dilution by Ly49C/I$^+$ (gray fill) and Ly49C/I$^-$ (black line) mixed bone marrow chimeric Ly49H$^+$ wild-type and SHP-1$^{Me\text{-}v}$ NK cells 5 days after MCMV infection. Data are representative of three experiments with 2-3 animals each.

Figure 5: Unlicensed NK cells control MCMV infection

MCMV titers in the (**a**) salivary glands and (**b**) liver one-week post infection in B6 wild-type mice either untreated or depleted of Ly49C/I$^+$ NK cells, Ly49G2$^+$ NK cells, or all NK cells. Data are representative of two experiments with five mice per group. MCMV titers in the (**c**) salivary glands and (**d**) liver one-week post infection of B6 wild-type mice and *B2m*$^{-/-}$ mice, both depleted of CD8$^+$ T cells. Data are representative of two experiments with five mice per group.

Figure 6: Licensed NK cells do not protect neonates from MCMV infection

Ly49h$^{-/-}$ neonates received (**a**) 1 x 10^5 Ly49H$^+$ NK cells sorted as either Ly49C/I$^+$ or Ly49G2$^+$Ly49C/I$^-$ or PBS or (**b**) 7.5 x 10^4 Ly49H$^+$ NK cells from donors pre-depleted of Ly49C/I$^+$ or Ly49G2$^+$ cells *in vivo* or PBS and challenged with 2 x 10^3 pfu of MCMV. Animals were monitored daily for morbidity.

Online Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute. *B2m*$^{-/-}$ mice on the C57BL/6 background were purchased from Jackson Labs. S. Vidal generously provided *Ly49h*$^{-/-}$ B6 mice, and C. Lowell generously provided *SHP-*

1$^{me\text{-}v}$ B6 mice. *H2K*^{b}*D*$^{b-/-}$ B6 mice were purchased from Taconic. Mixed bone marrow chimeras were generated by mixing bone marrow from CD45.1^{+} wild-type and SHP-1$^{Me\text{-}v}$ mice at a 1:4 ratio and injecting into CD45.1^{+} wild-type recipients following lethal (950 rads) irradiation. Chimeras were analyzed 8-10 weeks post-reconstitution. All mice were maintained in the UCSF specific pathogen-free animal facility. The UCSF Institutional Animal Care and Use Committee approved animal protocols.

Cell-based stimulation

Target cells were generated by retrovirally transducing MCMV m157 into RMA and RMA-S cell lines. H-2K^{b} was blocked by addition of AF6-88.5 mAb. Naïve splenic NK cells were incubated 1:1 with target cells for 6 h in the presence of brefeldin A and anti-CD107a (mAb 1D4B). Cells were then harvested and surface stained for Ly49C/I (mAb 5E6), NK1.1 (mAb PK136), TCRβ (mAb H57-597), and Ly49H (mAb 3D10) and for intracellular IFN-γ (mAb XMG1.2) by using the Intracellular Staining Kit from BD Biosciences. Antibodies were purchased from eBioscience, BD Biosciences, and BioLegend.

CFSE labeling

B6 CD45.1^{+} splenocytes were labeled for 10 min at 37˚C with 2 μM CFSE in PBS, and washed twice in PBS. 2x10^{7} labeled splenocytes were transferred i.v. into naïve recipient mice. Recipient mice were infected with 5 x 10^{4} pfu MCMV and sacrificed five days later. Adoptively transferred NK cells were analyzed for

Ly49H and Ly49C/I expression and CFSE dilution by flow cytometry. Alternatively, splenocytes from CD45.1$^+$ WT x SHP-1$^{Me\text{-}v}$ mixed bone marrow chimeras were labeled in the same way and transferred into CD45.1$^+$CD45.2$^+$ wild-type recipients.

Stability of Ly49C/I expression

Naïve DX5$^+$ TCRβ^- NK cells were sorted as Ly49C/I$^+$Ly49G2$^-$ or Ly49C/I$^-$ Ly49G2$^+$ and 1 x 10^5 cells were injected i.v. into CD45 congenically marked B6 recipients. Recipients were infected one day later and Ly49H$^+$ donor NK cells were analyzed for Ly49C/I expression six days post-infection.

MCMV infection

Six to eight week old mice were treated with 150 μg of depleting Ly49C/I (clone 5E6), Ly49G2 (clone 14D11), NK1.1 (clone PK136), or CD8α (clone 2.43) monoclonal antibodies or were undepleted and then infected i.p. with 5x10^4 pfu MCMV (Smith strain) one day later. Thirty-six hours to 5 days later splenic NK cells were surface stained for Ly49H, NK1.1, Ly49C/I, and CD69 (clone H1.2F3) and intracellularly for IFN-γ and granzyme B (clone GB11). For viral titers, salivary glands and livers were harvested and snap frozen on dry ice in RPMI-1640 with 2% FCS. Samples were stored at –80°C, thawed, homogenized, plated on M2-10B4 cells (ATCC) in RPMI-1640 without FCS, and incubated for 2 hrs at 37°C. RPMI-1640 with 10% FCS and 0.75% carboxymethyl cellulose was added and samples were incubated for 7-10 days. Plaques were visualized by

staining with crystal violet dye. For neonatal survival studies 2-3 day old mice were injected with $1x10^5$ Ly49H$^+$ NK cells sorted as either Ly49C/I$^+$ or Ly49C/I$^-$ Ly49G2$^+$ or received PBS only and infected one day later with $2x10^3$ pfu MCMV. Animals were monitored daily for morbidity. Alternatively, $7.5x10^4$ Ly49H$^+$ NK cells from adult donor mice pre-depleted of Ly49C/I$^+$ or Ly49G2$^+$ cells *in vivo* by treatment with depleting antibodies 5E6 or 4D11, respectively, were transferred into 2-3 day old *Ly49h*$^-$ B6 mice and infected one day later with $2x10^3$ pfu MCMV. Animals were monitored daily for morbidity.

Statistical analysis

Statistical differences in percentages of IFN-γ^+, CD107a$^+$, and Ly49C/I$^+$ NK cells were determined by the paired, two-tailed Student's t test. Statistical differences in viral titers were determined by the unpaired, two-tailed Mann-Whitney test. Statistical differences in neonatal survival were determined by the Mantel-Cox test. Statistics were determined with Prism software (GraphPad Software, Inc.).

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