# Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection

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**Natural killer (NK) cells have the potential to deliver both direct antimicrobial effects and regulate adaptive immune responses, but NK cell yields have been reported to vary greatly during different viral infections. Activating receptors, including the Ly49H molecule recognizing mouse cytomegalovirus (MCMV), can stimulate NK cell expansion. To define Ly49H's role in supporting NK cell proliferation and maintenance under conditions of uncontrolled viral infection, experiments were performed in *Ly49h*$^{-/-}$, perforin 1 *(Prf1)*$^{-/-}$, and wild-type (*wt*) B6 mice. NK cell numbers were similar in uninfected mice, but relative to responses in MCMV-infected *wt* mice, NK cell yields declined in the absence of *Ly49h* and increased in the absence of *Prf1*, with high rates of proliferation and Ly49H expression on nearly all cells. The expansion was abolished in mice deficient for both *Ly49h* and *Prf1* (*Ly49h*$^{-/-}$*Prf1*$^{-/-}$), and negative consequences for survival were revealed. The Ly49H-dependent protection mechanism delivered in the absence of *Prf1* was a result of interleukin 10 production, by the sustained NK cells, to regulate the magnitude of CD8 T cell responses. Thus, the studies demonstrate a previously unappreciated critical role for activating receptors in keeping NK cells present during viral infection to regulate adaptive immune responses.**

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Abbreviations used: HCMV, human CMV; HCV, hepatitis C virus; LCMV, lymphocytic choriomeningitis virus; MCMV, mouse CMV; Prf1, perforin 1; *wt*, wild type.

Classical (non–T) NK cells are generally found at low frequencies in leukocyte populations (Biron et al., 1999). They have the potential to mediate antiviral and immunoregulatory functions through a variety of mechanisms (Orange et al., 1995; Su et al., 2001; Lee et al., 2007; Robbins et al., 2007; Strowig et al., 2008). By altering cell availability, in vivo conditions changing NK cell numbers may indirectly influence all of their effects. Activating receptors on NK cells are linked to stimulatory pathways overlapping with those used by TCRs to drive cell expansions (Murali-Krishna et al., 1998; Pitcher et al., 2003; French et al., 2006; MacFarlane and Campbell, 2006; Biron and Sen, 2007; Lee et al., 2007) and can induce NK cell proliferation (Dokun et al., 2001; French et al., 2006). Although particular activating receptors have been reported to recognize microbial products (Lanier, 1998; Vidal and Lanier, 2006; Jonjic et al., 2008), dramatic NK cell expansion has not been observed during infections. Except under rare experimental conditions (Caligiuri et al., 1991; Yamada et al., 1996; Fehniger et al., 2001; Huntington et al., 2007a; Sun et al., 2009), NK cell division is generally induced for limited periods of time as a consequence of transient innate cytokine exposure (Biron et al., 1984; Biron et al., 1999; Dokun et al., 2001; Nguyen et al., 2002; Yokoyama et al., 2004). Increasing proportions of NK cell subsets with activating receptors recognizing particular viral ligands can be detected during certain infections (Dokun et al., 2001; Gumá et al., 2006), but this is observed without dramatic increases in overall NK cell numbers, and many viral infections



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induce striking reductions in NK cell functions, frequencies, and yields (Biron et al., 1999; Tarazona et al., 2002; Lehoux et al., 2004; Reed et al., 2004; Azzoni et al., 2005; Vossen et al., 2005; Morishima et al., 2006). Thus, particular conditions of viral challenges must result in differential regulation of NK cell proportions and numbers, with consequences for the delivery of NK cell functions.

An NK cell activating receptor in the mouse is Ly49H (Lanier, 1998; Gosselin et al., 1999; Smith et al., 2000; Vidal and Lanier, 2006). This molecule recognizes a mouse CMV (MCMV) ligand (Arase et al., 2002; Smith et al., 2002), is expressed on NK cell subsets in strains of particular genetic backgrounds, including C57BL/6 (B6) mice, and is reported to be an exclusive marker for the classical NK cell subset (Smith et al., 2000). Through an associated molecule, Ly49H stimulates using signaling pathways overlapping with those used by the TCR (MacFarlane and Campbell, 2006; Biron and Sen, 2007). Additional markers for all NK cells include CD49b, expressed on other activated cell types (Arase et al., 2001); NKp46, selectively expressed on classical NK cells (Gazit et al., 2006; Walzer et al., 2007a; Walzer et al., 2007b); CD122, the IL-2Rβ chain, expressed on all NK cells and activated T cells (Huntington et al., 2007b); and NK1.1, exclusively expressed on C57BL6 (B6) NK and NKT cells (Lian and Kumar, 2002; MacDonald, 2002; Yokoyama et al., 2004; Huntington et al., 2007b). The TCR with associated CD3 molecules is not expressed on their cell surfaces (Biron et al., 1999). The mechanisms for NK cell–enhanced resistance to MCMV infection are incompletely characterized (Lee et al., 2007), but Ly49H contributes to their protective effects (Scalzo et al., 1990; Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001; Lee et al., 2003). Engagement of the Ly49H receptor can lead to killing of target cells (Arase et al., 2002; Smith et al., 2002), and the correlation of increases in viral burdens resulting from the absence of Ly49H (Scalzo et al., 1990) as compared with those resulting from defects in cytotoxicity functions, such as mutation of the membrane pore-forming protein perforin 1 (*Prf1*; Tay and Welsh, 1997; Loh et al., 2005; van Dommelen et al., 2006), supports a role for Ly49H-dependent killing of virus-infected cells in the delivery of NK cell antiviral effects. The receptor may have other functions associated with its ability to stimulate proliferation, however, as the proportions of NK cells expressing Ly49H are increased during MCMV infection (Dokun et al., 2001).

The studies presented in this paper were undertaken to dissect the proliferative from the cytotoxic functions accessed through Ly49H, and to define the contribution of the regulation of NK cell numbers to protection during infection. To carry out the work, responses were evaluated, during MCMV infections, in wild-type (*wt*) B6 mice and mice deficient in *Ly49h* (Fodil-Cornu et al., 2008), *Prf1* (Kägi et al., 1994), or both. As expected, single $Ly49h^{-/-}$ and $Prf1^{-/-}$ mice had profoundly increased viral burdens, but significant differences in NK cell expansion were discovered. The NK cell populations were decreasing in infected $Ly49h^{-/-}$ mice, whereas infected $Prf1^{-/-}$ mice had an unexpected dramatic proliferation of NK cells uniformly expressing Ly49H. The expansion was proven to be dependent on Ly49H. During uncontrolled infection in the absence of Prf1, Ly49H beneficially promoted effects for survival, because the sustained NK cells produced IL-10 to control the magnitude of the CD8 T cell response and limit immunopathology. The data suggest that Ly49H-dependent cytotoxicity acts to control viral infection and NK cell expansion, but that in the absence of the killing function, Ly49H promotes a continued NK cell expansion critical for supporting life over death because the NK cells are available to regulate adaptive immune responses.

## RESULTS

### Consequences of Ly49H$^-$ as compared with Prf1 deficiency for maintenance of NK cells

NK responses were investigated in *wt*, *Ly49h*-deficient (Fodil-Cornu et al., 2008), and *Prf1*-deficient B6 mice after infection with 2,500 PFU MCMV. As expected and in contrast to *wt* mice, high virus replication was consistently observed in the absence of Ly49H receptor (Fig. 1 A). Interestingly, $Ly49h^{-/-}$ mice had decreasing frequencies and numbers of NK cells, identified as either $CD49b^+TCR\beta^-$ (Fig. 1, B and C) or $NK1.1^+TCR\beta^-$ (Fig. 1, D and E), in spleens. The differences in $Ly49h^{-/-}$ as compared with *wt* were greater than twofold reductions in percentages and fivefold reductions in yields on day 5 of infection. The frequencies of cells expressing TCRβ were comparable in *wt* and $Ly49h^{-/-}$ B6 mice (Fig. 1 F), indicating a differential decrease of NK cells. Consistent with earlier studies (Tay and Welsh, 1997; Loh et al., 2005) and similar to $Ly49h^{-/-}$ mice (Fig. 1 A), $Prf1^{-/-}$ mice showed higher viral loads after infection (Fig. 1 G). Surprisingly, however, $Prf1^{-/-}$ mice contained higher frequencies, ∼25% or fivefold increases, of splenic $CD49b^+TCR\beta^-$ NK cells at day 5 as compared with *wt* mice (Fig. 1 H), and the absolute numbers of these NK cells were significantly higher (by approximately threefold) than those of *wt* mice (Fig. 1 I). The trends were the same for $NK1.1^+TCR\beta^-$ NK cells (Fig. 1, J and K). The proportions of cells expressing TCRβ were comparable in *wt* and $Prf1^{-/-}$ mice (Fig. 1 L), suggesting selective expansion of NK cells. Thus, the absence of Ly49H results in conditions failing to sustain NK cells but the absence of *Prf1* results in profound NK cell expansion during MCMV infection. Because both $Ly49h^{-/-}$ and $Prf1^{-/-}$ mice had high viral burdens, the changes in NK cell frequencies and yields are not simply a consequence of secondary effects resulting from high viral replication.

The higher proportions and numbers of NK cells in $Prf1^{-/-}$ as compared with $Ly49h^{-/-}$ mice led us to examine expression of Ly49H on the CD49b cell subsets. After challenge with 2,500 PFU MCMV, ∼30–50% of splenic CD49b cells expressed Ly49H in uninfected *wt* and $Prf1^{-/-}$ mice as well as in day 1.5, 3, and 5 infected *wt* mice (Fig. 2 A). In contrast, the proportions of CD49b cells expressing Ly49H were increasing during infection of $Prf1^{-/-}$ mice such that >90% expressed Ly49H on day 5. To evaluate the effect of challenge dose, infections were initiated with 1,000, 2,000, or

