Supporting Information

Cooper *et al.* 10.1073/pnas.0813192106

SI Materials and Methods

Cytokine Stimulation and Intracellular Flow Cytometry. Splenic NK cells from *Rag*$^{-/-}$ hosts were enriched by negative selection and cultured for 15 h in media; low-dose IL-15 (10ng/mL); or IL-12 (10 ng/mL) + IL-18 (50 ng/mL) + low-dose IL-15. Brefeldin A was added for the last 2.5 h of culture and intracellular IFN-γ protein measured by flow cytometry gating on total NK1.1^{+} NK cells.

Preactivation of NK Cells With or Without IL-15. NK cells from *Rag1*$^{-/-}$ hosts were enriched by negative selection and cultured for 5 h with IL-12 (10ng/mL) + IL-18 (50ng/mL) + low-dose IL-15 (10ng/mL); IL-12 + IL-18; or low-dose IL-15 alone. At the end of the culture NK cells were washed a total of 4 times in PBS, labeled with 1μM CFSE and adoptively transferred by tail vein injection into C57BL/6 hosts (obtained from the National Cancer Institute). Splenic NK cells were harvested 7 days later and proliferation of transferred NK cells measured by CFSE dilution. Cells were cultured in IL-12 (10ng/mL) + IL-15 (100ng/mL) for 4 h with brefeldin A added for the last 3 h. IFN-γ production was measured by intracellular flow cytometry gating on donor (CFSE^{+}NK1.1^{+}) and host (CFSE^{-}NK1.1^{+}CD3^{-}) NK cells.

Quantitative RT-PCR of IFN-γ Transcript. Splenic donor NK cells (CFSE^{+}NK1.1^{+}) previously activated with IL-12 (10 ng/mL) + IL-18 (50 ng/mL) + low-dose IL-15 (10 ng/mL) and host (CFSE^{-}NK1.1^{+}) NK cells were purified by flow cytometry 7 days after adoptive transfer. Equal numbers of NK cells were immediately lysed and RNA purified according to manufacturer's instructions (RNeasy Plus Micro, Qiagen). cDNA was generated with random hexamers according to manufacturer's instructions (SuperScript III, Invitrogen). Quantitative RT-PCR was performed using primer-probe sets for murine *β-actin* (Applied Biosystems) and murine *IFNG* (F′ tcaagtggcatagatgtggaagaa; R′ tggctctgcaggattttcatg; probe FAM-tcaccatccttttgccagttcctccag-TAMRA, from ref. 1). Standard curves were created by inserting the PCR product from the *β-actin* and *IFNG* primers into the pCR2.1-TOPO vector (Invitrogen) which were used to create copy number standards. Reactions were performed on an ABI StepOne Plus machine (Applied Biosystems) and analyzed with StepOne software (Applied Biosystems).

