anti-Ly49H) (36) was generated and biotinylated in our laboratory. PerCP-conjugated anti-CD3 (145-2C11), allophycocyanin-conjugated anti-NK1.1 (PK136), and FITC-conjugated anti-BrdU Abs as well as PE-streptavidin were all purchased from BD Pharmingen. Goat polyclonal, affinity-purified, biotinylated murine anti-IL-15Rα, anti-IL-17R, anti-IL-18Rα, and anti-IL-21R Abs were purchased from R&D Systems, and control polyclonal goat biotinylated Abs were purchased from Caltag Laboratories. The generation and characterization of BaF3 cells transfected with m157 (BaF3/m157) or with an empty vector (BaF3) have been previously described (13). RMA cells transfected with m157 (RMA/m157) were generated following the protocol used in the generation of BaF3/m157 transfectants (13). The generation and characterization of anti-m157 F(ab′)$_2$ and isotype control F(ab′)$_2$ have been previously described (37).

CFSE in vitro proliferation assays

Single-cell suspensions of splenocytes were prepared using standard techniques (15). NK cells from B6 and DAP12^{KI} splenocytes were enriched from ~2.5 to 30–65% NK1.1$^+$CD3$^-$ lymphocytes following negative selection with magnetic beads (Miltenyi Biotec NK cell negative selection kit). RAG$^{-/-}$ or SCID splenocytes (typically ~30% NK1.1$^+$CD3$^-$) or enriched B6 or DAP12^{KI} splenocytes were labeled with 2 μM CFSE (Molecular Probes) for 6 min at 37°C. The reaction was quenched by adding RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (HyClone), and the cells were subsequently incubated for 30 min at 37°C per the manufacturer's instructions. The CFSE-labeled splenocytes were plated in 96-well plates and cultured for 3 days in various concentrations of murine IL-15 (PeproTech) or low concentrations of IL-15 with either IL-2 (Chiron) or murine IL-18 (R&D Systems). BaF3 or BaF3/m157 cells were added to the wells over a range of E:T ratios (typically 3:1; 1.6:1; 0.8:1).

MCMV and infection of mice

Smith and K181 strains of MCMV were gifts from H. Virgin (Washington University, St. Louis, MO). A salivary gland stock of MCMV was prepared from young BALB/c mice that had been i.p. injected with 1 $\times$ 10^6 PFU tissue culture-propagated MCMV, and the titer of the stock was determined via standard plaque assay (6) using permissive NIH 3T12 fibroblasts (American Type Culture Collection). For in vivo experiments, mice were injected i.p. with 2 $\times$ 10^3 to 1 $\times$ 10^5 PFU/mouse salivary gland Smith MCMV stock or salivary gland K181 MCMV stock.

Splenocyte preparation, intracellular staining, and flow cytometry

To minimize the complication of BrdU$^+$ NK cells from the bone marrow emigrating to the spleen, we used an acute pulse of BrdU (2 mg/mouse) injected i.p. 3 h before euthanizing the mice at various time points after infection rather than prolonged BrdU exposure in the drinking water. Single-cell suspensions of splenocytes were prepared using standard techniques (15). Splenocytes were incubated in 2.4G2 (anti-FcγRII/III) supernatants (hybridoma from American Type Culture Collection) before staining with labeled Abs to block nonspecific binding of Abs to FcRs. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen), treated with DNase, and then stained with FITC-conjugated anti-BrdU Abs. Analysis was performed with a FACSCalibur flow cytometer (BD Pharmingen) gating on the NK1.1$^+$CD3$^-$ lymphocyte subpopulation, and the data were analyzed with CellQuest (BD Pharmingen).

Selectivity index (SI)

An SI to assess the extent of preferential Ly49H$^+$ NK cell proliferation was defined as the ratio of the percentage of Ly49H$^+$ NK cells that were BrdU$^+$ (fraction of overall NK cell population that was Ly49H$^+$ and BrdU$^+$ divided by the fraction of the overall NK cell population that was Ly49H$^+$) compared with the percentage of Ly49H$^-$ NK cells that were BrdU$^+$ (fraction of overall NK cell population that was Ly49H$^-$ and BrdU$^+$ divided by the fraction of the overall NK cell population that was Ly49H$^-$). An SI value of 1 denoted equal proliferation in Ly49H$^+$ and Ly49H$^-$ NK cell subsets. An SI value greater than 1 represented preferential proliferation of Ly49H$^+$ NK cells, and conversely an SI value less than 1 denoted less proliferation occurring in the Ly49H$^+$ NK cell subset compared with the Ly49H$^-$ NK cell subset.

Results

Selective NK cell proliferation during MCMV infection

We previously observed two distinct phases of MCMV-induced NK cell proliferation with an early, nonspecific proliferative response, followed by preferential Ly49H$^+$ NK cell proliferation in both the spleen and liver (15). To more carefully delineate the time course of these responses as well as to demonstrate their robustness and reproducibility, we analyzed acute BrdU incorporation in splenic NK cells from infected B6 mice in more than 35 independent experiments performed over a 3-year period (Fig. 1*A*). Splenic NK cells from naive mice were relatively quiescent undergoing only low levels of proliferation. Following MCMV infection, NK cell proliferation rapidly increased, consistently peaked at days 4–5 p.i. when ~40% of the splenic NK cells incorporated BrdU over a 3-h period, and then waned after 6–7 days p.i. (Fig. 1*A*).