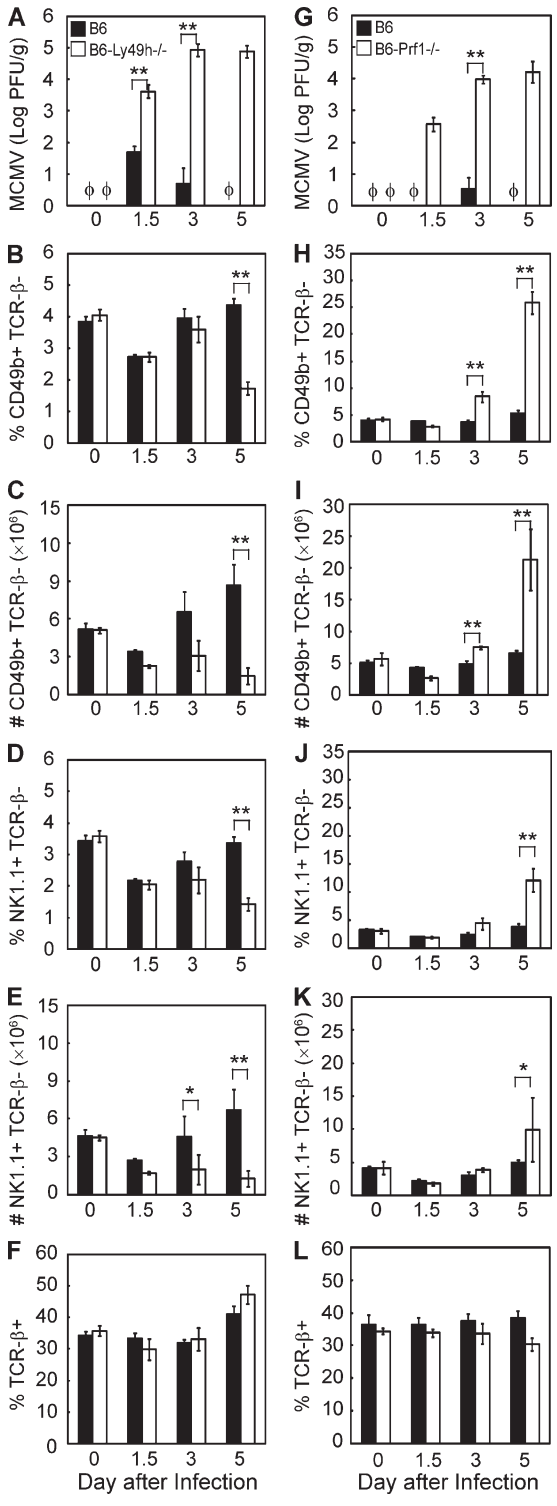


**Figure 1. Effects of *Ly49h* or *Prf1* gene deficiency on NK cell frequencies and numbers during MCMV infection.** (A–L) Virus titers in spleens of *wt* and *Ly49h*$^{-/-}$ (A) or *Prf1*$^{-/-}$ (G) mice were determined at days 0, 1.5, 3, and 5 after infection with 2,500 PFU MCMV. Percentages and absolute numbers of CD49b$^{+}$TCRβ$^{-}$ NK cells in splenic leukocytes isolated from *wt* and *Ly49h*$^{-/-}$ (B and C) or *Prf1*$^{-/-}$ (H and I) mice were measured at the indicated time points. Percentages and absolute numbers of NK1.1$^{+}$TCRβ$^{-}$ NK cells in splenic leukocytes isolated from *wt* and *Ly49h*$^{-/-}$ (D and E) or *Prf1*$^{-/-}$ (J and K) mice were measured at the indicated time points. Percentages of TCRβ$^{+}$ cells from *wt* and *Ly49h*$^{-/-}$ (F) or *Prf1*$^{-/-}$ (L) mice were measured at the indicated time points. Data are presented as means ± SD of three to six mice. Statistical significances between groups are indicated (*, $P < 0.05$; **, $P < 0.01$). Results are representative of two independent experiments with at least three mice per group. ϕ, not detected.

5,000 PFU MCMV. The shift to NK cell subsets predominantly expressing Ly49H was observed under all conditions, but the proportions of NK cells increased with increasing challenge dose such that 36% of the splenic leukocytes were Ly49H$^{+}$CD49b$^{+}$ cells on day 5 after infection with 5,000 PFU (Fig. 2 B). Thus, the dramatic increases in NK cells expressing Ly49H are observed in response to different doses of MCMV, but higher doses lead to higher proportions of the cells.

The phenotype of the expanding NK cells was characterized further. Several development or activation status markers were compared for their expression on CD49b$^{+}$TCRβ$^{-}$ NK cells from *wt* and *Prf1*$^{-/-}$ mice at day 4 of MCMV infection. Consistent with known NK cell lineage markers, NK cells from both mice showed high expression of CD122 (IL-2Rβ chain), and high expression of the NK cell activation markers CD69, CD11b, and CD11c (Fig. 2 C). Interestingly, markers thought to be specific for immature NK cells (Kim et al., 2002; Huntington et al., 2007b) were differentially expressed, with CD27 detected on both *wt* and *Prf1*$^{-/-}$ NK cell populations and CD51 (integrin αV) detected at high levels on NK cells from *Prf1*$^{-/-}$ mice. Investigation of other known lymphocyte activation markers, Ly6C and CD62L (L-selectin; Sinclair et al., 2008), demonstrated an unexpectedly higher expression of Ly6C and lower expression of CD62L on NK cells from *Prf1*$^{-/-}$ as compared with *wt* mice. Expression of another NK selective cell marker, NKp46, was also examined, and virtually all of the NKp46$^{+}$ cells coexpressed Ly49H on day 4 in the *Prf1*$^{-/-}$ mice, but only half expressed Ly49H under the other conditions. The cells were double positive for CD49b and NKp46 under all conditions (Fig. 2 D). Mixed populations of NK cells expressing cytoplasmic CD3 have been reported, and certain activated T cells can down-regulate CD3 expression while maintaining expression of markers overlapping with NK cells (Lanier, 2007; Stewart et al., 2007). Because cytoplasmic staining of CD3ε can identify such T cell populations, experiments were performed to evaluate intracellular CD3ε expression in the highly expanding NK cells. Intracellular CD3ε expression was not significantly expressed in any of the Ly49H-positive cells isolated from *wt* or *Prf1*$^{-/-}$ mice on either day 0 or 4 (Fig. 2 E). Thus, the NK cells expanded in the *Prf1*$^{-/-}$ mice are bona fide NK cells but enriched in proportions expressing Ly49H.

### Proliferation of Ly49H$^{+}$ NK cells

To measure proliferation of NK cells during infections of *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice, in vivo incorporation of the DNA analogue BrdU was analyzed. In *wt* mice, CD49b$^{+}$TCRβ

NK cell proliferation was increasing with a peak at day 3 of MCMV infection when 31% incorporated BrdU during a 2-h pulse in vivo (Fig. 3 A). In the case of *Ly49h*$^{-/-}$ mice, NK cell proliferation showed a severe defect at day 3. In sharp contrast to these two groups, greater and longer BrdU incorporation was observed in *Prf1*$^{-/-}$ mice during MCMV infection (Fig. 3 A). The peak was found at day 4, and at this time, the proportion of BrdU$^{+}$ cells among the NK cells was 2.3-fold higher than that in *wt* mice. Because differential NK cell proportions (5% in B6, 2% in *Ly49h*$^{-/-}$, and 15% in *Prf1*$^{-/-}$ mice) were found at day 4, the frequencies and numbers of proliferating NK cells among splenic leukocytes demonstrated even more dramatic differences. In *Prf1*$^{-/-}$ mice on day 4, >5- and 50-fold increases in the proportions of BrdU$^{+}$ NK cells, respectively, were observed in comparison to *wt* and *Ly49h*$^{-/-}$ mice. The absolute numbers of proliferating NK cells in *Prf1*$^{-/-}$ mice were found to be increased similarly and showed the greatest difference at day 4 of MCMV infection. Hence, in comparison to *wt* NK cell responses, NK cells have reduced BrdU incorporation in the absence of Ly49H but increased incorporation in the absence of Prf1, and these changes are accompanied by respective differences in NK cell yields.

Because a severe defect in NK proliferation was observed in the absence of *Ly49h*, and nearly all NK cells in *Prf1*$^{-/-}$

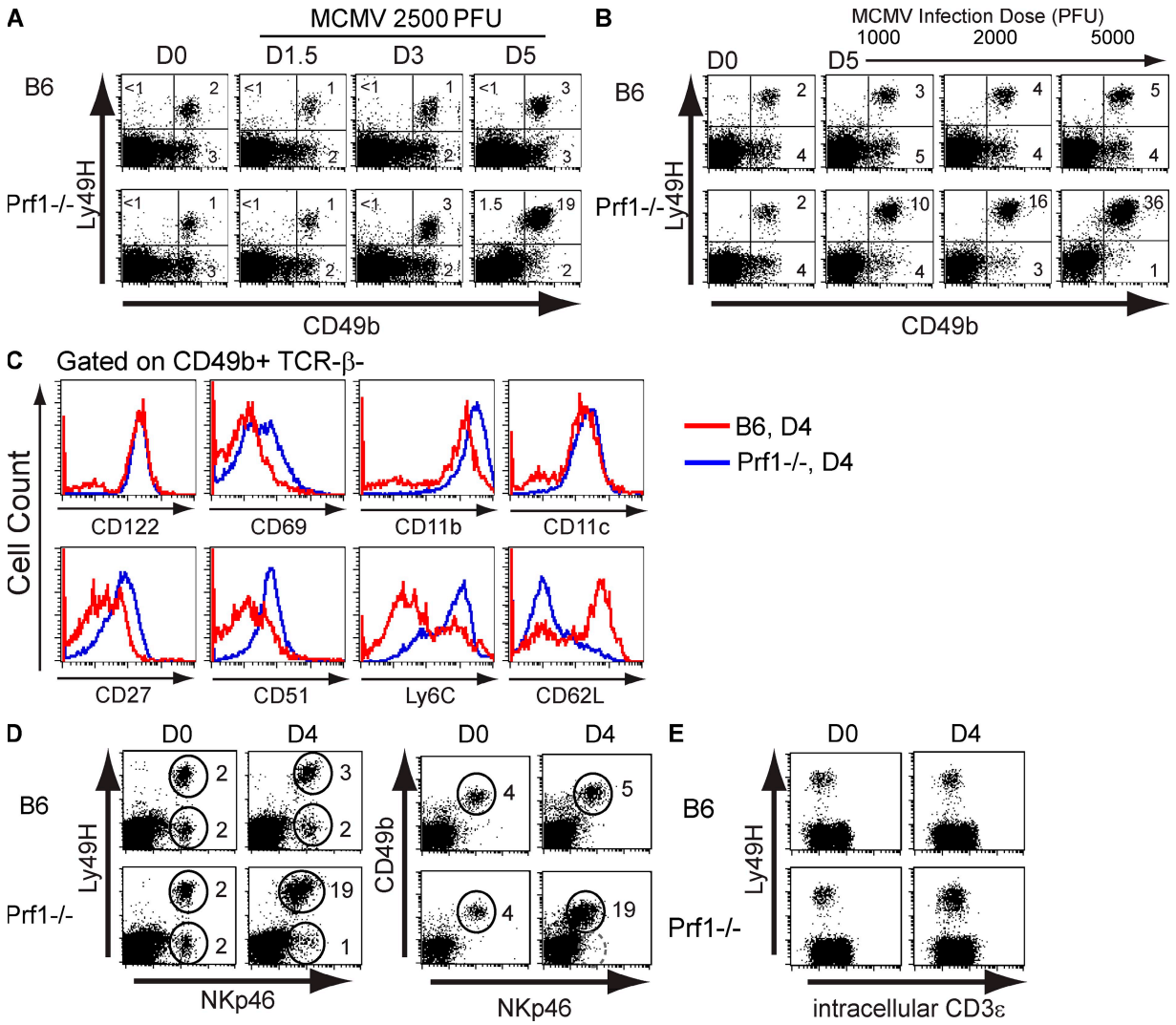


**Figure 2. Characterization of highly expanded NK cells in *Prf1*$^{-/-}$ mice.** (A) Expression of Ly49H and CD49b on splenic leukocytes at days 0, 1.5, 3, and 5 after infection of *wt* and *Prf1*$^{-/-}$ mice with 2,500 PFU MCMV is shown. (B) NK expansion in splenic leukocytes isolated from day 5 MCMV-infected *wt* and *Prf1*$^{-/-}$ mice using 1,000, 2,000, or 5,000 PFU is shown. (C) Histograms show overlays of CD122, CD69, CD11b, CD11c, CD27, CD51, Ly6C, and CD62L expressed on gated CD49b$^{+}$TCRβ$^{-}$ NK cells from day 4 MCMV-infected (2,500 PFU) *wt* (red line) and *Prf1*$^{-/-}$ (blue line) mice. (D) Dot plots show expression of Ly49H and CD49b with regard to NKp46 expression on splenic leukocytes isolated from day 4 MCMV-infected *wt* and *Prf1*$^{-/-}$ mice. Circles indicate the absence of specific populations. (E) Dot plots show expression of intracellular CD3ε chain with regard to Ly49H expression on splenic leukocytes isolated from day 4 MCMV-infected *wt* and *Prf1*$^{-/-}$ mice. The numbers indicate percentages of cells in each area. Results are representative of two independent experiments with three mice per group.

