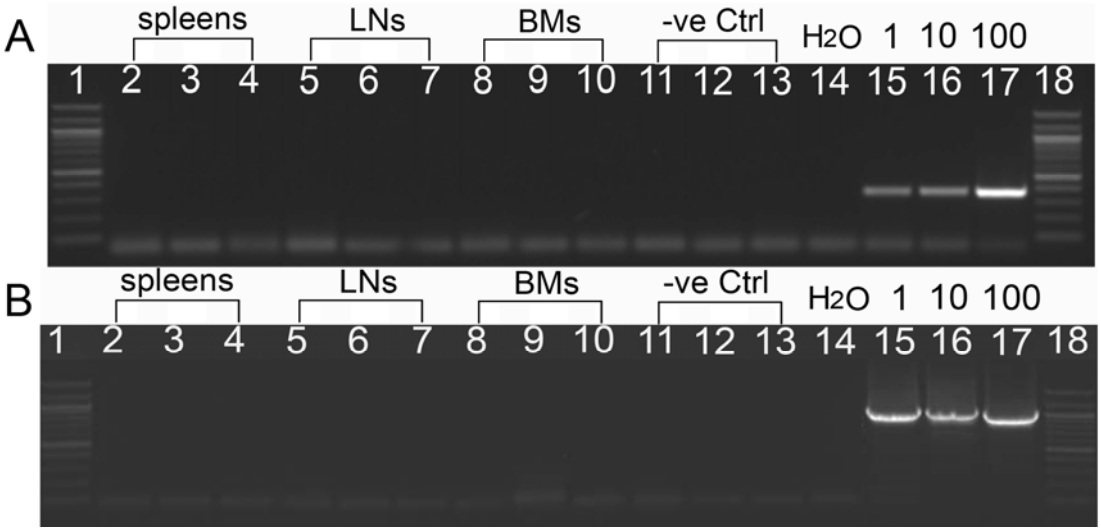
Supplemental Methods

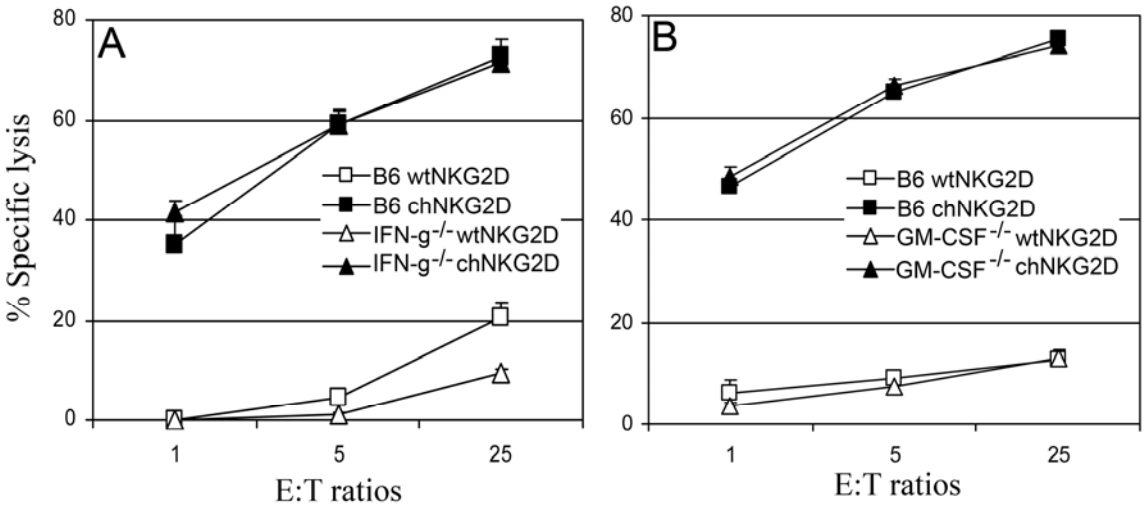
DNA isolation. Total DNA was isolated from lymphoid organs (spleens, lymph nodes and bone marrows) using a lysis buffer (50 mM Tris-HCl, 100mM NaCl, 0.1% Triton-X100, 1mg/ml proteinase K) at 10^7 cells/50μl. Briefly, lysis was carried out at 55°C overnight. The proteinase K was heat inactivated at 98°C for 10 mins. The lysed material was directly used as the template for PCR amplification.

