Adoptive Transfer of Gene-Modified Primary NK Cells Can Specifically Inhibit Tumor Progression In Vivo[1]

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NK cells hold great potential for improving the immunotherapy of cancer. Nevertheless, tumor cells can effectively escape NK cell-mediated apoptosis through interaction of MHC molecules with NK cell inhibitory receptors. Thus, to harness NK cell effector function against tumors, we used Amaxa gene transfer technology to gene-modify primary mouse NK cells with a chimeric single-chain variable fragment (scFv) receptor specific for the human erbB2 tumor-associated Ag. The chimeric receptor was composed of the extracellular scFv anti-erbB2 Ab linked to the transmembrane and cytoplasmic CD28 and TCR-ζ signaling domains (scFv-CD28-ζ). In this study we demonstrated that mouse NK cells gene-modified with this chimera could specifically mediate enhanced killing of an erbB2$^+$ MHC class I$^+$ lymphoma in a perforin-dependent manner. Expression of the chimera did not interfere with NK cell-mediated cytotoxicity mediated by endogenous NK receptors. Furthermore, adoptive transfer of gene-modified NK cells significantly enhanced the survival of RAG mice bearing established i.p. RMA-erbB2$^+$ lymphoma. In summary, these data suggest that use of genetically modified NK cells could broaden the scope of cancer immunotherapy for patients. *The Journal of Immunology,* **2008, 181: 3449–3455.**

Natural killer cells comprise 5–10% of PBLs and play an important role in the body's first line of defense against pathogen invasion and malignant transformation (1, 2). Unlike the exquisite Ag specificity observed for T and B lymphocytes, NK cells instead express a number of different activation and inhibition receptors which provide a balance of signals that dictate their overall response (3). This recognition system used by NK cells and the fact that they do not require prior sensitization provides a degree of flexibility to rapidly recognize different pathogens.

Several elegant studies have demonstrated that NK cells can effectively control tumor growth in mice mediated through release of perforin and cytokines (4–6). Their importance in anti-tumor immunity is further illustrated by the increased incidence of leukemia in patients with dysfunctional NK cells (7). The observation that NK cells can effectively respond to tumor cells exhibiting defective or altered MHC class I has made them promising effectors for immunotherapeutic strategies that target tumor escape variants (8). Nevertheless, tumor cells have developed several mechanisms to impede NK cell function, which include the expression of ligands that interact with NK cell inhibitory receptors (9).

Immunotherapeutic strategies to enhance NK cell anti-tumor activity have included the use of specific cytokines (10) or adoptive transfer of autologous ex vivo IL-2-activated lymphokine killer cells (LAK[4]; Ref.11). However, these approaches have only resulted in moderate success in restricted numbers of patients (12). More promising results have been recently achieved in the transplant setting with the use of allogeneic NK cells against acute myeloid leukemia (13, 14). Another emerging approach to address the problem of NK cell-mediated inhibition by tumors involves the genetic modification of NK cells with chimeric single-chain variable fragment (scFv) receptors that directly target tumor-associated Ags (TAA). This approach has successfully been used to enhance tumor recognition by primary T cells (15–24), and several studies have demonstrated specific killing of tumor target cells following redirection of NK cell lines (25–27) or primary human NK cells (28) with chimeric receptors. Nevertheless, investigation of whether these genetically engineered primary NK cells can specifically reject tumor in vivo has never been reported and has been hampered by lack of an efficient method for expressing transgenes in mouse NK cells.

In this study we have used Amaxa Nucleofector technology, an electroporation-based procedure, to genetically engineer primary mouse NK cells with an scFv anti-erbB2-CD28-ζ chimeric receptor. We and others have shown that this novel receptor design incorporating both costimulatory CD28 and TCR-ζ domains linked in the one intracellular domain could optimally trigger activation of transduced PBMC after Ag stimulation (15, 21, 29, 30). The Amaxa system uses optimized electrical parameters to enhance delivery of DNA to the cell nucleus, which increases transfection

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Received for publication April 17, 2008. Accepted for publication July 3, 2008.

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[1] This work was supported by a National Health and Medical Research Council Program Grant and a Cancer Council of Victoria research grant. M.H.K. and P.K.D. were supported by National Health and Medical Research Council of Australia R.D. Wright Research Fellowships. M.J.S. was supported by a National Health and Medical Research Council Senior Principal Research Fellowship.