mice were shown to be Ly49H expressing during MCMV infection, BrdU incorporation was specifically examined within the Ly49H-positive and -negative subsets (Fig. 3 B). As the peak of BrdU incorporation in *Prf1*$^{-/-}$ mice and the maximum difference in NK proliferation between *wt* and *Prf1*$^{-/-}$ mice were observed at day 4, this time point was chosen for the analysis. The Ly49H$^{+}$ NK cells proliferated at a higher rate than did Ly49H$^{-}$ NK cells in *wt* mice, and these cells were absent in *Ly49h*$^{-/-}$ mice. Interestingly, at this time point in *Prf1*$^{-/-}$ mice, most NK cells were expressing Ly49H, and these NK cells had the highest level of BrdU incorporation. As analyzed in Fig. 3 B, the frequency of Ly49H$^{-}$ NK cells incorporating BrdU was similar among B6, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice, whereas the frequency in Ly49H$^{+}$ NK cells

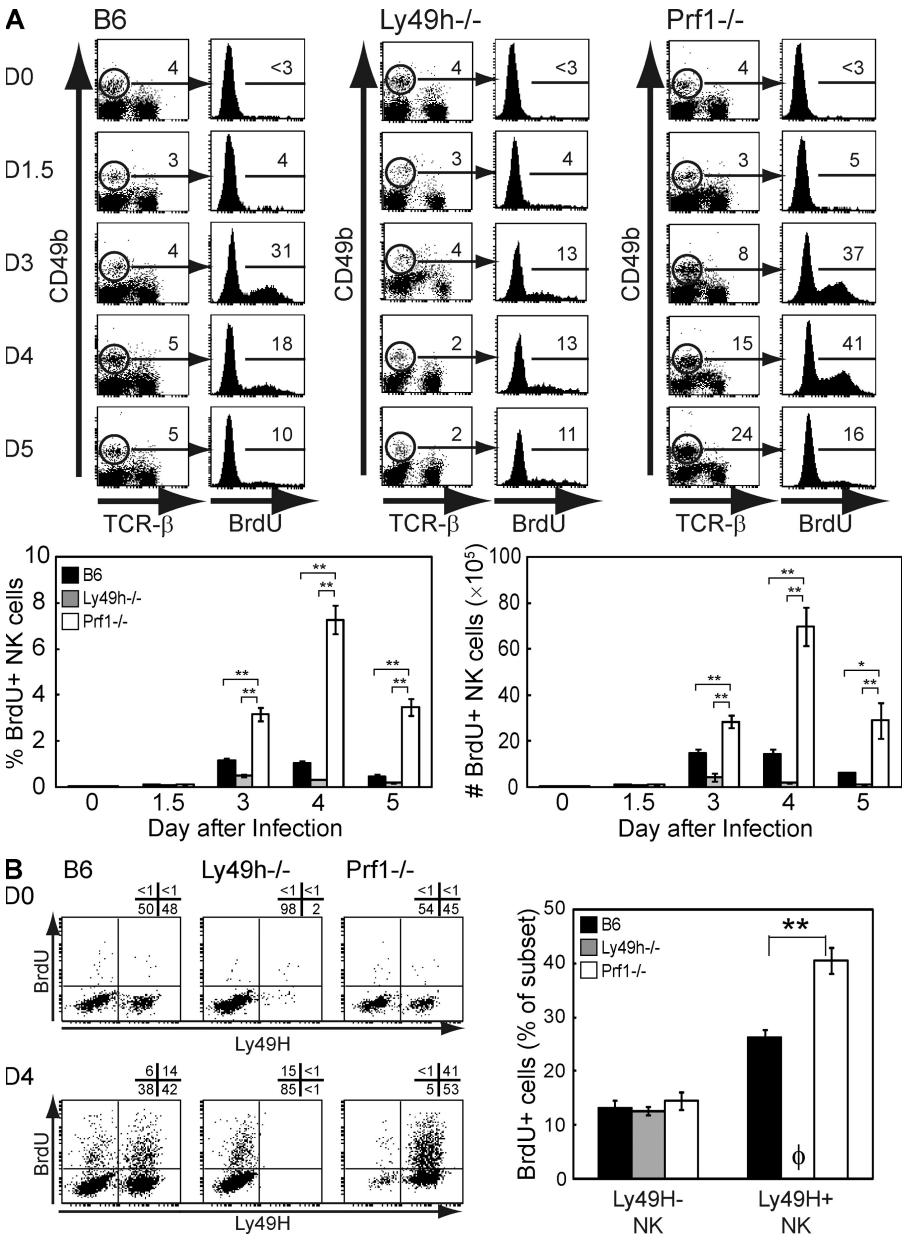


**Figure 3. Preferential proliferation of Ly49H$^{+}$ NK cells in *Prf1*$^{-/-}$ mice during MCMV infection.** (A) Percentages of and BrdU incorporation in CD49b$^{+}$TCRβ$^{-}$ NK cells isolated from *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice on indicated days after infection were measured. Infections were initiated with 2,500 PFU of virus, and BrdU was administered 2 h before harvest. The proportions of NK cells incorporating BrdU and their numbers in total splenic leukocytes are shown with SD from replicate samples. (B) Dot plots indicate BrdU incorporation in the context of Ly49H expression in NK cells from *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice at days 0 and 4 MCMV after infection. Dot plots gated on the CD49b$^{+}$CD3$^{-}$ population are shown. The numbers given in the top right indicate the percentage of cells in each quadrant. Percentages of BrdU$^{+}$ cells among Ly49H$^{-}$ and Ly49H$^{+}$ NK cell subsets at day 4 MCMV infection of *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice were determined. Data are presented as means ± SD of three to six mice. Statistical significances between groups are indicated (*, $P < 0.05$; **, $P < 0.01$). Results are representative of two independent experiments with at least three mice per group. ϕ, not detected.

was significantly higher in *Prf1*$^{-/-}$ mice. Therefore, preferential proliferation of Ly49H$^+$ NK cells accompanies the dramatic expansion of NK cell numbers in *Prf1*$^{-/-}$ mice.

Although the studies clearly demonstrated heightened proliferation of Ly49H$^+$ cells, particularly in *Prf1*$^{-/-}$ mice, they did not evaluate a potential contribution of cell-intrinsic differences to division. To determine the intrinsic proliferative potential of Ly49H$^+$ as compared with Ly49H$^-$ cells from *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$ mice, negatively enriched NK cells (40–50%) were stimulated with a growth factor capable of activating all cells, IL-2, and with control cells (BaF3) or cells expressing the Ly49H ligand m157 (BaF3/m157) ex vivo. The BrdU incorporation was evaluated after a 2-h pulse at the end of stimulation. After IL-2 treatment, BrdU incorporation in NK1.1$^+$TCR$\beta^-$ NK cells from *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice was comparable and irrespective of Ly49H expression (Fig. S1 A). In contrast, stimulation with either $\gamma$-irradiated BaF3 or BaF3/m157 cells demonstrated that Ly49H$^+$ NK cells from both *wt* and *Prf1*$^{-/-}$ mice specifically incorporated BrdU in response to the ligand and did so at comparable rates but that Ly49H$^-$ cells did not (Fig. S1 B). The populations from *Ly49h*$^{-/-}$ mice had extremely poor responses under these conditions. The results indicated that the intrinsic proliferative responsiveness of NK cells from *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice are equivalent but that the expression of the Ly49H selects for subset expansion in either *wt* or *Prf1*$^{-/-}$ populations.

### Requirement for the Ly49H receptor in sustaining NK cell proliferation and survival, and consequences for health

To directly test the hypothesis that the enhanced NK cell expansion in *Prf1*$^{-/-}$ mice is dependent on Ly49H, mice deficient in both *Prf1* and *Ly49h* (hereafter *Ly49h*$^{-/-}$*Prf1*$^{-/-}$) were bred and investigated for NK cell proliferation at day 4 of MCMV infection. As compared with *wt* mice, the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ and *Prf1*$^{-/-}$ mice were both highly susceptible to MCMV infection, and showed similar levels of uncontrolled virus replication (Fig. 4 A). The ablation of the *Ly49h* gene in *Prf1*$^{-/-}$ mice, however, resulted in dramatic reductions of both NK cell frequencies and numbers at day 4 (Fig. 4, B and C), with the absolute number of NK cells being lower by greater than two- and fivefold, respectively, as compared with *wt* and *Prf1*$^{-/-}$ mice (Fig. 4 C). The frequencies of cells expressing TCR$\beta$ were comparable among the groups (Fig. 4 D). To examine the consequences for proliferation in vivo, experiments were performed with 2-h BrdU pulses. The NK cells were identified as CD49b$^+$TCR$\beta^-$ (Fig. 4 E) and NKp46$^+$TCR$\beta^-$ (Fig. 4 F) populations. A severe reduction in the percentages of BrdU$^+$ NK cells was apparent in the absence of Ly49H. When examined as total numbers of NK cells incorporating, a significant reduction (up to greater than eightfold) was observed in *Ly49h*$^{-/-}$ *Prf1*$^{-/-}$ as compared with *Prf1*$^{-/-}$ mice at day 4 of MCMV infection (Fig. 4, G and H). Thus, the Ly49H NK receptor is required for NK cell proliferation and maintenance of NK cells during MCMV infections in *Prf1*$^{-/-}$ mice.

To evaluate the consequences of the Ly49H-dependent NK cell expansion for health, body weight changes and survival were evaluated for extended periods of time after infections in *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice. At a challenge dose of 2,500 PFU of virus, the *wt* mice started gaining weight at day 4 after infection. In contrast, both the *Prf1*$^{-/-}$ and the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were losing weight by day 4 of infection. The *Prf1*$^{-/-}$ mice had only ~6% weight loss at maximum. This was sustained through intermediate times, and the mice were returning to their preinfection weights at day 12 after infection. However, the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice continued losing weight after day 4 and reached weight losses of ~15% at intermediate times (day 10) before starting to then gain weight on day 12 (Fig. 5 A). After infection with 5,000 PFU MCMV, the *wt* and *Prf1*$^{-/-}$ mice survived, but *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were beginning to die at day 6 and 80% had succumbed by day 10 of infection (Fig. 5 A). Thus, the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice are significantly more sensitive to disease during MCMV infection, and the presence of Ly49H makes the difference between life and death.

Because the separation of body weight loss and survival after day 4 of infection was observed in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ as compared with *wt* and *Prf1*$^{-/-}$ mice, viral titers were measured. As expected, *wt* mice were clearing a challenge dose of 2,500 PFU MCMV by day 4 and 5 after infection. In contrast, both the *Prf1*$^{-/-}$ and the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice had substantially higher (>4 log) increases in viral titers at these times, but the groups did not differ significantly (Fig. 5 B). Thus, differences in disease susceptibility between the *Prf1*$^{-/-}$ and the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice could not be explained by altered sensitivity to infection. Because pathology during viral infections can also be a result of the magnitude of immune responses, circulating levels of cytokines were measured on days 4 and 5 of infection. *wt* mice had extremely low levels of IFN-$\gamma$ and TNF-$\alpha$, whereas both the *Prf1*$^{-/-}$ and the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice had elevated levels of these cytokines on days 4 and 5 of infection, with no statistically significant differences in TNF-$\alpha$ but higher IFN-$\gamma$ levels on day 5 in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice (Fig. 5 B). The differences in disease parameters in these two groups of mice were, however, also accompanied by significant differences in circulating levels of IL-10, a cytokine known to inhibit T cell responses. Serum IL-10 levels were undetectable in *wt* mice. High circulating levels of the cytokine were detectable in the *Prf1*$^{-/-}$ mice; on day 5 of infection, it reached a level of >3,600 pg/ml in individual mice. However, *Ly49h*$^{-/-}$ *Prf1*$^{-/-}$ mice only had IL-10 levels reaching 984 pg/ml (Fig. 5 B). Therefore, under conditions of viral challenge in *Prf1*$^{-/-}$ mice, there is high Ly49H-dependent NK cell expansion and better protection against disease, whereas *Prf1*$^{-/-}$ mice also deficient for Ly49H lose their NK cells, have poor health, and succumb to death. High endogenous IL-10 production is present in the first but not the second condition.