1. Overbergh L, *et al.* (2003) The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech* 14:33–43.

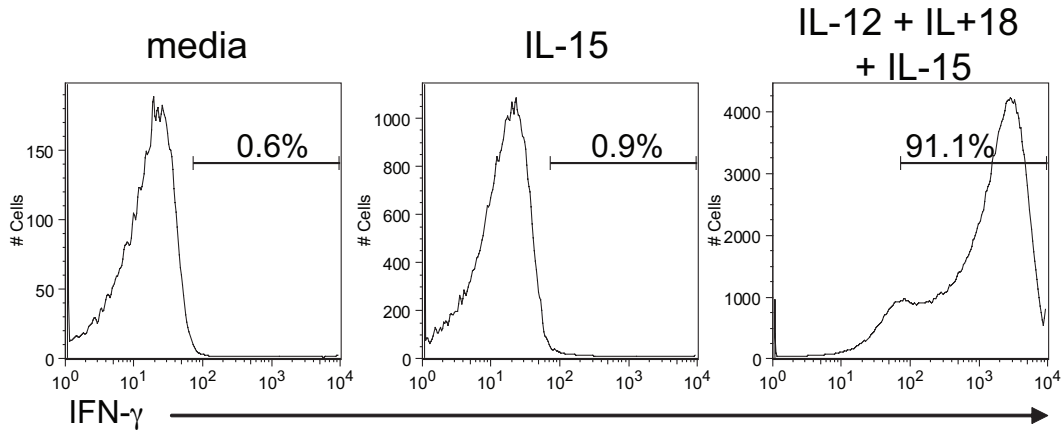

Fig. S1. Stimulation of NK cells with IL-12 + IL-18 with low-dose IL-15 induces abundant IFN-γ protein. Enriched splenic NK cells from *Rag1*$^{-/-}$ mice were cultured overnight in the presence of media, low-dose IL-15 (10 ng/mL), or IL-12 + IL-18 + low-dose IL-15 (10 ng/mL). IFN-γ production was measured by flow cytometry. Numbers indicate percentage of IFN-γ-positive NK cells. Flow histograms are gated on NK1.1^{+} lymphocytes.