H.J.P. performed the research, analyzed the data, and wrote first draft of the paper. J.T.J. performed the research. M.J.S. analyzed the data. M.H.K. designed the research and analyzed the data. P.K.D. designed the research, analyzed the data, and wrote the manuscript.

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[4] Abbreviations used in this paper: LAK, lymphokine killer cells; scFv, single-chain variable fragment; TAA, tumor-associated Ags; FasL, Fas ligand; WT, wild type.

efficiency and gene expression levels. NK cells expressing the chimeric receptor were demonstrated to enhance target cell killing following receptor ligation. Furthermore, adoptive transfer of scFv-receptor gene-modified NK cells led to significant growth inhibition of erbB2$^+$ T cell lymphomas in mice. These data suggest that gene-modified NK cells may have significant potential as an effective immunotherapy for cancer.

Materials and Methods

Cell lines

The C57BL/6 murine lymphoma cell lines RMA and RMA-S are T cell lymphomas derived from the Rauscher murine leukemia virus-induced RBL-5 cell line (8). The murine melanoma cell line B16-F10 was obtained from American Type Culture Collection. The erbB2-expressing cell lines RMA-erbB2 and B16-F10-erbB2 were generated by transduction with a retroviral vector (murine stem cell vector) encoding the cDNA for human erbB2. All cell lines were maintained in complete DMEM medium containing 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The murine lymphoma cell line YAC-1 was maintained in RPMI 1640 medium (Invitrogen), with 10% FCS (v/v), 2 mM L-glutamine, 0.1 mM non-essential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 5×10^{-5} mM 2ME.

Mice

C57BL/6, C57BL/6-*ptprc*^{a}, and C57BL/6 RAG-1-deficient (RAG-1$^{-/-}$) mice were purchased from the Walter and Eliza Hall Institute of Medical Research. C57BL/6 perforin (pfp)-deficient (C57BL/6-pfp$^{-/-}$) and C57BL/6 *gld* (Fas ligand (FasL) mutant) were bred at the Peter MacCallum Cancer Centre. All mice were housed in specific pathogen free conditions at the Peter MacCallum Cancer Centre and mice 6–12 wk of age were used in all experiments.

Isolation of NK cells

Dissected spleens from C57BL/6 mice were crushed into hypotonic lysis buffer and filtered to create a single cell suspension. NK cells were then selected using anti-DX5 Microbeads or an NK cell isolation beading kit (Miltenyi Biotec) according to manufacturer's specifications. The cells were then grown in RPMI 1640 medium containing 10% (v/v) FCS, 2 mM L-glutamine, 5×10^{-5} mM 2ME, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 2 mM HEPES, and 1000 IU/ml recombinant human IL-2 (Biological Resources Branch Preclinical Repository, National Cancer Institute).

Gene modification of NK cells

Seven-day IL-2-activated mouse NK cells were gene modified by electroporation using the Amaxa Nucleofector system (Amaxa Biosystems). In brief, NK cells were placed in 0.1 ml electroporation solution with either 4 μg pMAX plasmid DNA encoding the scFv α-erbB2-CD28-ζ chimeric receptor or GFP. Following electroporation, the cells were placed into 2 ml Amaxa recovery medium with 600 IU/ml recombinant human IL-2 for 24 h before being used in experiments.

Flow cytometry

Expression of the chimeric scFv receptor on the surface of NK cells was determined by indirect immunofluorescence with a primary *c-myc* tag Ab (Cell Signaling Technology), followed by staining with a secondary PE-labeled anti-mouse Ig mAb (BD Biosciences). Background fluorescence was determined by staining cells with an isotype control Ab followed by a secondary PE-conjugated anti-mouse Ig mAb. Direct detection of GFP by flow cytometry was examined in transfected vs non-transfected NK cells. Phenotyping of cell surface marker expression on NK cells was determined by staining cells with allophycocyanin-conjugated Abs specific for NK1.1, DX5, CD11b, and CD27 (eBioscience) and biotin-conjugated KLRG1, NKG2D (eBioscience), and biotin- conjugated CD94, CD25, and CD69 (BD Pharmingen). This was the followed by staining with a PerCPCy5.5-streptavidin (BD Pharmingen) Ab. MHC class I expression on tumor cells was determined by staining cells with a PE-conjugated Ab specific for mouse H2k^b (BD Biosciences).