Nested-PCR amplification. Primary PCR reactions used 1 μl of lysed material mentioned above as the template in a total volume of 20 μl. Primers were 5PFB (5′-ATCCTCTAGACTGCCGGATCG-3′) and 3PFB (5′-CACATTGCCAAAAGACGG-CAATATG-3′), which are specific for the retroviral vector pFB-neo (Stratagene, La Jolla, CA). Cycling conditions involved an initial 3-min denaturation at 94°C, followed by 30 cycles, each consisting of a 30-s denaturation at 94°C, a 30-s annealing at 56°C, and a 45-s extension at 72°C. These 30 cycles were followed by a 3-min extension at 72°C. Nested amplifications used 1 μl of the primary PCR product as the template in a total volume of 20 μl. The nested-PCR primers were 5GFP (5′-CACATGAAGC-AGCACGACTT-3′) and 3GFP (5′-TGCTCAGGTAGTGGTTGTCG-3′), which are specific for *gfp* gene. Nested cycling conditions were: initial denaturation at 95°C for 3 mins, 30 cycles of amplification (each consisting of a 30-s denaturation at 95°C, a 45-s annealing at 59°C, and a 45-s extension at 72°C), and final 3-min extension at 72°. PCR detection of chNKG2D DNA in samples was performed in the similar way, except that extension reaction was lengthened to 90-s and the primary and nested-PCRs used the same amplification conditions. The nested-PCR primers for chNKG2D DNA were 5chNKG2D (5′-GGCGTCGACACCATGAGAGCAAAATTCAGCAGGAG-3′) and

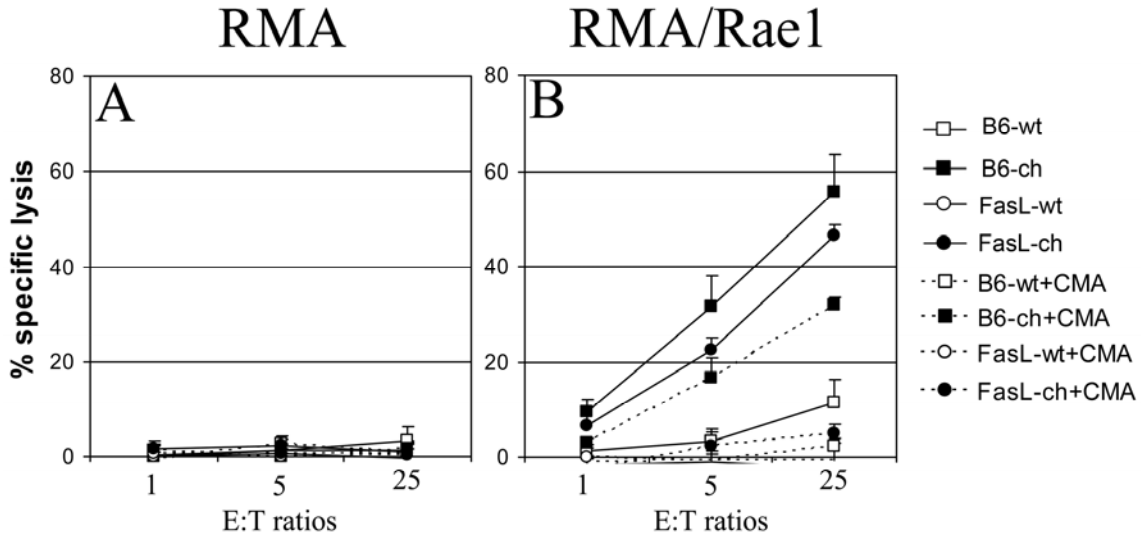
3chNKG2D (5′-GGCGCTCGAGTTACACCGCCCTTTTCATGCAGAT-3′), which are specific for *chNKG2D* gene. Each PCR amplification contained 200 μM each dNTP, 1 U of *Taq* polymerase (NEB, Ipswich, MA), and 0.2 μM of primers. The PCR products were run on agarose gels and visualized by staining with SYBR safe (Invitrogen, Carlsbad, CA).