### IL-10 production by NK cells

To determine whether or not the expanded NK cells were producing IL-10, NK cells were purified and their ex vivo

production of the factor was measured. For these studies, *wt* and *Prf1*$^{-/-}$ mice were infected for 4 d. In the first set of experiments (Fig. 6 A), total, NK and non–NK cells were prepared by positive selection using CD49b$^{+}$ magnetic bead isolation to compare responses in *wt* and *Prf1*$^{-/-}$ mice. As reported in Fig. 5 B, the *Prf1*$^{-/-}$ but not the *wt* mice had high circulating levels of IFN-γ and IL-10 on day 4. Ex vivo production of IFN-γ but not IL-10 was detectable with NK but not non–NK cells enriched from the *wt* mice. High production of both of these cytokines, however, was enriched in the

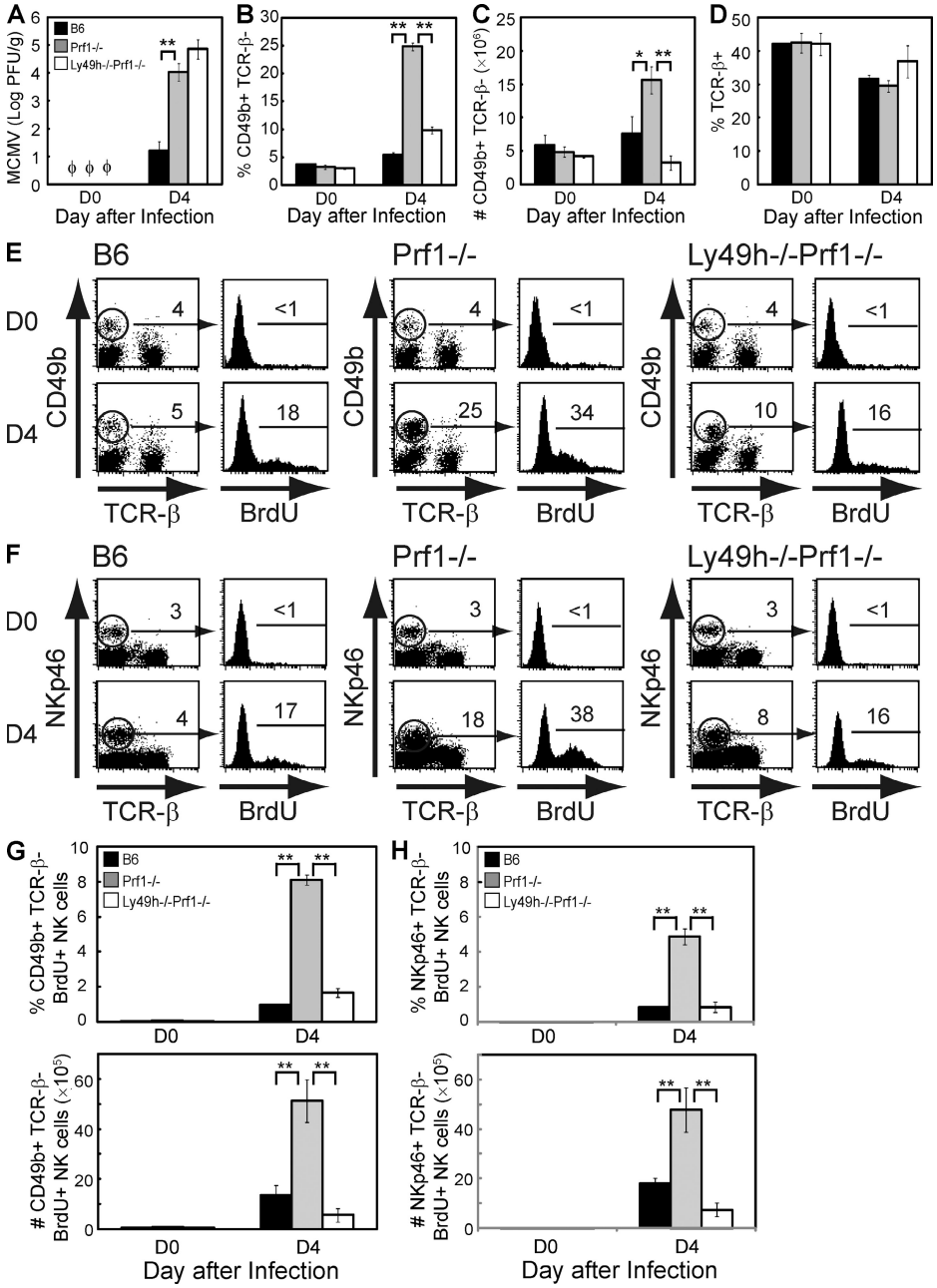


**Figure 4. Ly49H NK receptor requirement for enhanced NK proliferation in *Prf1*$^{-/-}$ mice.** (A) Virus titers in spleen were determined at day 4 after infection of *wt*, *Prf1*$^{-/-}$, and *Prf1*$^{-/-}$*Ly49h*$^{-/-}$ mice with 2,500 PFU MCMV. (B–D) Percentages (B) and absolute numbers (C) of CD49b$^{+}$TCRβ$^{-}$ NK cells and percentages (D) of TCRβ$^{+}$ cells in splenic leukocytes were determined at days 0 and 4. (E) Percentages of and BrdU incorporation in CD49b$^{+}$TCRβ$^{-}$ NK cells from *wt*, *Prf1*$^{-/-}$, and *Prf1*$^{-/-}$*Ly49h*$^{-/-}$ mice at day 4 after infection are shown. (F) Percentages of and BrdU incorporation in NKp46$^{+}$TCRβ$^{-}$ NK cells at day 4 after infection are shown. (G and H) The proportions and numbers of proliferating CD49b$^{+}$TCRβ$^{-}$ NK cells (G) or NKp46$^{+}$TCRβ$^{-}$ NK cells (H) in total splenic leukocytes of *wt*, *Prf1*$^{-/-}$, and *Prf1*$^{-/-}$*Ly49h*$^{-/-}$ mice at day 4 after infection were determined. Data are presented as means ± SD of three to six mice. Statistical significances between groups are indicated (*, $P < 0.05$; **, $P < 0.01$). Results are representative of two independent experiments with at least three mice per group. ϕ, not detected.

NK but not the non–NK cells from the *Prf1*$^{-/-}$ mice. As a second approach, highly purified NK cells were prepared from the *Prf1*$^{-/-}$ mice by FACS sorting of the CD49b$^{+}$Ly49H$^{+}$ cells on day 4 of infection (Fig. 6 B). Again, ex vivo production of IFN-γ and IL-10 was greatly enriched in the NK but not the non–NK cell populations. The total and sorted populations from day 4 MCMV-infected *Prf1*$^{-/-}$ mice were also examined for IL-10 expression at the mRNA level using RT-PCR, and the signaling for IL-10 was increased in the NK but not the non–NK cells (Fig. 6 C). Thus, high IL-10 is being produced in the *Prf1*$^{-/-}$ but not the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice at intermediate times after infection, and the greatly expanded NK cells are the source of the factor.

The cytokine IL-21 has been reported to induce IL-10 mRNA expression within NK cells (Brady et al., 2004). It was not possible to detect IL-21 protein production in the serum (unpublished data), but the mRNA was detected using samples from the populations isolated on day 4 after infection of the *Prf1*$^{-/-}$ mice. The signal was increased in the samples prepared from non–NK rather than NK cells (Fig. 6 C). Many culture experiments evaluating NK cell responses are enhanced by the addition of IL-15, and this cytokine was being expressed at the mRNA level in samples prepared from non–NK as compared with NK cells (Fig. 6 C) on day 4 after infection of the *Prf1*$^{-/-}$ mice. To evaluate the pathways stimulating IL-10 on day 4 in the *Prf1*$^{-/-}$ mice, ex vivo production

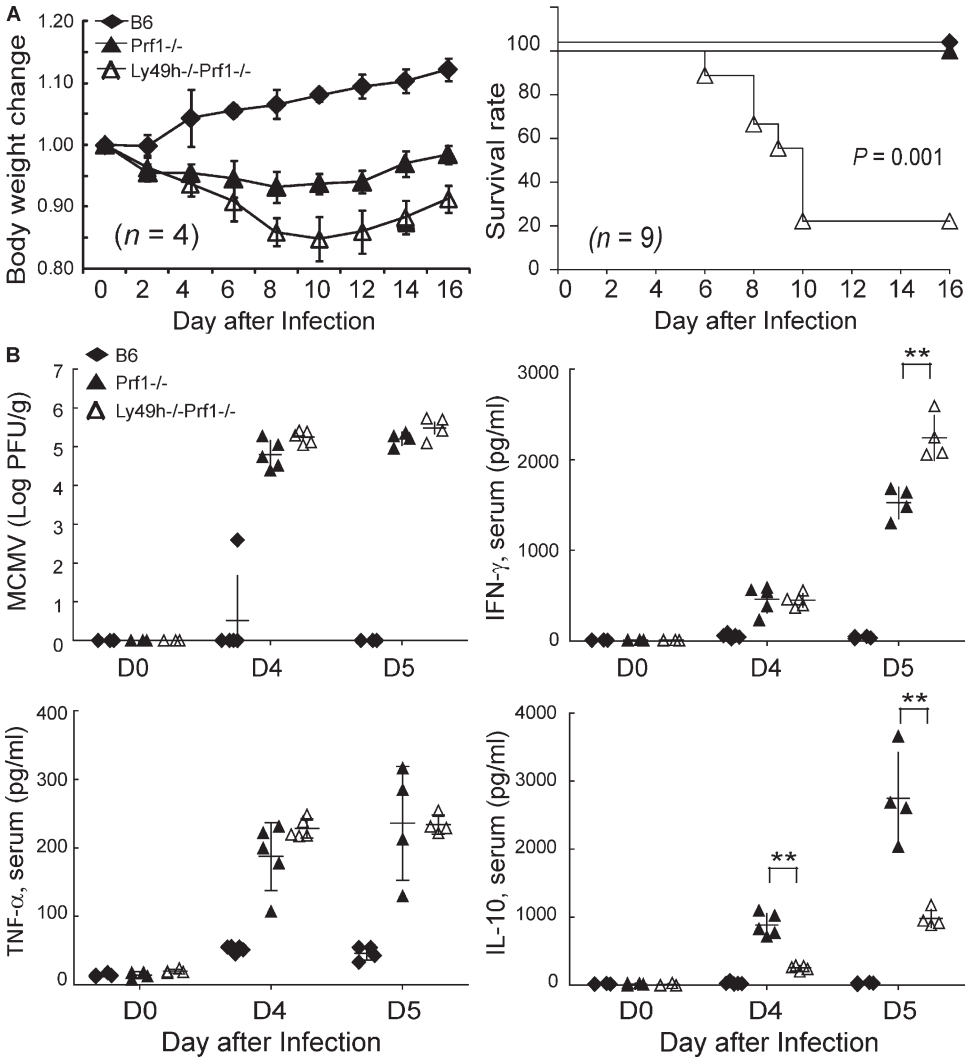


**Figure 5. Ly49H receptor requirements for better protection during infections of *Prf1*$^{-/-}$ mice.** (A) Body weight change after infection with 2,500 PFU MCMV and survival after infection with 5,000 PFU MCMV were evaluated in *wt*, *Prf1*$^{-/-}$, *and Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice. Results of body weight changes are representative of two independent experiments. Survival data were compiled from two independent experiments, and the p-value was determined by the log-rank survival test. (B) Virus titers in spleens, and IFN-γ, TNF-α, and IL-10 levels in serum from *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice after MCMV infections were measured at indicated time points. Symbols provide results from individual mice (*wt*, closed diamonds; *Prf1*$^{-/-}$, closed triangles; *Ly49h*$^{-/-}$*Prf1*$^{-/-}$, open triangles). Horizontal line data presented are means, and vertical lines indicate the ± SD of three to five mice. Statistical significances between groups are indicated (**, P < 0.01). Results are representative of at least two independent experiments with at least three mice per group.