Cytotoxicity

The ability of gene-modified mouse NK cells from wild-type (WT) and/or

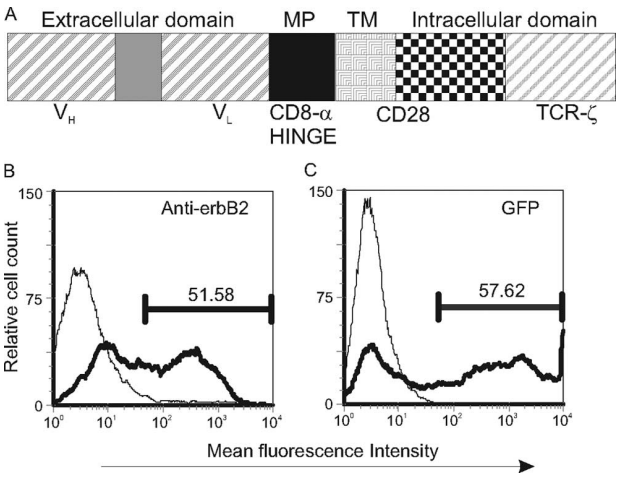

FIGURE 1. Gene modification of primary murine NK cells with the anti-erbB2 chimeric receptor. *A*, Schematic representation of the scFv-anti-erbB2-CD28-ζ receptor. The chimeric receptor consisted of the V_H and V_L regions of the anti-erbB2 mAb joined by a flexible linker, a CD8-α membrane-proximal hinge region (MP), and the transmembrane (TM) and cytoplasmic regions of the mouse CD28 signaling chain fused to the intracellular domain of human TCR-ζ. *B*, The expression of the chimeric scFv anti-erbB2 receptor in mouse NK cells was analyzed following staining with an anti-tag Ab mAb and PE-labeled sheep anti-mouse Ig (thick line) or a secondary PE-conjugated Ab (thin line). *C*, Expression of GFP in transfected (thick line) vs nontransfected (thin line) NK cells was analyzed by flow cytometry. Results shown are representative of seven experiments performed.

^{51}Cr-release assay. In brief, NK cells were incubated with 1×10^5 ^{51}Cr-labeled tumor targets at various E:T ratios in triplicate wells of a 96-well round-bottom plate (in 200 μl of complete DMEM). The percentage of specific release of ^{51}Cr into the supernatant was assessed as described previously (31).

Adoptive transfer

The ability of gene-modified NK cells expressing the α-erbB2-CD28-ζ receptor to enhance the survival of tumor bearing mice was investigated in the following model. C57BL/6 RAG-1$^{-/-}$ mice were injected i.p. with 2×10^5 RMA-parental or RMA-erbB2 tumor cells. Mice were then treated on days 0, 1, days 0, 1, 2, 3 (early model), or days 3, 4 (delayed model) with 2×10^6 (per injection) of α-erbB2 NK cells or GFP-NK control cells delivered i.p. In some experiments gene-modified NK cells were coadministered with high dose IL-2 (200,000 IU/ml) injected i.p. on days 0, 1, and 2. To investigate the persistence of NK cells in vivo, 2×10^6 donor gene-modified NK cells from congenic C57BL/6- *ptprc*^{a} (CD45.1$^+$) mice were transferred into RMA-erbB2 tumor-bearing RAG-1$^{-/-}$ recipient mice (CD45.2$^+$) cell on days 0 and 1. Three mice at each time point were then sacrificed on days 1, 2, and 5 following tumor injection, and spleens were harvested and i.p. washes were performed to determine the number of CD45.1$^+$ cells present.

Statistical analysis

The Mann-Whitney *U* test was used for statistical analysis. Values of $p < 0.05$ were considered significant.

Results

Expression of the chimeric anti-erbB2 receptor in primary mouse NK cells

The genetic modification of primary mouse NK cells with scFv chimeric receptors using retroviral-based transduction methods has proven difficult. To address this, we used the Amaxa Nucleofector system to gene modify mouse NK cells with the scFv α-erbB2-CD28-ζ chimeric receptor (Fig. 1*A*). Using this method, high level