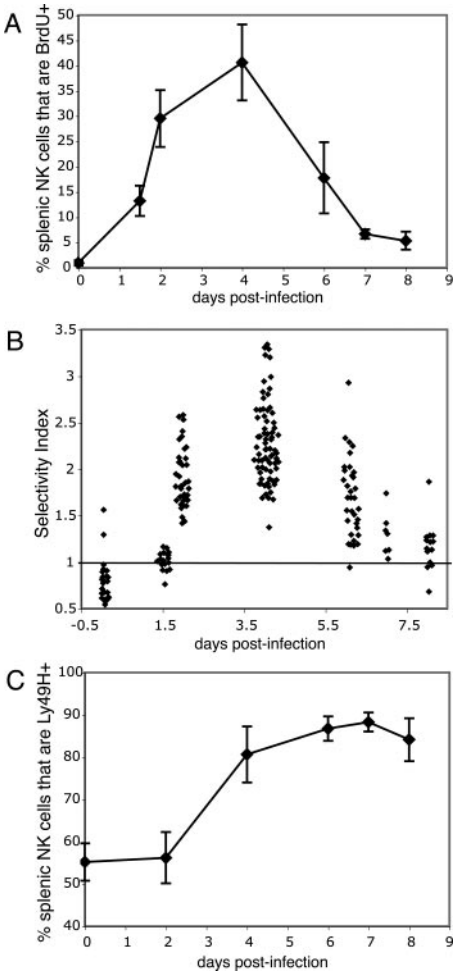

FIGURE 1. Selective NK cell proliferation during MCMV infection. *A*, Cumulative results (average ± SD) are shown of splenic NK cell (NK1.1$^+$CD3$^-$) proliferation assessed by intracellular staining for acute BrdU incorporation at various times following MCMV infection (5×10^4 PFU/mouse) in B6 mice from 36 independent experiments ($n = 25$ mice at day 0, $n = 18$ mice at day 1.5, $n = 38$ mice at day 2, $n = 73$ mice at day 4, $n = 34$ mice at day 6, $n = 7$ mice at day 7, and $n = 14$ mice at day 8). *B*, Cumulative representation of splenic SI values for 209 individual B6 mice in 36 experiments at different days following MCMV infection. SI was defined as the ratio of the percentage of Ly49H$^+$ NK cells that were BrdU$^+$ compared with the percentage of Ly49H$^-$ NK cells that were BrdU$^+$. An SI value >1 represents preferential proliferation of Ly49H$^+$ NK cells. *C*, Selective expansion of splenic Ly49H$^+$ NK cells (average ± SD) during MCMV infection illustrated by the percentage of splenic NK cells that are Ly49H$^+$ at various days during the course of MCMV infection in B6 mice from 36 independent experiments.

We were particularly interested in characterizing the selective phase of NK cell proliferation. To more precisely describe and quantify the extent of preferential Ly49H$^+$ NK cell proliferation that occurred during MCMV infection in these separate experiments, an SI was defined by comparing the percentage of the Ly49H$^+$ NK cells that were proliferating with the percentage of Ly49H$^-$ NK cells that were proliferating. SI values were then calculated for splenic NK cells from individual mice at various time points during the course of MCMV infection (Fig. 1*B*). In naive mice, Ly49H$^+$ NK cells were slightly less likely to be proliferating than Ly49H$^-$ NK cells (SI = 0.8 ± 0.23; $n = 25$ mice). Early during infection (day 1.5 p.i.), the nonspecific phase of NK cell proliferation was represented by SI values of 1 (1.02 ± 0.1; $n = 18$ mice), illustrating that Ly49H$^+$ and Ly49H$^-$ NK cells were proliferating to similar extents. However, as early as 48 h and extending to 6 days p.i., the preferential proliferation of Ly49H$^+$ NK cells was demonstrated by SI values >1. The average SI value of 2.3 ± 0.42 ($n = 73$ mice) on day 4 p.i. illustrated that Ly49H$^+$ NK cells were proliferating at greater than twice the frequency of Ly49H$^-$ NK cells. The preferential Ly49H$^+$ NK cell proliferation observed during MCMV infection contrasted with the absence of preferential proliferation seen in Ly49D$^+$ NK cells, as evidenced by an average Ly49D$^+$ NK cell SI value of 1.05 ± 0.08 ($n = 15$ mice from four independent experiments) on day 4 p.i. This strongly biased proliferation of Ly49H$^+$ NK cells during MCMV infection provides a plausible explanation for the expansion of the Ly49H$^+$ NK cell population in the spleen (Fig. 1*C*) and liver (data not shown) during days 2–6 p.i., which is not observed with Ly49D$^+$ NK cells (15) (data not shown). The accumulation of Ly49H$^+$ NK cells during MCMV infection is unlikely to arise secondary to the up-regulation of Ly49H on mature Ly49H$^-$ cells given the stability of Ly-49 receptor expression profiles observed both in vivo and in vitro on dividing NK cell subpopulations (36, 38–40).

Selective NK cell proliferation is mediated through DAP12 signaling

To test whether or not Ly49H signaling mediated via the ITAM-bearing adaptor DAP12 was necessary for stimulating viral-induced NK cell proliferation, we studied MCMV infection in loss-of-function DAP12^{KI} mice. These mice express a signaling-deficient DAP12 molecule that facilitates relatively normal frequencies and surface expression levels of DAP12-dependent activation receptors, such as Ly49H, on NK cells when compared with wt mice (17, 33).

Following MCMV infection, the early nonspecific proliferative responses in the DAP12^{KI} mice (15.0 ± 2.4% of NK cells were BrdU$^+$) were similar to wt B6 mice (15.4 ± 2.7% of NK cells were BrdU$^+$) with comparable increased BrdU incorporation in both the Ly49H$^+$ and Ly49H$^-$ NK cell compartments, as illustrated by SI values of 0.97 ± 0.16 in DAP12^{KI} mice ($n = 5$) and 1.03 ± 0.08 in B6 mice ($n = 5$) at day 1.5 p.i. (Fig. 2*A*). These data demonstrate that the nonspecific NK cell proliferative response observed early during MCMV infection is DAP12 independent.

In contrast to wt B6 mice, DAP12^{KI} mice at later time points during MCMV infection displayed no preferential Ly49H$^+$ NK cell proliferation (Fig. 2*B*), as evidenced by SI values of 0.94 ± 0.42 at day 2 p.i. ($n = 4$) and 1.06 ± 0.05 at day 3 p.i. ($n = 5$) (Fig. 2*A*). Due to the inability of the DAP12^{KI} mice to effectively control MCMV infection (17), it was difficult to assess later time points, as there was a paucity of remaining viable splenic NK cells.