by the total, NK, and non–NK cells was evaluated after stimulation with antibodies against Ly49H or with cytokines. Treatment with plate-bound anti-Ly49H did induce increases in IL-10 production by the NK cells, with levels increased approximately from 230 to 307 pg/$10^6$ cells (Fig. 6 D). This was an extremely modest response, however, when compared with cytokine stimulation. In this case, IL-21 treatment alone increased the levels to ~3,451 pg/$10^6$ cells, and IL-21 with IL-15 treatment increased the levels to ~8,582 pg/$10^6$ cells (Fig. 6 D). Thus, NK cells activated during MCMV infection of *Prf1*$^{-/-}$ mice produce IL-10, and IL-21 is a potent inducer of elevated IL-10 production by these cells.

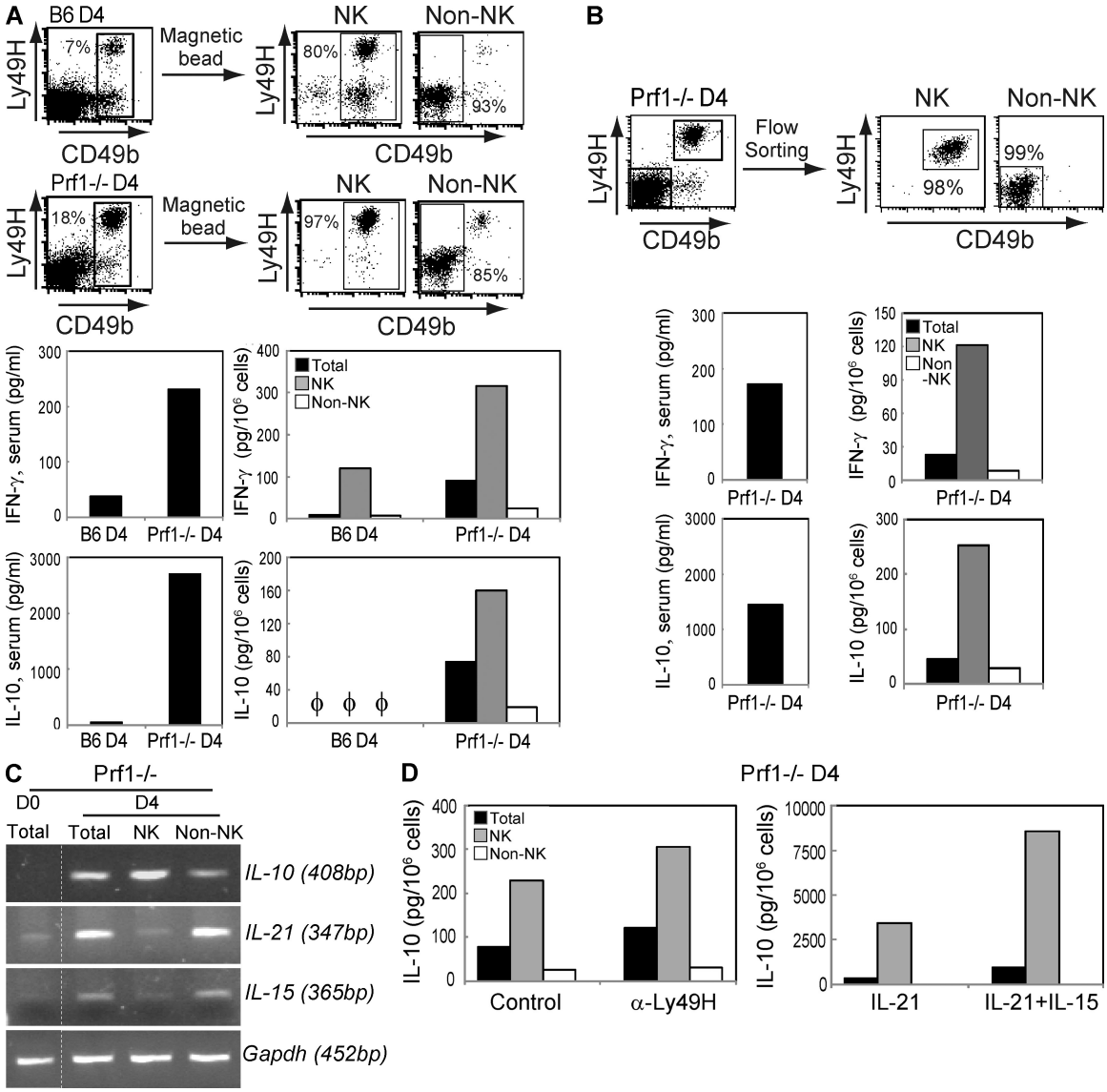


**Figure 6. IL-10 production by NK cells from MCMV-infected *Prf1*$^{-/-}$ mice.** (A) The top dot plots show the purity of NK cells (CD49b$^+$) and non–NK cells (CD49b$^-$) isolated from day 4 MCMV-infected *wt* and *Prf1*$^{-/-}$ mice after CD49b$^+$ magnetic isolation. The numbers indicate percentages of cells in each box. Cytokine production of IFN-γ and IL-10 in the serum of infected *wt* and *Prf1*$^{-/-}$ mice, or in 24 h conditioned media prepared with total splenic leukocytes and isolated subsets were determined. (B) The top dot plots show purity of NK cells (CD49b$^+$Ly49H$^+$) and non–NK cells (CD49b$^-$Ly49H$^-$) cells of day 4 MCMV-infected *Prf1*$^{-/-}$ mice after flow sorting. The numbers indicate percentages of cells in each box. Cytokine production of IFN-γ and IL-10 in serum of infected *Prf1*$^{-/-}$ mice, or in 24 h conditioned media from total splenic leukocytes and isolated subsets were determined. (C) Expression of *IL-10*, *IL-21*, and *IL-15* in the total and sorted populations from day 4 MCMV-infected *Prf1*$^{-/-}$ mice was analyzed by semiquantitative RT-PCR. *Gapdh* was used as an internal control. (D) Total splenic leukocytes, highly purified NK, and non–NK subsets from day 4 MCMV-infected *Prf1*$^{-/-}$ mice were stimulated either on anti-Ly49H antibody or with cytokines. After 24 h of incubation, IL-10 production was determined in conditioned media. Results are representative of at least two independent experiments using a total of two to four individual mice. The studies in A were repeated twice for *wt* and four times for *Prf1*$^{-/-}$ mice. Those in B were repeated three times, and those in C were repeated twice. The studies in D with anti-Ly49H antibody were repeated twice, and those with cytokines were repeated four times. The dashed white line indicates that intervening lanes have been spliced out. ϕ, not detected.

### Role for Ly49H and IL-10 in regulating CD8 T cell responses

To examine the T cell responses in the *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice, mice were infected with MCMV for 7 d. Although the proportions of NK cells (CD49b$^{+}$TCR$\beta^{-}$) in *Prf1*$^{-/-}$ mice were increased and those in *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were severely reduced, the frequencies of CD8$\alpha^{+}$TCR$\beta^{+}$ cells reached 25–30% in all three groups of mice (Fig. 7 A). The activation states, however, were higher in the *Prf1*$^{-/-}$ mice and highest in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice. The proportions of CD8 T cells from these mice expressing IFN-$\gamma$ were <1, 6, and 22%, respectively, and those expressing granzyme B were <4, 47, and 71%, respectively. When the proportions and yields of CD8$\alpha^{+}$IFN-$\gamma^{+}$ cells in splenic populations were calculated, the order and differences between the groups were similar. In terms of overall yields of CD8 T cells expressing IFN-$\gamma$, however, the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice had >45-fold increases as compared with *wt* mice and >4.2-fold increases as compared with *Prf1*$^{-/-}$ mice (Fig. 7 B). The MCMV titers were higher in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ as compared with the *Prf1*$^{-/-}$ mice, but the difference was not significant (Fig. 7 C). In contrast, the circulating levels of IFN-$\gamma$, TNF-$\alpha$, and IL-10 were all substantially and significantly increased in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice, with values at least 3.4-fold higher. Thus, the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice lacking NK cell expansion and dramatically reduced NK cell production of IL-10 at intermediate times after infection have profoundly increased CD8 T cell responses to MCMV infection with only modest increases in viral burdens.

IL-10 has a wide range of immunoregulatory functions including controlling T cell responses (Maynard and Weaver, 2008). To examine its role in the regulation of CD8 T cell responses in the *Prf1*$^{-/-}$ mice, the consequences of neutralizing the cytokine were evaluated. Mice were given control antibody or antibody-neutralizing IL-10. The treatment protocol used was developed to deliver maximum effects while allowing reappearance of day 7 circulating levels of IL-10 equivalent to those in the *Prf1*$^{-/-}$ mice, i.e., concentrations of 200–300 mg/ml (Fig. 7 C, bottom right). It required antibody delivery on days 2 and 4 of infection (see Materials and methods). Both control and anti–IL-10 antibody groups of mice still had increasing proportions of NK cells (CD49b$^{+}$ TCR$\beta^{-}$; Fig. 8 A). They were very different, however, in their CD8 T cell responses. Neutralization of IL-10 resulted in elevated endogenous CD8 T cell responses (Fig. 8, A and B). In this case, the viral burdens were increased by ~1.5 logs, and this difference was significant (Fig. 8 C). The circulating levels of IFN-$\gamma$, TNF-$\alpha$, and IL-10 were, however, increased by 12.6-, 10-, and 5.5-fold, respectively (Fig. 8 C). Overall, the differences in the parameters examined after IL-10 neutralization during MCMV infection of *Prf1*$^{-/-}$ mice paralleled those observed in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice. Therefore, early IL-10 production by the Ly49H-sustained NK cells regulates CD8 T cell responses.