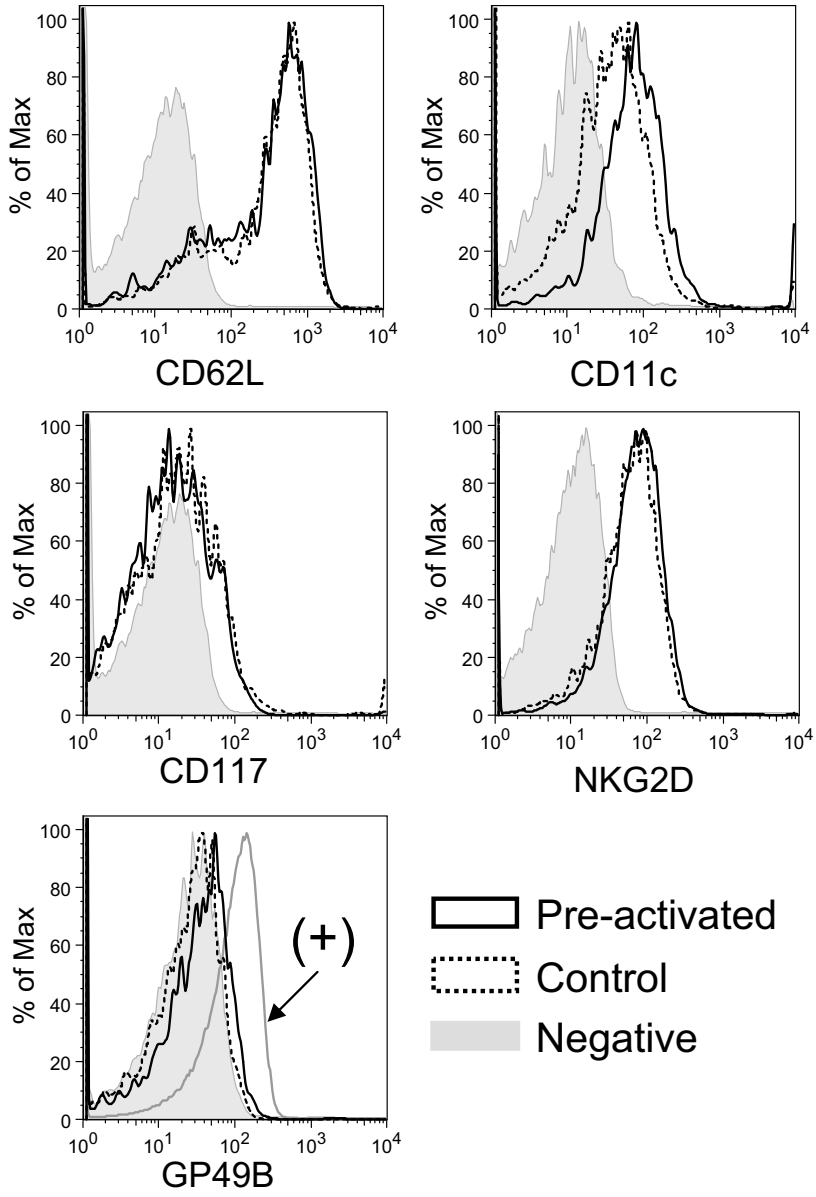

Fig. S2. Expression of NK markers on adoptively transferred cells at 7 days. Previously activated (IL-12 + IL-18 with 10ng/mL IL-15) or control-treated (IL-15 alone) NK cells were transferred into *Rag1*$^{-/-}$ hosts and one week later donor-derived splenic NK cells (CFSE^{+}NK1.1^{+}) were stained for expression of NK receptors and activation markers. Solid line, preactivated cells; dashed line, control-transferred NK cells; filled histogram, negative staining control; gray line, positive staining control (for gp49B only).

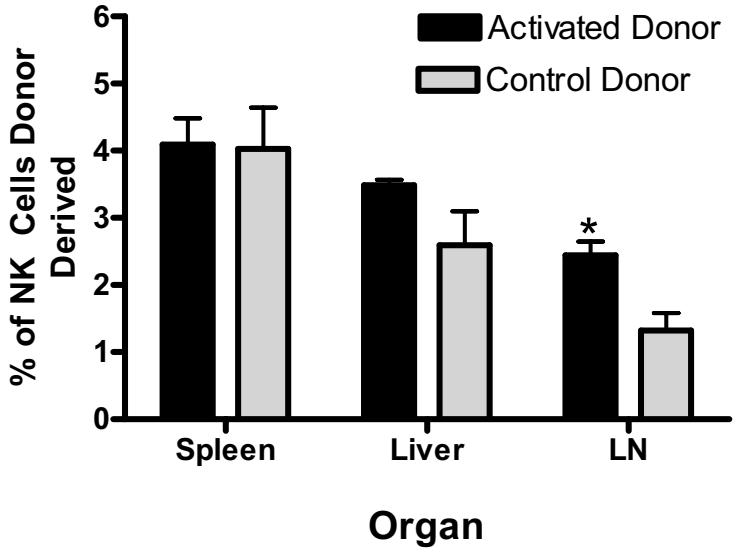

Fig. S3. Adoptively transferred NK cells are present in the spleen, liver, and lymph nodes. CFSE-labeled preactivated (IL-12 + IL-18 with low-dose IL-15) and control-treated (low-dose IL-15) NK cells were adoptively transferred into *Rag1*$^{-/-}$ hosts. At 7 days the spleen, liver, and lymph nodes (LN) were evaluated for CFSE^{+}NK1.1^{+} donor and CFSE^{-}NK1.1^{+} host NK cells. Data represent the percentage of total NK cells present in that organ that were donor-derived. Results indicate the mean of 4 independent experiments, error bars represent SEM (spleen, 4.1 ± 0.4% preactivated and 4.0 ± 0.6% control; liver, 3.5 ± 0.08% preactivated and 2.6 ± 0.5% control; LN 2.4 ± 0.2% preactivated and 1.3 ± 0.3% control NK cells). *, $P = 0.02$).