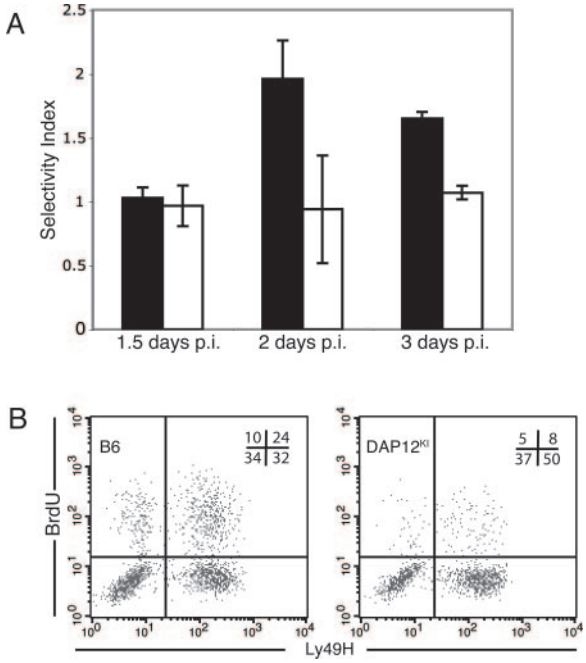

FIGURE 2. Preferential proliferation of Ly49H$^+$ NK cells is compromised in DAP12^{KI} mice. *A*, SI values of wt B6 (■) and DAP12^{KI} (□) splenic NK cells at 1.5, 2, and 3 days p.i. with MCMV (5×10^4 PFU/mouse) with four to five mice per group. *B*, Proliferation of splenic Ly49H$^+$ and Ly49H$^-$ NK cells was assessed by intracellular staining for acute BrdU incorporation on day 2 p.i. with 5×10^4 PFU/mouse MCMV in wt and DAP12^{KI} mice. Flow cytometry analysis was gated on NK1.1$^+$CD3$^-$ splenic lymphocytes. The percentage of NK cells in each quadrant is denoted in the *upper right-hand corner* of the scatterplots. Plots are representative of four individual mice within each group.

However, the observed absence of preferential Ly49H$^+$ NK cell proliferation at day 3 p.i. was supported by the lack of expansion and accumulation of the splenic Ly49H$^+$ NK cell subpopulation (50.7 ± 5.2% in DAP12^{KI} mice vs 74.3 ± 2.3% in B6 mice). Therefore, selective Ly49H$^+$ NK cell proliferation is directly dependent on signaling mediated via DAP12.

Ly49H signaling through DAP12 does not alter NK cell responsiveness to a panel of candidate cytokines

We hypothesized that Ly49H signaling through DAP12 would result in differential responsiveness to cytokines not involved in mediating early nonspecific NK cell proliferation, and that this differential cytokine responsiveness would lead to preferential Ly49H$^+$ NK cell proliferation during MCMV infection. We tested this hypothesis by directly examining the surface expression of candidate cytokine receptors on splenic NK cells and the in vivo NK cell proliferative responses in cytokine and cytokine receptor knockout mice during MCMV infection. We focused on cytokines, such as IL-2, IL-12, and IL-18, which have been shown to stimulate in vitro NK cell responses as well as a number of T cell-derived cytokines, including IL-4, IL-10, IL-17, and IL-21.

Expression of candidate cytokine receptors (common γ-chain (IL-2/15Rγ), IL-2/15Rβ, IL-2Rα, IL-4Rα, IL-12Rβ, IL-17R, IL-18Rα, and IL-21Rα) was evaluated on splenic NK cells from naive and infected (day 4 p.i.) B6 mice. Although several cytokine receptors were slightly up-regulated following MCMV infection, including the common γ-chain, IL-12Rβ, IL-18Rα, and IL-21Rα, only the surface expression of IL-2Rα (and IL-15Rα; see below) was significantly up-regulated (Fig. 3*A*). In addition, there was no differential expression of any of these receptors (including IL-2Rα) on Ly49H$^+$ or Ly49H$^-$ NK cell subsets following MCMV infection (with the exception of very subtle changes in IL-4Rα and IL-18Rα expression on Ly49H$^-$ NK cells) (Fig. 3*B*). Thus, differential expression of these candidate cytokine receptors does not account for the selective Ly49H$^+$ NK cell proliferation observed during MCMV infections.

Preferential NK cell proliferation during MCMV infection could result from altered Ly49H$^+$ NK cell cytokine responsiveness in the absence of differential cytokine receptor expression. We, therefore, investigated NK cell proliferation during MCMV infection in B6 mice with targeted deletions of candidate cytokines or cytokine receptors. Preferential BrdU incorporation in the Ly49H$^+$ NK cell subset was present in IL-2$^{-/-}$, IL-4$^{-/-}$, IL-10$^{-/-}$, IL-12$^{-/-}$, IL-18$^{-/-}$, IL-18R$^{-/-}$, and IL-21R$^{-/-}$ mice and was comparable to that observed in wt B6 mice on day 4 p.i. with MCMV (Fig. 3*C*). Furthermore, all these mice had SI values of 1.85 or greater, demonstrating significant preferential proliferation of Ly49H$^+$ NK cells (Fig. 3*D*). Therefore, selective NK cell proliferation during MCMV infection is not due to differential cytokine responsiveness to IL-2, IL-4, IL-10, IL-12, IL-17, IL-18, or IL-21.

Our results with IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice conflict with a previous report implicating IL-18 in the specific expansion of Ly49H$^+$ NK cells during MCMV infection (30). Due to this discrepancy, we examined NK cell proliferation in IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice in greater detail and found no significant differences in overall NK cell proliferation over the course of MCMV infection in either IL-18$^{-/-}$ (Fig. 4*A*) or IL-18R$^{-/-}$ (Fig. 4*B*) mice compared with IL-18$^{+/-}$ littermates and/or wt B6 mice. Furthermore, we found no deficits in the selective expansion and accumulation of splenic Ly49H$^+$ NK cells in IL-18$^{-/-}$ (Fig. 4*C*) or IL-18R$^{-/-}$ (Fig. 4*D*) mice following MCMV infection. The extent of the selective expansion of splenic Ly49H$^+$ NK cells is dependent on the inoculum dose of MCMV; however, we observed no differences in splenic Ly49H$^+$ NK cell expansion and accumulation in IL-18$^{-/-}$ mice compared with IL-18$^{+/-}$ littermates or wt B6 mice following infection over a range of lower inoculum doses ($2 \times 10^3 - 1 \times 10^4$ PFU/mouse; data not shown) comparable to those used by Andrews et al. (30). The absence of proliferation defects in IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice demonstrates that IL-18 either does not participate or plays a noncritical, redundant role in the specific proliferation and subsequent accumulation of Ly49H$^+$ NK cells during infection with Smith strain MCMV.

Impact of IL-15Rα on NK cells in modulating selective proliferation

IL-15 is crucial for NK cell development, homeostasis, and survival, and has been implicated in stimulating NK cell proliferation during viral infections (19). Given the inherent difficulties in blocking IL-15 in vivo, we focused our investigations on the role of IL-15Rα in viral-driven, selective NK cell proliferation. Because IL-15Rα$^{-/-}$ mice lack NK cells (26), we used IL-15Rα-deficient chimeric mice in which bone marrow from IL-15Rα-deficient mice was used to reconstitute irradiated wt recipients. In these chimeric mice, host cells expressing IL-15Rα efficiently present IL-15 in *trans* to IL-15Rα-null NK cells promoting their development and survival (29, 35).