To examine IL-10's role in controlling detrimental effects during infections in the *Prf1*$^{-/-}$ mice, the effects of IL-10 neutralization were evaluated under conditions of infection with 5,000 PFU MCMV. The first experiments examined the consequences of blocking IL-10 with antibodies against the IL-10 receptor (anti–IL-10R). Although all *wt* mice treated with anti–IL-10R survived the infection, *Prf1*$^{-/-}$ mice treated

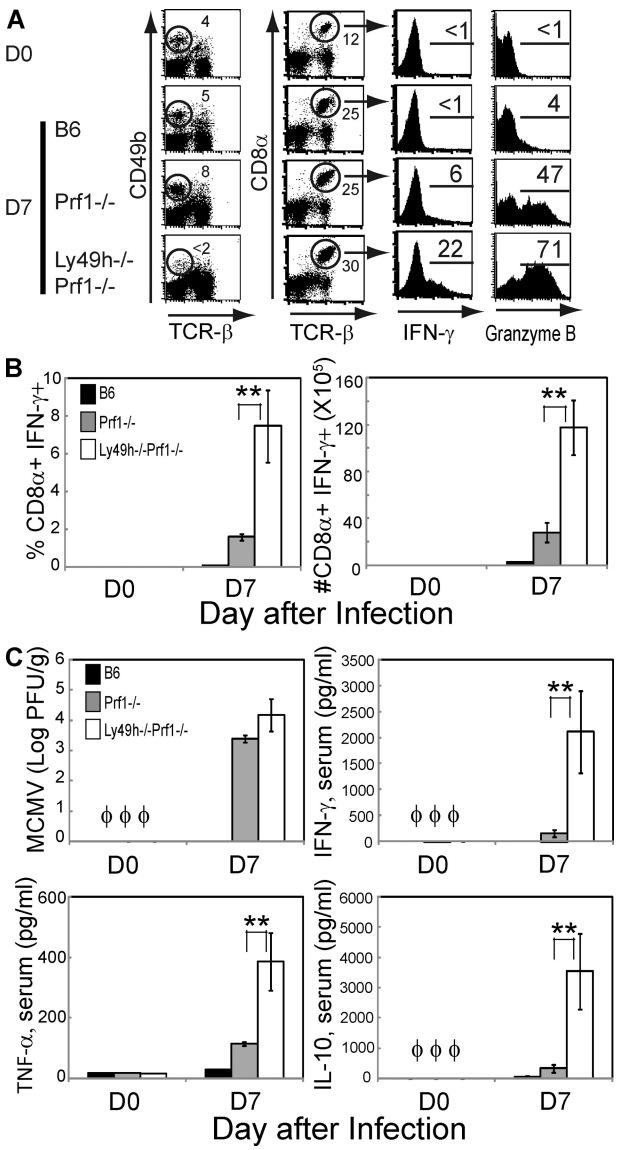


**Figure 7. Effect of Ly49H on CD8 T cell responses in MCMV-infected *Prf1*$^{-/-}$ mice.** (A) Splenic leukocytes from day 0 and 7 MCMV-infected *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were analyzed for frequencies of NK cells and CD8 T cells, and expression of IFN-$\gamma$ and granzyme B in CD8 T cells. (B) Percentages and absolute numbers of CD8$\alpha^{+}$IFN-$\gamma^{+}$ in total splenic leukocytes isolated from day 0 and 7 MCMV-infected *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were determined. (C) Virus titers in spleens, and IFN-$\gamma$, TNF-$\alpha$, and IL-10 in serum samples from day 0 and 7 MCMV-infected *wt*, *Prf1*$^{-/-}$, and *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were measured. The numbers indicate the percentages of cells in each area. Data are presented as means ± SD of three to six mice. Statistical significances between groups are indicated (*, $P < 0.05$; **, $P < 0.01$). Results are representative of at least two independent experiments with at least three mice per group. $\phi$, not detected.

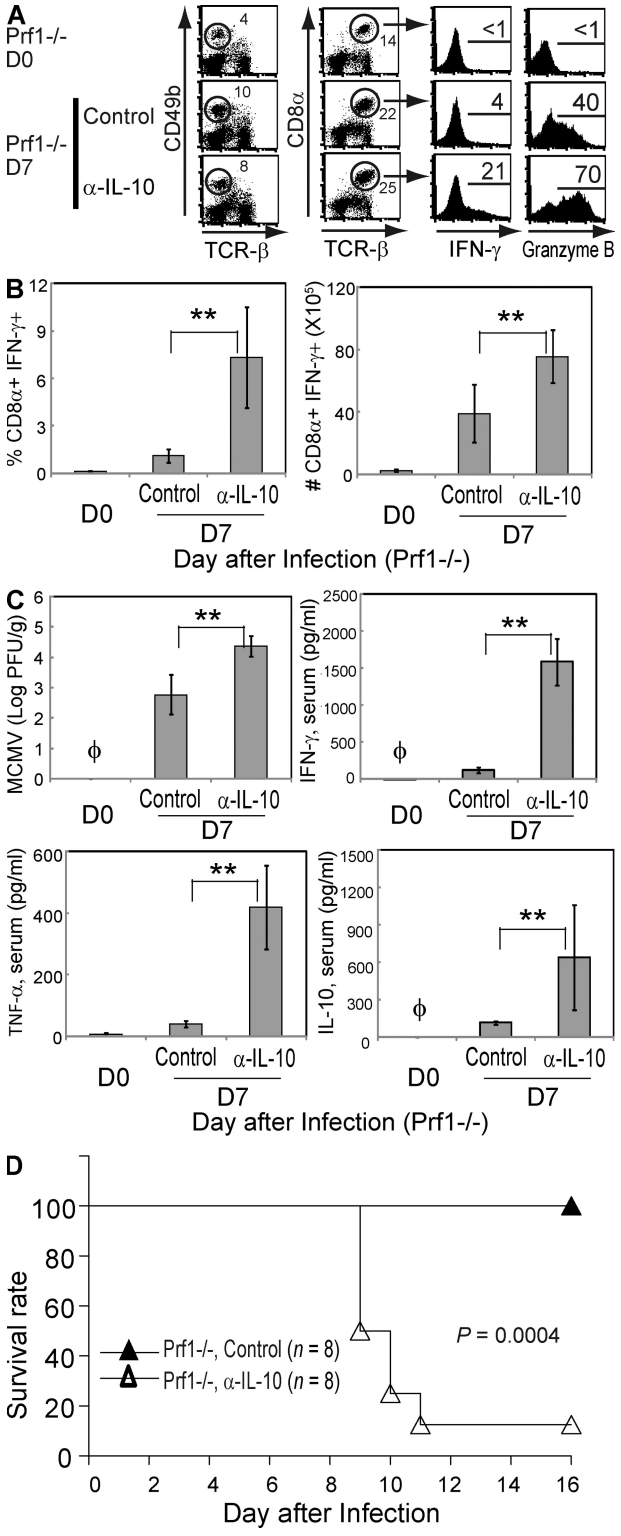


**Figure 8. Effect of blocking of IL-10 on CD8 T cell responses in and survival of MCMV-infected *Prf1*$^{-/-}$ mice.** (A) Splenic leukocytes from day 0 and 7 MCMV-infected *Prf1*$^{-/-}$ mice treated with either control antibody or anti–IL-10 antibody were analyzed for frequencies of NK cells and CD8 T cells, and expression of IFN-γ and granzyme B in CD8 T cells. (B) Percentages and absolute numbers of CD8α$^+$IFN-γ$^+$ in total splenic leukocytes from day 0 and 7 MCMV-infected *Prf1*$^{-/-}$ mice treated with either control antibody or anti–IL-10 antibody are shown. (C) Virus titers in spleens, and IFN-γ, TNF-α, and IL-10 levels in serum samples from day 0 and 7 MCMV-infected *Prf1*$^{-/-}$ mice treated with either control antibody or anti–IL-10 antibody were measured. The numbers indicate the percentages of cells in each area. Data are presented as means ± SD of three to six mice. Statistical significances between groups are indicated (**, P < 0.01). Results are representative of at least two independent experiments with at least three mice per group. (D) Survivals of *Prf1*$^{-/-}$ mice infected with 5,000 PFU and treated with either control antibody or anti–IL-10 antibody were evaluated. Data were compiled from two independent experiments, and the p-value was determined by the log-rank survival test. ϕ, not detected.

with anti–IL-10R all died by day 11 of infection (unpublished data). As an independent approach, *Prf1*$^{-/-}$ mice were treated with control antibody or antibody against the cytokine itself (αIL-10) on days 2 and 4 of infection, as described in the previous paragraph. Administration of αIL-10 resulted in death of >80% of the *Prf1*$^{-/-}$ mice (Fig. 8 D). Thus, blocking early IL-10 effects in *Prf1*$^{-/-}$ results in a increased sensitivity to infection and a phenotype comparable to *Ly49h*$^{-/-}$ *Prf1*$^{-/-}$ mice.

### Role for CD8 T cell responses in late cytokine responses and death during infections of *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice

To conclusively demonstrate the contribution of the CD8 T cell response to cytokine production and death after MCMV challenge in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice, mice were control treated or treated with antibodies against CD8 (αCD8). After challenge with 2,500 PFU of virus, blocking the CD8 T cell response had no effect on the day 7 viral titers in the *Ly49h*$^{-/-}$ *Prf1*$^{-/-}$ mice (Fig. 9 A) but did significantly inhibit the elevated circulating levels of cytokines (Fig. 9 A). Moreover, the αCD8α treatment protected against the lethal consequences of infection with 5,000 PFU MCMV (Fig. 9 B). Thus, the presence of the CD8 T cell responses was not effective at controlling the viral infection but did result in elevated cytokine responses and death. Collectively, the studies demonstrate that expanded NK cells and early IL-10 production is regulating the magnitude of endogenous CD8 T cell responses and protecting from a CD8 T cell–dependent death under these conditions of sustained viral infections.

## DISCUSSION

These studies demonstrate an unexpected role for an activating receptor in sustaining NK cells and, as a result, protecting against detrimental consequences during viral infection. In particular, they define the Ly49H receptor's contribution to the proliferation and maintenance of NK cells during MCMV infections. In *wt* B6 mice, viral replication was controlled. NK cell proliferation peaked at day 3 after challenge, with the proportions of Ly49H$^+$ populations increasing without profound cell expansion. Deficiencies in *Ly49h*, *Prf1*, or both all resulted in significant increases in MCMV burdens, but the consequences for NK cell expansion were profoundly

different; NK cell proportions, numbers, and division were decreased in the absence of *Ly49h* but dramatically increased in the absence of *Prf1*. The Ly49H receptor was required for the responses in infected *Prf1*$^{-/-}$ mice because (a) nearly all NK cells expressed Ly49H, (b) the Ly49H$^{+}$ cells were preferentially proliferating, and (c) ablation of the *Ly49h* gene in addition to the *Prf1* gene (*Ly49h*$^{-/-}$*Prf1*$^{-/-}$) abolished NK cell proliferation. The Ly49H-dependent effects were important because the absence of *Ly49h* in the *Prf1*$^{-/-}$ mice resulted in increased sensitivity to disease and death. The expanded Ly49H$^{+}$ NK cells in *Prf1*$^{-/-}$ mice were making IL-10, and CD8 T cell responses were being regulated by the NK cells and IL-10; CD8 T cell responses were dramatically elevated in *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ and *Prf1*$^{-/-}$ mice having had IL-10 function neutralized. Elevated CD8 T cell responses in the *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice were responsible for death after MCMV challenge. Thus, the Ly49H activating receptor contributes to the proliferation and maintenance of NK cells, the NK cells are induced to produce IL-10 under these conditions, and the NK cells and IL-10 are important for limiting the magnitude of CD8 T cell responses to make the difference between life and death (Fig. 10).