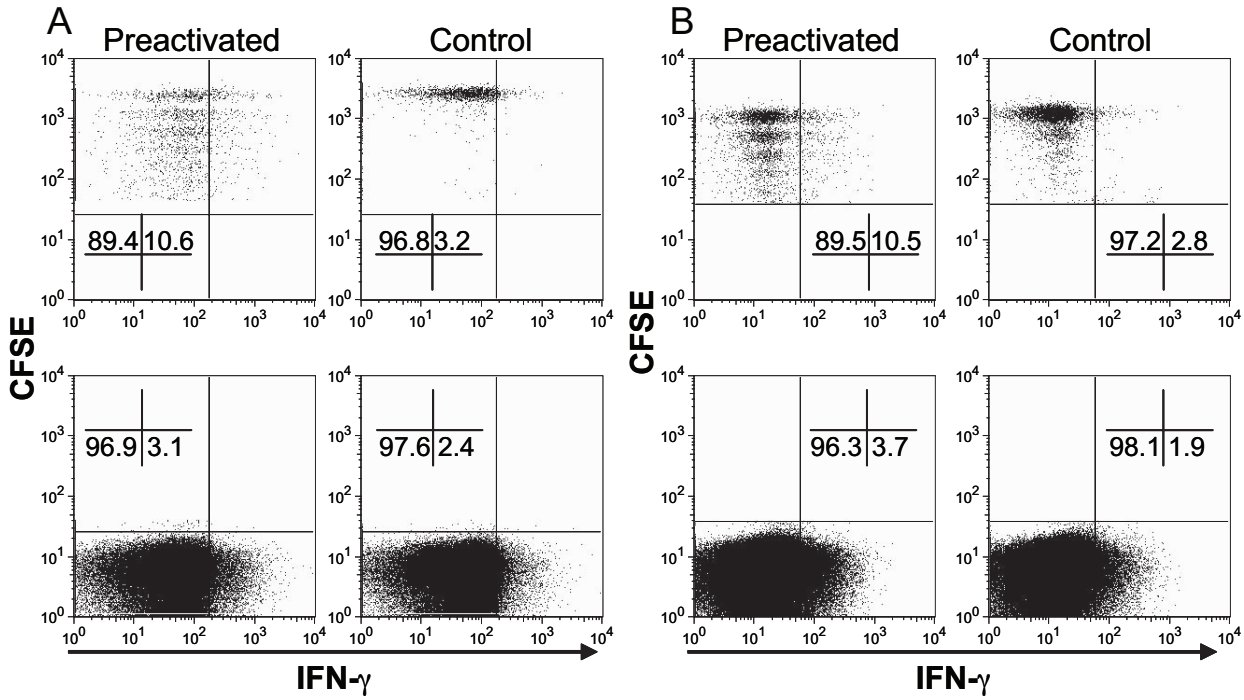

Fig. S4. Previously activated NK cells produce more IFN-γ following adoptive transfer of flow cytometry-sorted NK cells and as late as 22 days after adoptive transfer. NK cells were preactivated with IL-12 + IL-18 with low-dose IL-15 (10 ng/mL) or control-treated (IL-15 alone) prior to adoptive transfer into *Rag1*$^{-/-}$ hosts. (*A* and *B*) FACS plots of NK cell IFN-γ production following restimulation with IL-12 + IL-15 7 days after adoptive transfer of donor NK cells purified by flow cytometry (*A*) and 22 days after adoptive transfer of enriched NK cells (*B*). All plots are gated on NK1.1^{+} NK cells. The numbers indicate the percentage of CSFE^{+} donor NK cells (*Upper*) and CSFE^{-} host NK cells (*Lower*) in the corresponding gates, demonstrating that significantly more preactivated donor NK cells produce IFN-γ 7 days after adoptive transfer of flow cytometry-purified donor NK cells (*A*) and 22 days after adoptive transfer of NK cells (*B*).