In contrast to most of the cytokine receptors that we have examined, IL-15Rα is up-regulated on splenic NK cells from wt mice following MCMV infection (day 4 p.i.), as evidenced by the increase in mean fluorescence intensity of IL-15Rα from 26 on NK cells from naive wt mice to 131 on NK cells from MCMV-infected wt mice (Fig. 5*A*). However, as was seen with IL-2Rα, no differential expression of IL-15Rα was observed on Ly49H$^+$ compared with Ly49H$^-$ NK cells from infected mice (data not shown). The

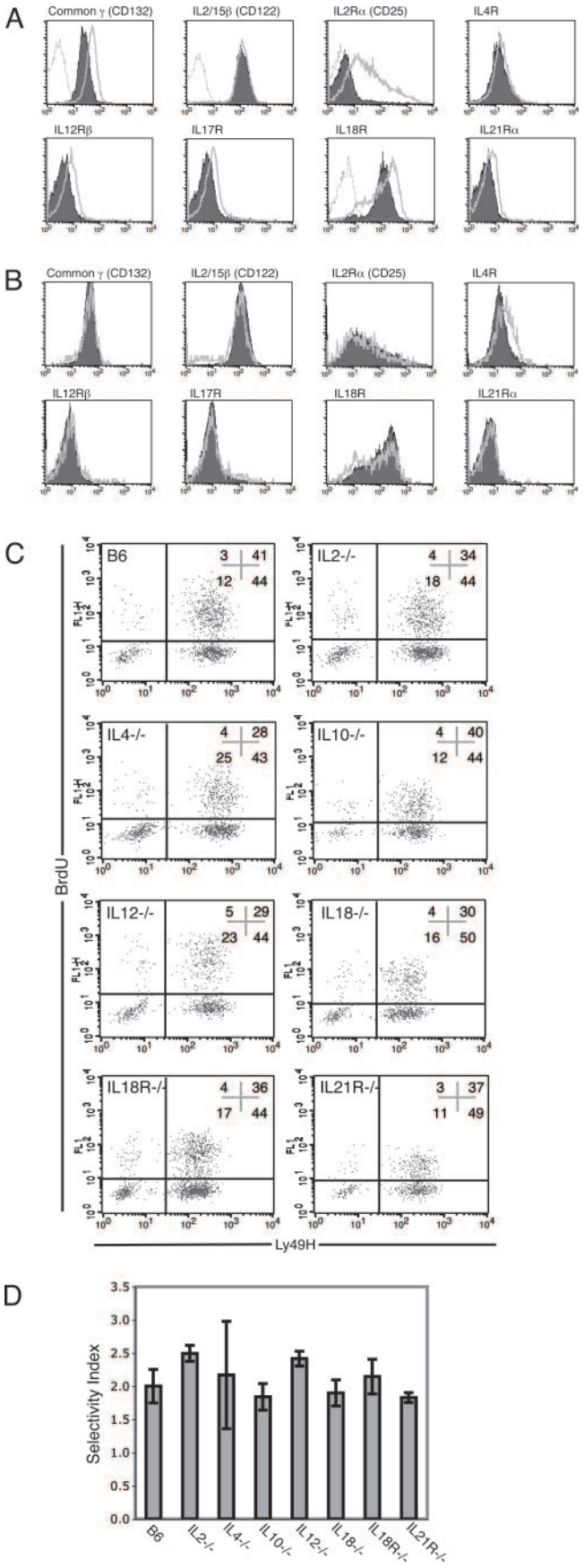

FIGURE 3. Cytokine receptor expression and in vivo NK cell proliferative responses in cytokine knockout mice during MCMV infection. *A*, Cytokine receptor expression was assessed on splenic NK cells from naive and infected B6 mice (day 4 p.i. with 5×10^4 PFU/mouse). Flow cytometry analysis was gated on NK1.1$^+$CD3$^-$ splenic lymphocytes. Filled histograms represent staining of anti-cytokine receptor Abs on NK cells from naive mice; gray-outlined unfilled histograms represent staining of anti-cytokine receptor Abs on NK cells from MCMV-infected mice, and dotted-outlined unfilled histograms represent staining of control Abs. *B*, Cytokine receptor expression on Ly49H$^+$ and Ly49H$^-$ splenic NK cells from infected B6 mice (day 4 p.i. with 5×10^4 PFU/mouse). Flow cytometry analysis was again gated on NK1.1$^+$CD3$^-$ splenic lymphocytes. Filled histograms represent staining of anti-cytokine receptor Abs on Ly49H$^+$ NK cells, whereas gray-outlined, unfilled histograms represent staining of anti-cytokine receptor Abs on Ly49H$^-$ NK cells. Histograms from Ly49H$^-$ NK cells were scaled to allow comparison with Ly49H$^+$ NK cells. *C*, Splenic NK cell proliferation was assessed by intracellular staining for acute BrdU incorporation at day 4 p.i. with 5×10^4 PFU/mouse MCMV in a panel of cytokine and cytokine receptor knockout mice. Flow cytometry analysis was gated on NK1.1$^+$CD3$^-$ splenic lymphocytes. The percentage of NK cells in each quadrant is denoted in the *upper right-hand corner* of the scatterplots. Plots are representative of three to five individual mice within each group. *D*, SI values for a panel of cytokine and cytokine receptor knockout mice at day 4 p.i. with 5×10^4 PFU/mouse MCMV. Knockout mice were infected in a number of independent experiments (3–5 mice per group) with wt B6 controls, and representative B6 SI values are shown from one experiment. Results for each knockout strain of mice are representative of at least two independent experiments.

up-regulation of IL-15Rα following MCMV infection could also be observed on NK cells from wt chimeric mice (generated by reconstituting irradiated wt recipients with wt bone marrow; Fig. 5*A*).