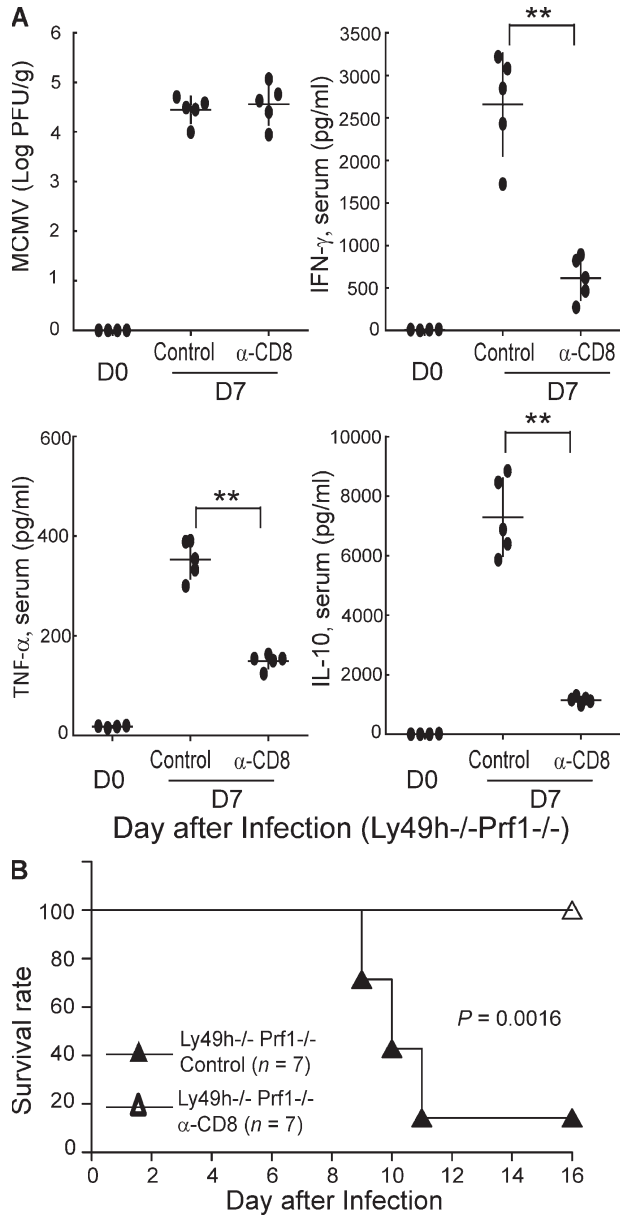


**Figure 9. Effect of blocking CD8 T cell responses during MCMV infection of *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice.** (A) Virus titers in spleens, and IFN-γ, TNF-α, and IL-10 levels in serum samples from day 0 and 7 MCMV-infected *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice treated with either control antibody or anti-CD8α antibody were measured. Symbols provide results from individual mice. Horizontal line data presented are means, and the vertical lines indicate ± SD of five mice. Statistical significances between groups are indicated (*, $P < 0.05$; **, $P < 0.01$). Results are representative of at least two independent experiments. (B) Survivals of *Ly49h*$^{-/-}$*Prf1*$^{-/-}$ mice MCMV infected with 5,000 PFU and treated with either control antibody or anti-CD8α antibody were evaluated. Data were compiled from two independent experiments, and the p-value was determined by the log-rank survival test.

The results have a broad significance to our understanding of the regulation and function of NK cells during viral infections. Before the clear demonstration of a role for NK cells in defense against herpes group viruses, there were numerous reports of defects in NK cell functions after a wide range of viral infections (Biron et al., 1999; Lee et al., 2007). More recently, decreases in NK cell numbers and maturation states have been reported during infections of humans with HIV (Tarazona et al., 2002; Azzoni et al., 2005), hepatitis C virus (HCV; Morishima et al., 2006), and varicella zoster virus (Vossen et al., 2005); of monkeys with Ebola virus (Reed et al., 2004); and of mice with mouse hepatitis virus 3 (Lehoux et al., 2004). It is important to note, however, that there are genetic differences between individuals and strains, and that there are also reports of increases in proportions of NK cells expressing particular activating receptors during viral infections (Dokun et al., 2001; Gumá et al., 2006; De Maria et al., 2007; Alter et al., 2009). In our study, an activating receptor is shown to play a critical role in sustaining NK cells. In fact, the extreme expansion of these "MCMV-specific" NK cells in the absence of cytotoxic function is demonstrated. Analogues of Ly49H recognition of virus-infected cells have been proposed to exist for viruses in humans. One good candidate identified during human CMV (HCMV) infections is the NKG2C/CD94-activating NK receptor. Significantly increased proportions of NK cells expressing NKG2C are observed in HCMV seropositive individuals compared with HCMV seronegative individuals (Gumá et al., 2006). Increases in the proportions of NK cells expressing the NKp30 activating receptor have been observed during chronic HCV infection (De Maria et al., 2007), and in the proportions of NK cells expressing the KIR3DS1 activating receptor have been observed during acute HIV infection (Alter et al., 2009). Thus, studies in both mice and men indicate that activating receptors may be helping to select NK cell subsets during infections and that as a consequence, the frequencies of NK cells expressing particular activating receptors are changing. Our study also demonstrates, however, that activating receptors

are required for sustaining NK cells because the absence of Ly49H results in decreasing NK cell yields during MCMV infections. Collectively, the studies show that activating receptors promote the expansion of particular NK cell subsets and that without such a pathway, NK cells are not sustained during prolonged infection.

The work adds to the growing literature characterizing conditions supporting NK cell expansion (Biron et al., 1984; Caligiuri et al., 1991; Yamada et al., 1996; Lanier, 1998; Biron et al., 1999; Dokun et al., 2001; Fehniger et al., 2001; Nguyen et al., 2002; Yokoyama et al., 2004; French et al., 2006; Huntington et al., 2007a; Sun et al., 2009). In this case, the importance for the expansion is clearly demonstrated. Keeping the NK cells around is likely to promote defense and immunoregulatory mechanisms mediated through the activating receptor supporting expansion, and allow access of those induced through stimulation of other activating receptors, cytokine receptors, and/or engagement of the TNF-related apoptosis-inducing ligand expressed on activated NK cells (Lee et al., 2007). The latter three mechanisms have the potential to promote effects even if stimulation of the original activating receptor through its ligand is blocked. The NK cells expanded in the *Prf1*$^{-/-}$ mice during MCMV infection are shown in this paper to be producing IL-10. Although stimulation through Ly49H itself could modestly (<1.5-fold) enhance IL-10 production, mRNA expression of IL-21 is also apparent, and addition of this cytokine causes dramatic (>15-fold) increases in IL-10 production by the NK cells. Thus, the system demonstrates a mechanism using the activating NK cell receptor to promote the continued presence of the cells for activation by other cytokines. Keeping the cells around is likely to enhance resistance and health under a wide range of viral infections, and learning how to manage this during infections failing to directly stimulate an activating receptor to support NK cell expansion will have important therapeutic applications.

The cytokine IL-10 was first shown to be a product of T cells, but the list of cellular sources is growing (Brooks et al., 2006; Ejrnaes et al., 2006; Maris et al., 2007; O'Garra and Vieira, 2007). Before our study, the characterization of NK cell IL-10 production had been largely limited to expression at the mRNA level during *Toxoplasma* infection of mice (Maroof et al., 2008), after treatment of mouse cells with IL-21 (Brady et al., 2004), or in cells prepared from HIV-infected individuals (Brockman et al., 2009). There are recent reports, however, that human regulatory NK cells can be mitogen stimulated to produce IL-10 (Deniz et al., 2008) and that NK cells recovered during chronic HCV infections produce IL-10 (De Maria et al., 2007). Although the factor can also enhance inflammatory responses under particular conditions (Herrero

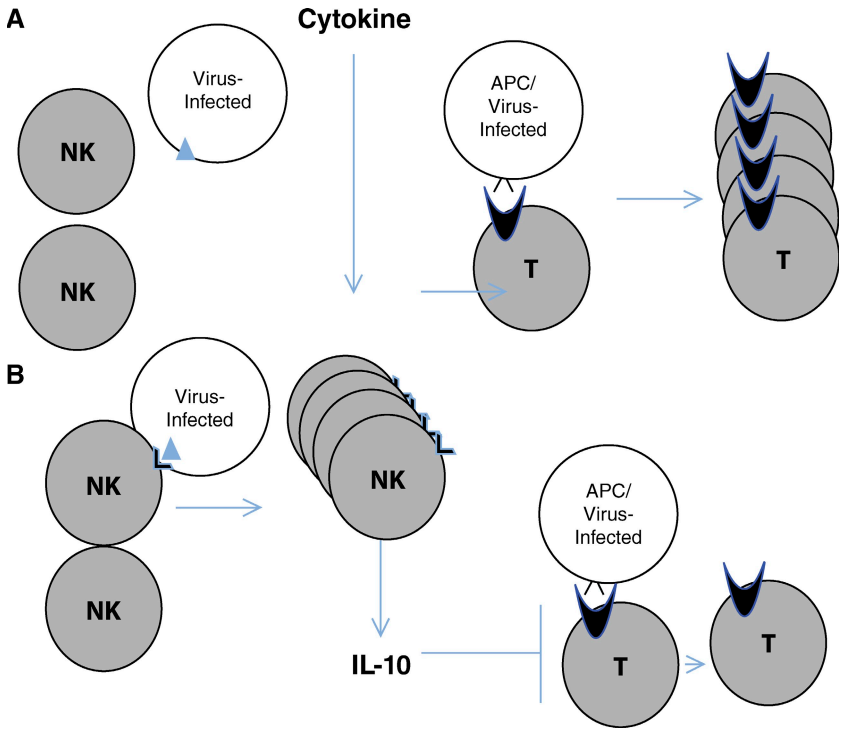


**Figure 10. Model for sustaining NK cells for regulation of adaptive immune responses during viral infections.** (A) In the absence of stimulation through activating receptors, NK cells decrease during extended viral infections. As a result, they are not available to contribute antiviral and/or immune regulatory functions for extended periods. If the virus is unchecked, unregulated downstream adaptive immune responses can lead to immune pathology and death. (B) In the presence of stimulation through activating receptors, NK cell proliferation is supported and the cells are available to contribute a wide range of functions for extended periods. The studies in this paper demonstrate that during profound viral infections, the conditions can result in induction of NK cell IL-10 production to regulate the magnitude of adaptive CD8 T cell responses and protect from CD8 T cell–dependent death.

et al., 2003; Sharif et al., 2004), IL-10 has largely been appreciated for its direct and indirect inhibitory effects on several T cell responses (Deniz et al., 2008; Maynard and Weaver, 2008). Therefore, the production of IL-10 during infections has been thought to present a situation for interfering with viral defense (Brooks et al., 2006; Ejrnaes et al., 2006; De Maria et al., 2007; Maris et al., 2007). However, IL-10 has also been shown to contribute to regulation of immunopathology (Gazzinelli et al., 1996), and the expansion of NK cells and their early production of IL-10 reported in this paper is demonstrated to be important for limiting CD8 T cell response and CD8 T cell–dependent disease. Thus, an NK cell contribution to regulating adaptive immunity for protection against immune-mediated disease is demonstrated for the first time. The pathway may be in place at some level during infections of immunocompetent mice because our laboratory has previously shown NK cell–dependent regulation of T cell responses to MCMV in *wt* mice (Su et al., 2001), and challenges with extremely high doses of the virus have been reported to induce low levels of IL-10 and IL-10–dependent protection against disease (Oakley et al., 2008).