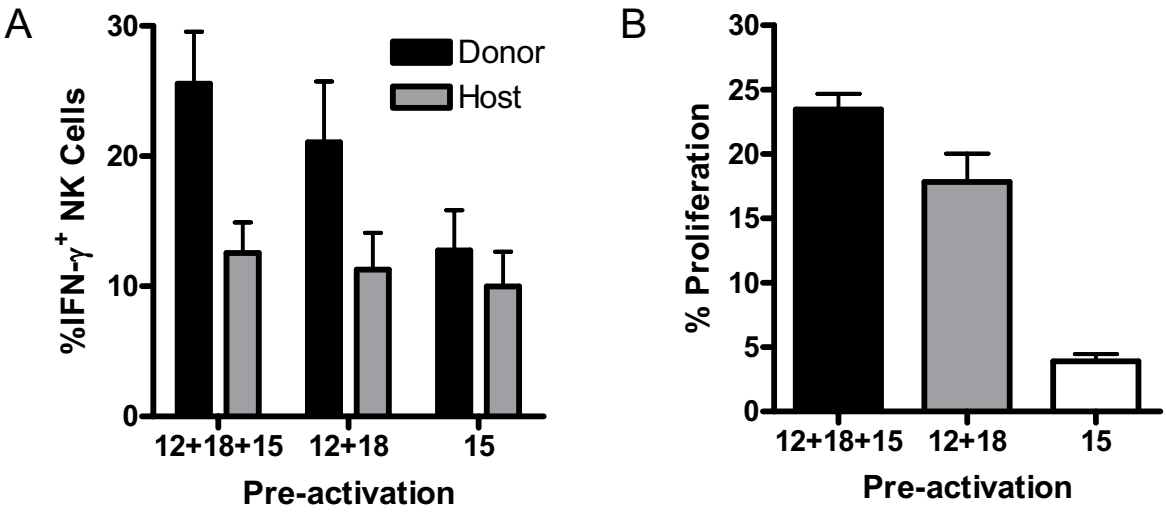

Fig. S5. NK cells previously activated for 5 h proliferate in vivo and produce IFN-γ upon restimulation regardless of prior low-dose IL-15 stimulation. NK cells were cultured in vitro with IL-12 + IL-18 with low-dose IL-15; IL-12 + IL-18; or IL-15 alone for 5 h and then adoptively transferred into C57BL/6 hosts. (*A*) IFN-γ production by splenic donor-derived ($CFSE^+NK1.1^+$, black bars) and host NK cells ($CFSE^-NK1.1^+CD3^-$, grey bars) in response to IL-12 + IL-15 stimulation in vitro for 4 h one week after adoptive transfer. Results indicate 3 independent experiments, error bars indicate SEM. (*B*) Proliferation of recovered splenic donor NK cells was measured 7 days after adoptive transfer by CFSE dilution. Results represent the mean ± SEM of 3 independent experiments (23.5 ± 1.2%, IL-12 + IL18 + IL-15; 17.8 ± 2.2%, IL-12 + IL-18; 3.9 ± 0.6%, low-dose IL-15 alone).

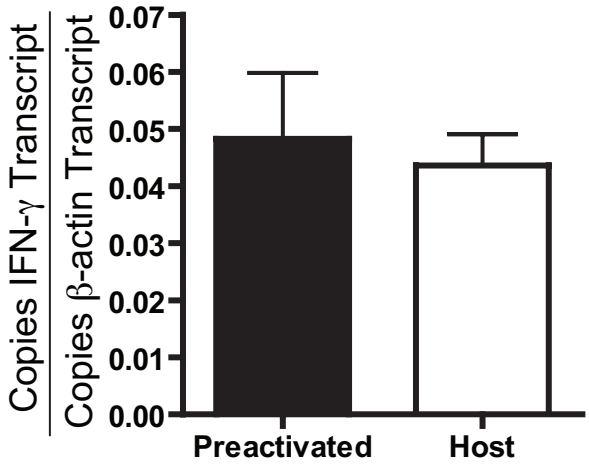

Fig. S6. Memory-like NK cells express similar levels of IFN-γ transcript as host NK cells. Splenic memory-like donor (CFSE$^+$) NK cells and naïve, host (CFSE$^-$) NK cells were purified by flow cytometry 7 days after adoptive transfer. IFN-γ and β-actin transcript copy number was measured by quantitative RT-PCR. Results represent the ratio of IFN-γ to β-actin transcript number. Data indicate mean $\pm$ SEM of triplicate wells from 2 separate experiments.