We hypothesized that the up-regulation of IL-15Rα on NK cells during MCMV infection could enhance NK cell proliferation by facilitating *cis* presentation of IL-15 on NK cells potentially prolonging IL-15 stimulation. However, no deficits were observed in NK cell proliferation (Fig. 5*B*) or the expansion of Ly49H$^+$ NK cells (Fig. 5*C*) in the IL-15Rα-deficient chimeric mice compared with the wt chimeric mice on day 4 or 5 p.i. with MCMV. The SI values of 1.8 $\pm$ 0.14 and 2.2 $\pm$ 0.5 in the IL-15Rα-deficient chimeric mice on day 4 or 5 p.i. demonstrate that selective Ly49H$^+$ NK cell proliferation was not compromised by the absence of IL-15Rα on NK cells (Fig. 5*C*). Therefore, up-regulation of IL-15Rα on wt NK cells did not facilitate selective NK cell proliferation during MCMV infection.

m157 recognition in the context of low concentrations of proproliferative cytokines is sufficient to stimulate selective NK cell proliferation

Although we observed no evidence that up-regulation of IL-15Rα on NK cells contributed to preferential NK cell proliferation, the possibility remained that Ly49H stimulation could directly enhance IL-15-dependent NK cell proliferation. We tested this hypothesis in vitro by incubating enriched, CFSE-labeled B6 NK cells with target cells in varying concentrations of IL-15. Low IL-15 concentrations stimulated preferential proliferation of Ly49H$^+$ NK cells as measured by CSFE dilution when the NK cells were incubated with BaF3 cells transfected with m157, but no preferential proliferation when the NK cells were incubated with BaF3 targets lacking m157 expression (Fig. 6*A*). Incubation of NK cells without targets in the same low concentrations of IL-15 promoted only low levels of NK cell proliferation. At low IL-15 concentrations, we consistently saw a small decrease in NK cell proliferation with the addition of target cells presumably reflecting competition for cytokines or other components in the medium, but this did not obscure the differences between Ly49H$^+$ and Ly49H$^-$ NK cell proliferation in the presence of BaF3/m157 targets. Preferential proliferation of Ly49H$^+$ NK cells was also observed when

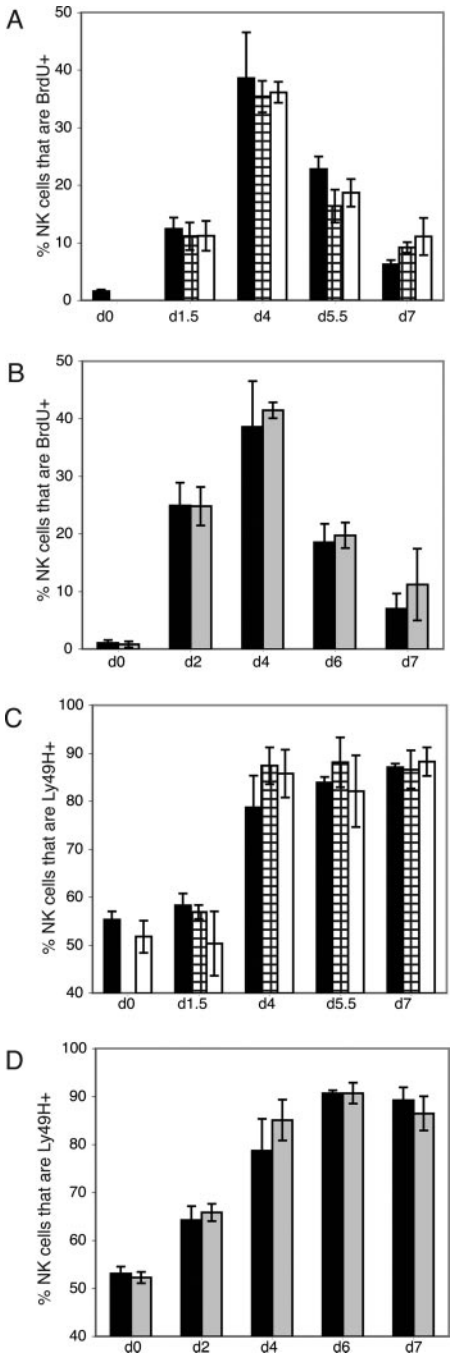

FIGURE 4. IL-18 does not play a critical role in selective NK cell proliferation and accumulation during MCMV infection. *A*, Splenic NK cell proliferation assessed by intracellular staining for acute BrdU incorporation at various times following infection with 5×10^4 PFU/mouse MCMV in IL-$18^{-/-}$ mice. ■, Represent wt B6 mice; hatched bars, represent IL-$18^{+/-}$ littermates; and □, represent IL-$18^{-/-}$ mice (four mice per group). *B*, Splenic NK cell proliferation assessed by intracellular staining for acute BrdU incorporation at various times following infection with 5×10^4 PFU/mouse MCMV in IL-$18R^{-/-}$ mice. ■, Represent wt B6 mice; ▨, represent IL-$18R^{-/-}$ mice (four mice per group). Note wt B6 cohort at day 4 p.i. in *A* and *B* are from the same experiment. *C*, Percentage of splenic NK cells ($NK1.1^+CD3^-$) that are $Ly49H^+$ assessed by flow cytometry at various days during the course of MCMV infection (5×10^4 PFU/mouse) in IL-$18^{-/-}$ mice. ■, Represent wt B6 mice; hatched bars, represent IL-$18^{+/-}$ littermates; and □, represent IL-$18^{-/-}$ mice (four mice per group). *D*, Percentage of splenic NK cells that are $Ly49H^+$ at various times following infection with 5×10^4 PFU/mouse MCMV in IL-$18R^{-/-}$ mice. ■, Represent wt B6 mice; ▨, represent IL-$18R^{-/-}$ mice (four mice per group). Note wt B6 cohort at day 4 p.i. in *C* and *D* are from the same experiment.