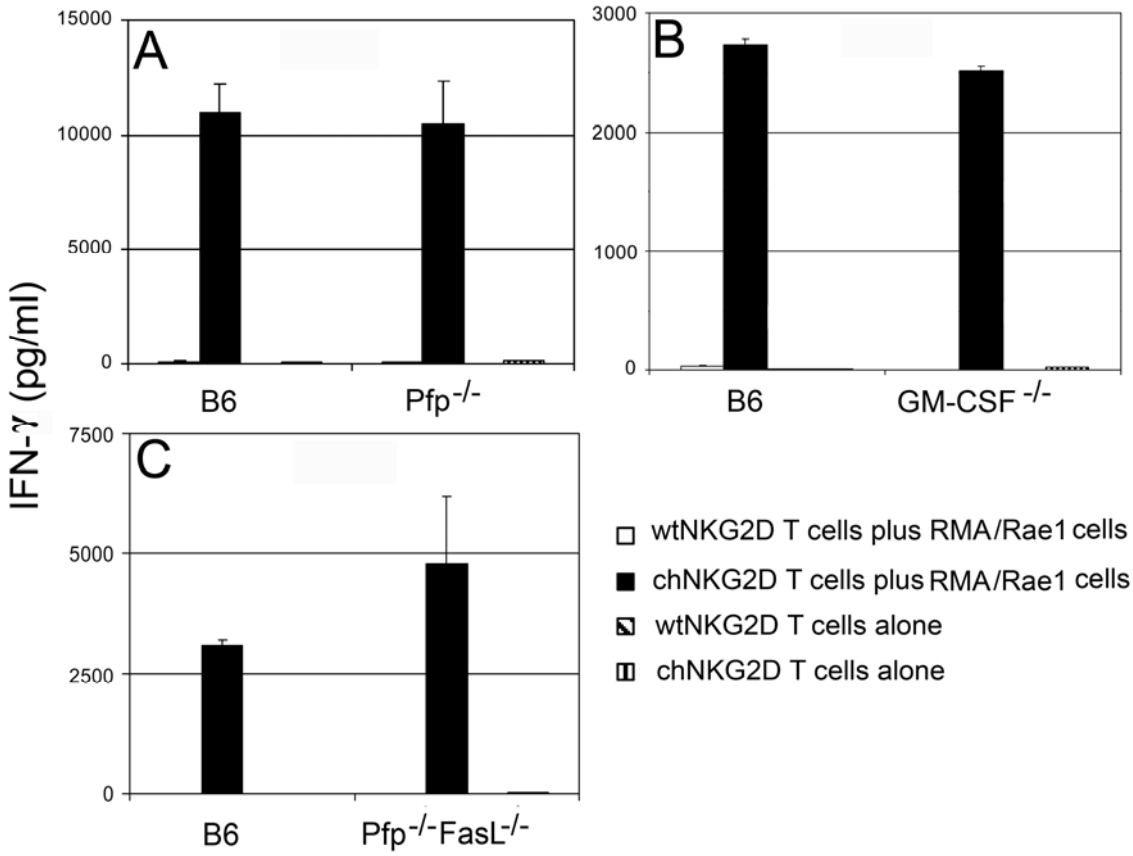
Supplemental Fig. 1 No detection of RMA/RG tumor cells (A) and chNKG2D T cells (B) in lymphoid organs of long-term survivors by nested-PCR. PCR products amplified from DNA obtained from 3 long-term survivor (125 days after tumor inoculation) spleens (lanes 2 to 4), lymph node (lanes 5 to 7) and bone marrow (lanes 8 to 10). The DNA samples from a naïve mouse spleen (lane 11), lymph node (lane 12) and bone marrow (lane 13) were used as negative controls. Lanes 1 and 18 are size standards (100 bp marker). Lanes 15 to 17 in (A) show expected amplicons (379bp) with RMA/RG cells numbering 1, 10, and 100, respectively. Lanes 15 to 17 in (B) show expected amplicons (~1 kb) with chNKG2D T cells numbering 1, 10, and 100, respectively. Lanes 14 in both (A) and (B) represent the H_2O control (negative control).



Supplemental Fig. 2 ChNKG2D modified T cells that are deficient in either IFN-γ or GM-CSF have equivalent cytotoxic activity against RMA/Rae1 tumor cells. Effector T cells derived from B6 (squares), IFN-$\gamma^{-/-}$ (triangles in A), or GM-CSF$^{-/-}$ (triangles in B) mice that were modified with either wtNKG2D(wt, open symbols) or chNKG2D(ch, filled symbols) were co-cultured with target cells RMA/Rae-1 cells at ratios from 1:1 to 25:1 in 5-hr ^{51}Cr release assays. The data are presented as mean ± SD of two independent experiments.



Supplemental Fig. 3 Concanamycin A (CMA) inhibited the chNKG2D T cell-mediated cytotoxicity against RMA/Rae1 tumors. T cells were pre-treated with an inhibitor of perforin-based cytotoxic activity, concanamycin A (CMA, 20nM, broken lines) for 2 hr before equal volume of target cells were added to co-culture. Blocking perforin pathway by CMA in combination with FasL deficiency significantly reduced the cytotoxicity of chNKG2D-transduced T cells against RMA/Rae1 cells ($P<0.05$) at all ratios compared with vehicle controls (ethanol). The data shown in A & B are presented as mean ± SD and are representative of two independent experiments.



Supplemental Fig. 4 ChNKG2D modified T cells that are deficient in perforin, GM-CSF or perforin and FasL ($Pfp^{-/-}FasL^{-/-}$) produced similar amounts of IFN-γ as B6 in response to stimulation by RMA/Rae1 tumor cells. Effector T cells derived from B6, $Pfp^{-/-}$(A), GM-$CSF^{-/-}$(B) or $Pfp^{-/-}FasL^{-/-}$ (C) mice that were modified with either wtNKG2D (□) or chNKG2D (■) were cocultured with RMA/Rae-1 cells at a ratio of 1:1 for 24 h. The amounts of IFN-γ were determined by ELISA as described in Methods and Materials. T cells alone were used as controls. The data are presented as mean ± SD and each sample was done as triplicates.