The work also conclusively separates, for the first time, the beneficial functions of Ly49H-dependent expansion from Ly49H-dependent cytotoxicity. The activating receptor has been reported to induce NK cell killing and proliferation in culture, but only delivery of cytotoxic function has been thought to be important in protection against MCMV (Scalzo et al., 1990; Tay and Welsh, 1997; Loh et al., 2005; van Dommelen et al., 2006). Although other factors such as cytokines may contribute (Nguyen et al., 2002; French et al., 2006), the requirement for Ly49H in supporting NK cell proliferation and health are definitively established in the absence of cytotoxic function. The results suggest a model whereby NK cells are sustained during periods of intense receptor stimulation but delivery of cytotoxic function to regulate viral replication acts to limit expansion by eliminating virus-infected cells. In the absence of cytotoxicity or in the presence of overwhelming viral replication failing to be limited by cytoxicity, however, NK cell expansion is increased through an Ly49H-dependent pathway to promote alternative beneficial effects. This identification of the importance of the activating receptor in the context of infection is distinct from another report investigating the role of these molecules in NK cell maturation and tolerance (Sun and Lanier, 2008; Tripathy et al., 2008). In this study, the activating receptor is shown to be critical for maintaining NK cells during acute infectious challenge.

Given these results, it is interesting to consider CD8 T cell expansion during infections with lymphocytic choriomeningitis virus (LCMV), a virus inducing dramatic expansion of CD8 T cells in *Prf1*$^{-/-}$ mice (Matloubian et al., 1999). Collectively, the responses to MCMV and LCMV indicate that linking stimulation for killing with stimulation for proliferation may be important for regulating proportions of both NK and CD8 T cells. It is remarkable, however, that in contrast to the known MCMV expression of a ligand for the NK cell activating receptor Ly49H, there is no evidence for NK cell activating receptor recognition of LCMV. Thus, LCMV infections of *Prf1*$^{-/-}$ mice may present a condition where NK cells are not expanded for maintenance, and as a result, they are not available to critically contribute to the regulation of the CD8 T cell response. This model would explain the dramatic magnitude of CD8 T cell responses during LCMV infections of both *wt* and *Prf1*$^{-/-}$ mice (Matloubian et al., 1999), as well as the apparent major contribution of the CD8 T cell responses to hemophagocytic lymphohistocytosis diseases under conditions of LCMV (Jordan et al., 2004) but not MCMV (Crozat et al., 2007) infections in systems with blocked cytotoxic functions. The model also suggests that the balance of NK and CD8 T cell responses might be controlled in a wide range of infection by the presence or absence of a ligand for an NK cell activating receptor.

In summary, this paper discovers a novel and important new pathway for sustaining NK cells during infection, proves its critical role in protection against disease, and defines the mechanism for the beneficial effects dependent on this pathway as NK cell regulation of adaptive immunity.

## MATERIALS AND METHODS

**Mice.** Specific pathogen-free C57BL/6 (C57BL/6NTac) mice were purchased from Taconic. *Ly49h*-deficient (*Ly49h*$^{-/-}$) mice with a B6 background were generated by a marker-assisted congenic approach (Fodil-Cornu et al., 2008). Perforin-deficient (*Prf1*$^{-/-}$) mice (Kagi et al., 1994) were originally obtained from the Jackson Laboratory to establish breeding colonies at Brown University. Mice deficient in both *Ly49h* and *Prf1* (*Ly49h*$^{-/-}$*Prf1*$^{-/-}$) were generated by crossing *Ly49h*$^{-/-}$ and *Prf1*$^{-/-}$ mice. All mouse colonies were kept and maintained in strict isolation under specific pathogen-free conditions. Mice used in experiments were 6–14 wk old. Animals obtained from sources outside of Brown University were housed in the animal care facility for at least 1 wk before use. Handling of mice and experimental procedures were conducted in accordance with institutional guidelines for animal care and use, and protocols were approved by the Brown University Institutional Animal Care and Use Committee.

**In vivo manipulation.** Unless otherwise indicated in the figures, infections were initiated on day 0 by i.p. injection of 2,500 PFU of salivary gland–derived MCMV prepared, as previously described (Orange et al., 1995; Dalod et al., 2002). Experiments were repeated with independently derived Smith strain MCMV from American Type Culture Collection, as previously described (Depatie et al., 1997). The infectious virus in tissue homogenates was quantified by plaque assay using mouse embryonic fibroblasts (Orange et al., 1995). To evaluate in vivo DNA synthesis, mice were injected with 2 mg BrdU (BD) on the days indicated in the figures and sacrificed 2 h later. For CD8 T cell blocking, 1 mg anti-CD8α antibody (clone 53.6.72; Bio-Express) was administered by i.p. injection at day 5. For controls, an equal amount of rat IgG (Sigma-Aldrich) was given. For blocking of the IL-10 receptor, 500 μg of blocking anti–IL-10R antibody (clone 1B1.3A; Bio-Express) was administered by i.p. injection at day 3. For neutralization of IL-10, 600 μg anti–IL-10 antibody (clone JES5-2A5; Bio-Express) was administered by i.p. injection at days 2 and 4. An equal amount of isotype control rat IgG1 mAb (clone HRPN; Bio-Express) was used as a control for anti–IL-10R antibody and anti–IL-10 antibody. The anti–IL-10 protocol was developed by modifying treatment conditions to maximize effects, ensure neutralization of the IL-10 produced at intermediate times after infection, and allow circulating levels of the cytokine on day 7. For the survival assay, mice were infected with 5,000 PFU MCMV.

**Preparation of leukocyte populations.** For preparation of splenic leukocytes, spleens were ground between the rough surfaces of glass slides and treated with Red Blood Cell Lysing Buffer (Sigma-Aldrich) to remove red

blood cells. Viable cell yields were determined by Trypan blue exclusion. The frequencies of NK and CD8 T cells among leukocytes were determined by flow cytometric analysis, and the absolute numbers were calculated by multiplying the frequencies by the total number of leukocytes.

**Flow cytometric and immunofluorescence analyses.** Single splenic leukocytes were prepared as described in the previous section and incubated for 20 min with 2.4G2 antibody to reduce nonspecific staining. Cell-surface staining was performed as previously described (Dalod et al., 2002), using antibodies directed against the following: CD49b-FITC, CD49b-PE, TCRβ-PE, TCRβ-allophycocyanin (APC), Ly6C-FITC, CD122-PE, CD69-PE, CD11c-APC, CD27-PE, CD51-PE, CD62L-APC, CD11b-PE, NK1.1-PE, NKp46-PE, and CD8α-PE. Antibodies were purchased from BD and eBioscience. Polyclonal anti–NKp46-FITC antibody was purchased from R&D Systems. The specific monoclonal antibody, directed against Ly49H (a gift from W. Yokoyama, Washington University School of Medicine, St. Louis, MO; Smith et al., 2000), was biotinylated and used with streptavidin fluorochromes (PE or APC) to identify Ly49H-expressing cells. For the detection of incorporated BrdU, cells were first stained for surface antigens and then fixed, permeabilized, treated with DNase I, and stained with FITC–anti-BrdU antibody (clone 3D4; BD) according to the manufacturer's protocol. For intracellular staining of IFN-γ and granzyme B, the cells were incubated with 5 μg/ml brefeldin A for 6 h and stained for surface markers. The cells were fixed and permeablized (Fix/Perm Buffer; BD) and labeled with either IFN-γ–APC or granzyme B–APC. Samples were acquired using a FACSCalibur with the CellQuest Pro software package (both from BD). Laser outputs were 15 mW at 488- and 635-nm wavelengths.

**Cytokine production analysis.** Cytokine production of IFN-γ, TNF-α, and IL-10 in serum or conditioned media was measured using cytometric bead assays (mouse inflammation kit; BD). Cytokine production was presented as pg/ml for serum and pg/$10^6$ cells for conditioned media.

**NK cell isolation.** For isolation of NK cells expressing CD49b, splenic leukocytes from *wt* and *Prf1*$^{-/-}$ mice at day 4 MCMV infection were processed using anti-CD49b magnetic beads and positive selection with the Possel_s program on the AutoMACS according to the manufacturer's instructions (Miltenyi Biotec). The enriched populations contained >80% CD49b$^+$ cells and >85% CD49b$^-$ cells. For FACS sorting of the NK cell subsets expressing CD49b and Ly49H and the non–NK cell subset not expressing CD49b and Ly49H, splenic leukocytes from *Prf1*$^{-/-}$ mice at day 4 of MCMV infection were stained with PE-CD49b and APC-Ly49H. Cell sorting was performed on a cell sorter (FACSAria; BD) at the Flow Cytometer Facility at Brown University. The purity of the subsets was >98%.

**Ex vivo NK cell manipulations.** To evaluate IL-10 and IFN-γ production from isolated subsets, $10^5$ cells were incubated for 24 h at $10^6$ cells/ml in complete RPMI 1640 medium. For further stimulation, $10^5$ highly purified cells were incubated either on control antibody (mouse IgG1)– and anti-Ly49H antibody–coated plates or with 100 ng/ml of mouse recombinant IL-21 (>97% purity; R&D Systems) in the presence or absence of 50 ng/ml IL-15 (>95% purity; R&D Systems).

**RT-PCR analysis.** Total RNA was isolated from total splenic leukocytes at days 0 and 4, and highly purified subsets at day 4 (>98%) using RNeasy (QIAGEN). 1 μg RNA was reverse-transcribed as described previously (Dalod et al., 2002). One tenth of the product from the RT reaction was used as a template for PCR amplification using primers specific for *IL-10*, *IL-21*, and *IL-15* synthesized by Operon in a programmable thermal cycler (PTC-200; MJ Research). Sequences of oligonucleotides are as follows: *IL-10* (forward, 5′-TGCTATGCTGCCTGCTCTTACTGA-3′; reverse, 5′-CCTGCTCCACTGCCTTGCTCTTAT-3′; Brady et al., 2004), *IL-21* (forward, 5′-CCCTTGTCTGTCTGGTAGTCATC-3′; reverse, 5′-ATCACAGGAAGGGCATTTAGC-3′; Brandt et al., 2003), and *IL-15* (forward, 5′-CATATGGAATCCAACTGGATAGATGTAAGATA-3′; reverse, 5′-CATATGCTCGAGGGACGTGTTGATGAACAT-3′; Zhang et al., 1998). *Gapdh* (forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′) was used as an internal control. The data shown in the figures were obtained before the amplification products reached saturation.

**Evaluation of the body weight and survival.** Mice were weighed and monitored for death once a day at the same time on every day during infection from day 0 (before infection) to day 16. For clarity, body weight changes at 2-d intervals are shown in the figures.

**Statistical analyses.** Statistical analyses were performed using an unpaired Student's two-tailed *t* test. Means ± SD are shown. The Kaplan-Meier log-rank test was used to compare survival between groups of mice using Prism 5 software (GraphPad Software, Inc.).

**Ex vivo NK cell proliferation assay.** For evaluation of intrinsic proliferative activity of NK cells, NK cells were enriched (30–50% of NK1.1$^+$TCRβ$^-$ cells) from splenocytes of *wt*, *Ly49h*$^{-/-}$, and *Prf1*$^{-/-}$ mice using negative selection with magnetic beads (Miltenyi Biotec). $5 \times 10^4$ enriched NK cells were stimulated on 96-well plates with either 150 ng/ml IL-2 or γ-irradiated Ba/F3 cells and Ba/F3 cells expressing m157 (effector/target ratio = 2:1; a gift from L. Lanier, University of California, San Francisco, San Francisco, CA). At 40 h after stimulation, cells were incubated with BrdU at a 20-μM concentration for an additional 2 h and were analyzed for BrdU incorporation.

**Online supplemental material.** Fig. S1 shows intrinsic NK cell proliferative potentials. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082387/DC1.

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