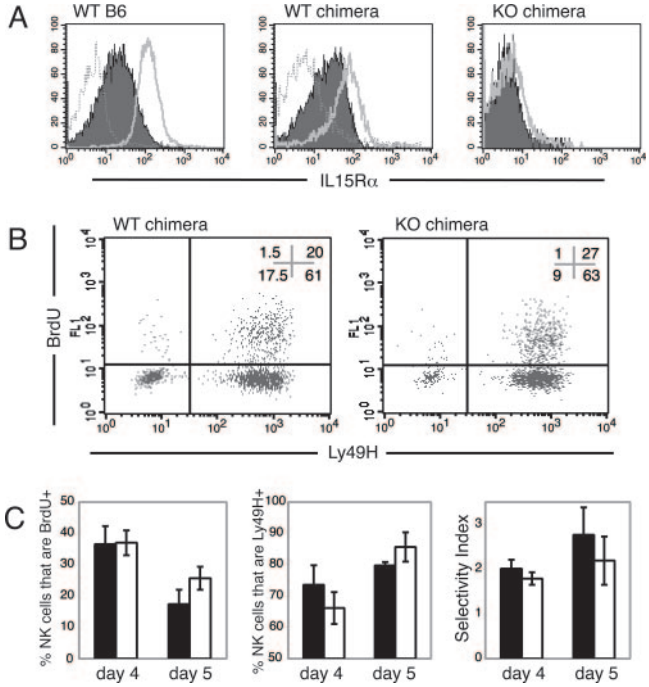

FIGURE 5. Preferential $Ly49H^+$ NK cell proliferation during MCMV infection does not depend on the expression of IL-15Rα on NK cells. *A*, IL-15Rα expression on splenic NK cells from naive and infected wt B6, wt chimeric, and IL-15Rα-deficient chimeric mice. (Infected wt B6 mice were day 4 p.i. with 5×10^4 PFU/mouse MCMV, whereas infected chimeric mice were day 5 p.i. with 7.5×10^3 PFU/mouse MCMV.) Flow cytometry analysis was gated on $NK1.1^+CD3^-$ splenic lymphocytes. Filled histograms represent staining of anti-IL-15Rα Abs on NK cells from naive mice, outlined unfilled histograms represent staining of anti-IL-15Rα Abs on NK cells from MCMV-infected mice, and dotted-outlined histograms represent staining of control Abs. Histograms were scaled for purposes of comparison. *B*, Proliferation of splenic $Ly49H^+$ and $Ly49H^-$ NK cells assessed by intracellular staining for acute BrdU incorporation at 5 days p.i. following MCMV infection (7.5×10^3 PFU/mouse) in wt chimeric and IL-15Rα-deficient chimeric splenic NK cells. Flow cytometry analysis was gated on $NK1.1^+CD3^-$ splenic lymphocytes. Plots are representative of three to four individual mice within each group. *C*, Average percentage of $BrdU^+$ splenic NK cells, percentage of $Ly49H^+$ splenic NK cells, and SI values of wt chimeric (■) and IL-15Rα-deficient chimeric (□) splenic NK cells, respectively, at either day 4 or 5 p.i. with MCMV (7.5×10^3 PFU/mouse) with three to four mice/group.

CFSE-labeled B6 NK cells were incubated in low IL-15 concentrations with syngeneic RMA cells transfected with m157, but not with RMA cells lacking m157 expression (Fig. 6*B*). As IL-15 concentrations were increased, broad-based, nonspecific stimulation of NK cell proliferation was observed regardless of Ly49H expression or the presence of m157 on the targets (Fig. 6*A*). Preferential proliferation of $Ly49H^+$ NK cells was abrogated in the presence of $F(ab')_2$ of anti-m157 Abs, demonstrating the specificity and nonredundancy of the interaction between Ly49H and m157 in stimulating preferential NK cell proliferation (Fig. 6*C*). Together with previous studies demonstrating that m157 is only recognized by Ly49H and not other NK cell receptors on B6 NK cells (12, 13), these results demonstrate that the interaction between Ly49H and m157 in the appropriate cytokine milieu is sufficient to mediate selective NK cell proliferation.

Interestingly, the stimulation of preferential NK cell proliferation in the presence of m157-transfected target cells was not limited to IL-15. Incubation of SCID splenocytes with BaF3/m157 targets in very low concentrations of IL-15 (which alone did not

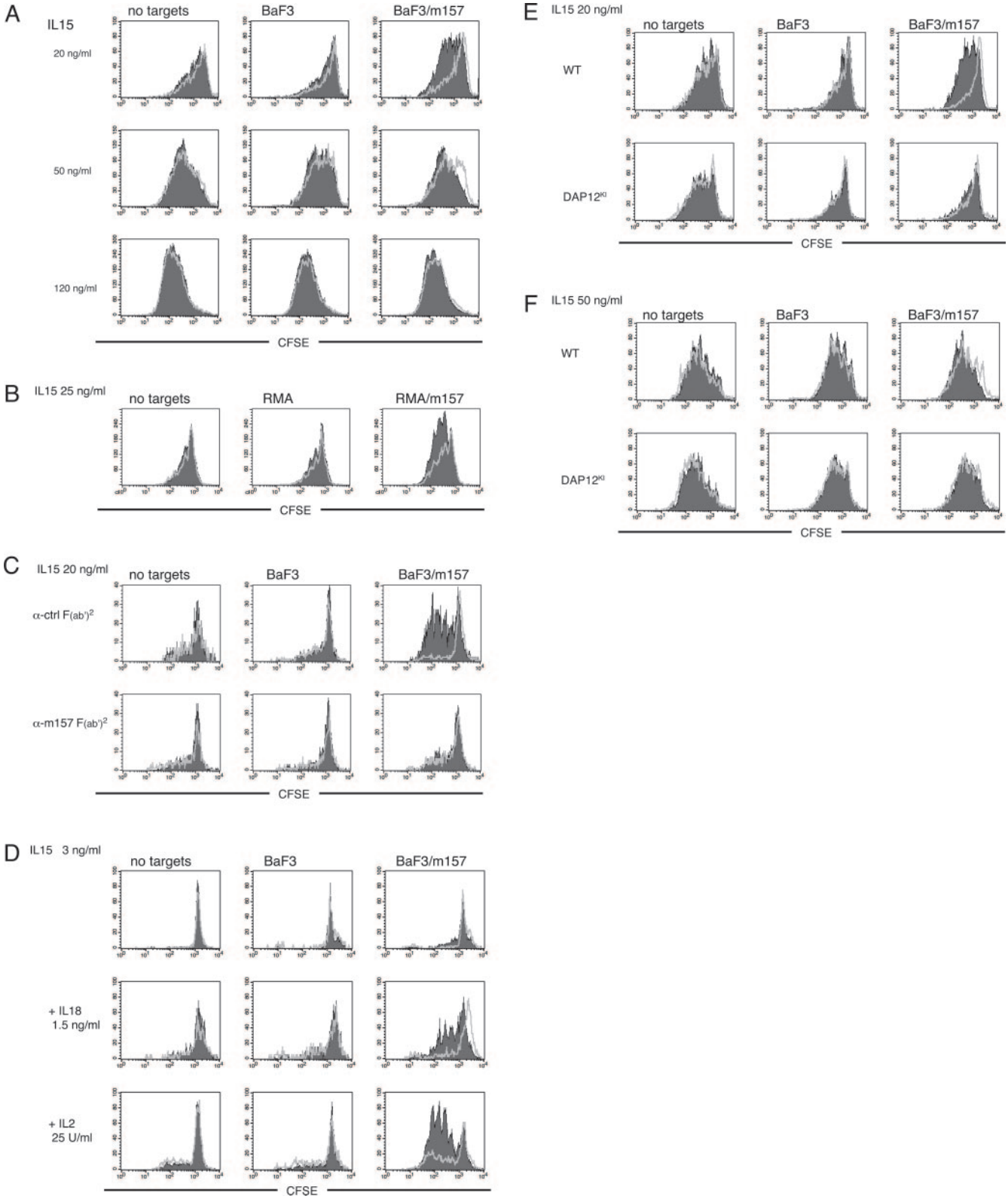

FIGURE 6. Ly49H signaling through DAP12 amplifies stimulation by proproliferative cytokines. *A*, Enriched, splenic B6 NK cells were labeled with CFSE and incubated in 20, 50, or 120 ng/ml murine IL-15 for 3 days either without targets or with BaF3 or BaF3/m157 targets. Flow cytometry analysis was gated on $NK1.1^+CD3^-$ splenic lymphocytes with filled histograms representing CFSE dilution of $Ly49H^+$ NK cells and gray-outlined, unfilled histograms representing CFSE dilution of $Ly49H^-$ NK cells. Histograms were scaled to facilitate comparisons between $Ly49H^+$ and $Ly49H^-$ NK cells. *B*, Enriched, CFSE-labeled B6 NK cells were incubated in 25 ng/ml IL-15 for 3 days without targets or with RMA or RMA/m157 targets. Analysis as described for *A*. *C*, Enriched B6 NK cells were labeled with CFSE and incubated in 20 ng/ml IL-15 for 3 days without targets or with BaF3 or BaF3/m157 targets in the presence of 50 μg/ml of either anti-m157 or isotype control $F(ab')_2$. Analysis as described for *A*. *D*, CFSE-labeled SCID spenocytes were incubated either with or without targets in very low concentrations of IL-15 (3 ng/ml) alone or supplemented with low concentrations of IL-18 (1 ng/ml) or IL-2 (25 U/ml). Analysis as described for *A*. *E* and *F*, Enriched, splenic B6, and $DAP12^{KI}$ NK cells were labeled with CFSE and incubated either without targets or with BaF3 and BaF3/m157 targets in 20 ng/ml (*E*) or 50 ng/ml (*F*) IL-15. Analysis as described for *A*.

stimulate NK cell proliferation) and either low concentrations of IL-2 or IL-18 also resulted in preferential proliferation of Ly49H$^+$ NK cells (Fig. 6*D*). However, high concentrations of IL-2 or IL-18 in the context of low IL-15 concentrations drove all NK cells to proliferate regardless of Ly49H expression or the presence of m157 on the target cells (data not shown). Ly49H signaling appears to selectively lower the response threshold of Ly49H$^+$ NK cells to proproliferative cytokines such as IL-15 or IL-2. This effect is masked by high concentrations of proproliferative cytokines that drive all NK cells to proliferate irrespective of Ly49H stimulation. Therefore, different patterns of in vitro NK cell proliferation analogous to what was found in vivo during MCMV infection (selective vs nonspecific, broad-based proliferation) can be observed in the presence of m157-transfected targets and varying concentrations of proproliferative cytokines.

Ly49H signaling through DAP12 directly augments stimulation by proproliferative cytokines

To verify the role of DAP12 in mediating Ly49H stimulation of preferential NK cell proliferation, the experiments described above were repeated with enriched splenic B6 and DAP12^{KI} NK cells in parallel. When B6 and DAP12^{KI} splenocytes were incubated in low IL-15 concentrations with BaF3/m157 targets, the B6 splenocytes underwent preferential proliferation of Ly49H$^+$ NK cells, whereas no preferential NK cell proliferation was observed in the DAP12^{KI} splenocytes (Fig. 6*E*). This verifies the nonredundant role of DAP12 in Ly49H-mediated stimulation of selective NK cell proliferation. The DAP12^{KI} NK cells were not deficient in their ability to proliferate in response to IL-15 stimulation, as demonstrated by the nearly identical proliferative responses of both DAP12^{KI} and B6 NK cells at higher IL-15 concentrations (Fig. 6*F*). Although Ly49H signaling mediated through DAP12 was necessary for selective NK cell proliferation, DAP12 signaling in the absence of proproliferative cytokine stimulation was not sufficient to stimulate NK cell proliferation or even to support NK cell survival, as demonstrated by the incubation of B6 NK cells with BaF3/m157 targets in the absence of IL-15 for 3 days (data not shown) or by the lack of preferential NK cell proliferation observed in the coculture of B6 NK cells and BaF3/m157 targets in the presence of very low concentrations of IL-15, which alone did not stimulate NK cell proliferation (Fig. 6*D*). Taken together, these results illustrate that the interaction between Ly49H and m157 results in signaling mediated via DAP12 that amplifies the response of Ly49H$^+$ NK cells to stimulation by low concentrations of proproliferative cytokines such as IL-15.

Discussion

Normally quiescent NK cells vigorously proliferate during viral infections. This response is rapidly dominated by the preferential proliferation of NK cells recognizing infected cells. In this study, we directly demonstrate that Ly49H recognition of m157 and subsequent signaling through DAP12 are sufficient in the context of low IL-15 concentrations to stimulate selective NK cell proliferation. High concentrations of proproliferative cytokines minimize the impact of Ly49H signaling through DAP12 by providing a strong stimulus to all NK cells, resulting in nonspecific, broad-based NK cell proliferation. DAP12 has previously been shown to play a crucial role in mediating NK cell cytotoxicity and cytokine production (10, 11, 17); however, this is the first report demonstrating that it is directly involved in mediating NK cell proliferation. We also establish that the early, nonspecific phase of MCMV-induced NK cell proliferation occurs in a DAP12-independent manner in contrast to the later phase of preferential Ly49H$^+$ NK cell proliferation that is dependent on DAP12 signaling.

We found no evidence that Ly49H signaling via DAP12 resulted in the selective up-regulation of cytokine receptors or increased responsiveness to cytokines that are different from those involved in stimulating nonspecific NK cell proliferation. Several candidate cytokine receptors, including IL-2Rα and IL-15Rα, were strongly up-regulated on NK cells during MCMV infection. However, our studies detected no deficits in NK cell proliferation or Ly49H$^+$ NK cell accumulation in IL-15Rα-deficient chimeric mice compared with wt chimeric mice during MCMV infection, indicating that *cis* presentation of IL-15 by IL-15Rα on NK cells is not crucial to MCMV-induced selective NK cell proliferation. In addition, the lack of differential expression of IL-2Rα on Ly49H$^+$ and Ly49H$^-$ NK cells, the normal preferential Ly49H$^+$ NK cell proliferation observed in IL-2$^{-/-}$ mice, and the normal Ly49H$^+$ NK cell expansion and accumulation in IL-2$^{-/-}$ mice during MCMV infection (data not shown) demonstrate that IL-2 and IL-2Rα do not play critical, nonredundant roles in mediating preferential NK cell proliferation during MCMV infection. We have observed subtle, but reproducible decreases in the extent of preferential proliferation of splenic Ly49H$^+$ NK cells in RAG$^{-/-}$ mice (A. French, unpublished data), raising the possibility that a different T cell-derived factor, such as IL-21, could play a minor, nonredundant role in this process. However, we found no deficits in preferential NK cell proliferation or Ly49H$^+$ NK cell expansion in IL-21R$^{-/-}$ mice following MCMV infection. In addition, we found no evidence that three other T cell-derived cytokines (IL-4, IL-10, and IL-17) play a role in MCMV-induced NK cell proliferation.

A previous report noted that Ly49H$^+$ NK cell accumulation was deficient in IL-18$^{-/-}$ mice and concluded that IL-18 may play a role in supporting the selective expansion of Ly49H$^+$ NK cells during MCMV infection (30). It is difficult to reconcile this conclusion with our results. One potential explanation is that our experiments were performed with Smith strain MCMV, while the experiments of Andrews et al. (30) were performed with K181 Perth strain MCMV. However, we observed similar results when we repeated the experiments with 10^4 PFU/mouse K181 strain MCMV (data not shown), although it is possible that differences exist in the Perth strain K181 used in Andrews' study and the K181 strain that we used. Another potential explanation lies in the completeness of the backcrossing of the IL-18$^{-/-}$ mice. For example, the DAP12 locus in the Australian IL-18$^{-/-}$ mice may remain from the 129 parental strain, resulting in a dysfunctional molecule that does not effectively transmit Ly49H signals (41). Alternatively, one could argue that we saw normal Ly49H$^+$ NK cell expansion in our IL-18$^{-/-}$ mice because they were not true knockouts. However, based on PCR genotyping, RNA protection assay for IL-18 RNA following MCMV infection, and decreased IFN-γ production following MCMV infection (data not shown), our IL-18$^{-/-}$ mice were IL-18 deficient and still manifested normal preferential NK cell proliferation and Ly49H$^+$ NK cell accumulation following infection with Smith strain MCMV over a wide range of MCMV inoculum doses. Furthermore, our data with IL-18$^{-/-}$ mice were identical with results in IL-18R$^{-/-}$ mice strongly supporting the conclusion that IL-18 plays a dispensable or redundant role in stimulating NK cell proliferation during MCMV infection.

Our findings indicate that Ly49H signaling mediated via DAP12 amplifies the response of Ly49H$^+$ NK cells to low concentrations of proproliferative cytokines. The question of how DAP12 signaling augments proproliferative cytokine stimulation is currently under investigation. It is possible that DAP12

signaling directly influences proproliferative cytokine stimulation by enhancing JAK/STAT phosphorylation or by suppressing SOCS (suppressor of cytokine signaling) proteins. Recent work by Ortaldo et al. (42) demonstrated that ITAM-mediated signaling in NK cells enhanced IL-12-stimulated IFN-γ production through modulation of p38 MAPK- and ERK-dependent signaling pathways; however, further study is needed to determine the intermediaries in the cross-talk between DAP12 and proproliferative cytokine signaling.

Our observations support a model in which viral-induced, selective NK cell proliferation is a function of both proproliferative cytokine concentrations and the effective activation receptor ligand "concentration" in the NK cell environment. The interplay between these two variables dictates the observed NK cell proliferative response, as illustrated in the case of MCMV infection. In the absence of m157, low IL-15 concentrations promote the survival of NK cells without stimulating significant proliferation. When proproliferative cytokine concentrations are high, such as early during viral infections or following poly(I:C) injection, NK cells are stimulated to proliferate regardless of Ly49H expression (poly(I:C); A. French, unpublished results). Under conditions of high proproliferative cytokine concentrations, m157 stimulation of $Ly49H^+$ NK cells is effectively masked by the strong cytokine-mediated proliferative stimulus to all NK cells (although it is possible that early during infection, low m157 expression also contributes to the lack of specific $Ly49H^+$ NK cell proliferation). As the proproliferative cytokine concentrations fall and/or the effective concentration of m157 increases, the impact of Ly49H recognition of m157 and subsequent signaling via DAP12 becomes manifest in preferential proliferation of $Ly49H^+$ NK cells. Ly49H signaling appears to alter the response threshold of NK cells, allowing them to respond to lower concentrations of proproliferative cytokines than unstimulated NK cells. Therefore, the impact of m157 will be most significant as the proproliferative cytokine concentrations fall to levels that alone provide little proliferative stimulus. Eventually, the NK cell population contracts presumably as m157 (i.e., MCMV infection) is cleared and proproliferative cytokine levels wane, resulting in steady-state IL-15 concentrations that are not able to support the expanded numbers of NK cells.

In many ways, the viral-induced preferential proliferation of $Ly49H^+$ NK cells resembles the clonal expansion seen in T cells following infections. Although NK cell receptors like Ly49H are not clonally restricted, they associate with ITAM-containing signaling molecules and use Syk family tyrosine kinase activation pathways in a similar manner to TCRs. In the appropriate cytokine milieu, specific receptor stimulation of NK cell receptors as well as TCRs results in preferential proliferation and selective expansion of the receptor-specific NK or T cells. Although the specific proliferation of $Ly49H^+$ NK cells leads to relatively prolonged (i.e., 4-wk) perturbations in splenic NK cell populations (A. French, unpublished observation), it remains to be determined whether this selective expansion of virus-specific NK cells results in functional immunological memory with a more vigorous NK cell response upon re-exposure to the infective stimulus in an analogous manner to T cells.

Disclosures

The authors have no financial conflict of interest.

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