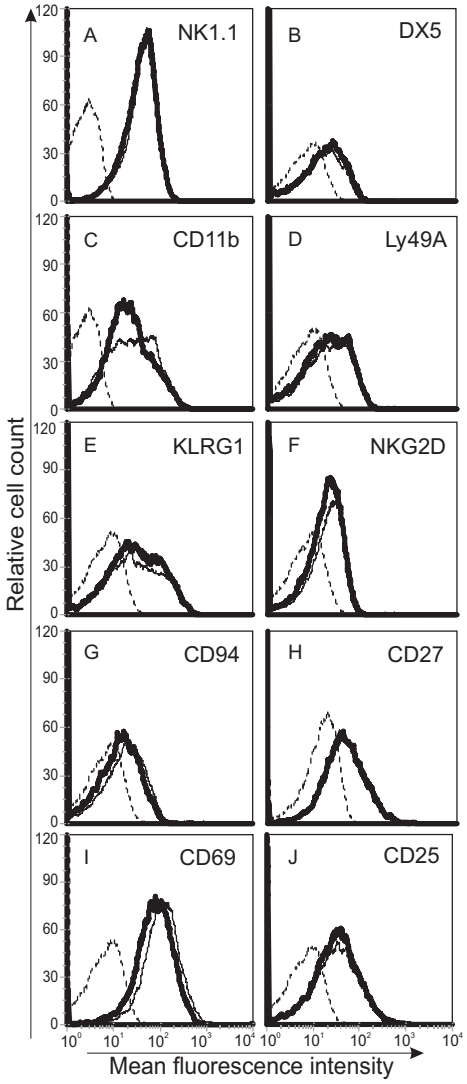

FIGURE 2. Phenotypic characterization of gene-modified primary mouse NK cells. The surface expression of various NK cell markers, activation markers, and activation/inhibition receptors was analyzed by flow cytometry following staining with appropriate Abs. Cells used for analysis were gated on either anti-tag positive cells (representing anti-erbB2 receptor expressing cells; thick line) or gated on GFP positive cells (representing control NK cells; thin line), or on unstained anti-erbB2-NK cells (dotted line). There was no significant difference in expression of the following molecules between anti-erbB2 or GFP transfected NK cells; NK1.1 (*A*), DX5 (*B*), CD11b (*C*), Ly49A (*D*), KLRG1 (*E*), NKG2D (*F*), CD94 (*G*), CD27 (*H*), CD69 (*I*), or CD25 (*J*). Results shown are representative of three independent experiments.

cells following staining with a *c-myc* tag mAb specifically recognizing a *c-myc* tag epitope incorporated into the extracellular domain of the chimeric receptor (46 ± 10%, $n = 7$; Fig. 1*B*). Equivalent levels of expression of autonomous GFP were also observed in control mouse NK cells (49 ± 11%, $n = 7$; Fig. 1*C*). Importantly, the transfected NK cell populations were TCRβ negative (data not shown). Cell viability ranged between 60 and 90% following electroporation.

Phenotypic characterization of gene-modified primary mouse NK cells

We next investigated whether expression of the chimeric scFv receptor altered the phenotype of primary mouse NK cells. Flow cytometry was used to compare expression of a number of molecules expressed by α-erbB2-NK and control GFP-NK cells, including activation and inhibition receptors. In three independently performed experiments, we observed no difference in the expression of NK cell markers NK1.1 or DX5 between GFP-NK and α-erbB2-NK cells (Fig. 2, *A* and *B*). There was also no difference in the level of expression of the CD11b marker between transfected NK cells (Fig. 2*C*). In addition, comparable expression of inhibitory receptors Ly49A, KLRG1, CD94, and the activatory receptor NKG2D, was observed between α-erbB2-NK and GFP-NK cells (Fig. 2, *D–F*). The levels of expression of the costimulatory receptor CD27 and activation markers CD69 and CD25 were also expressed at similar levels between the transfected NK cell types (Fig. 2, *H–J*). These data indicated that transfection of mouse NK cells with the scFv chimeric receptor has not phenotypically altered expression of a number of important NK cell-associated markers.

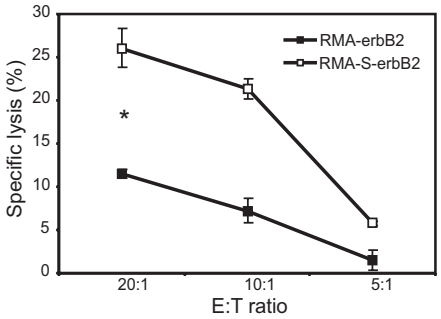

FIGURE 3. MHC class I inhibition of NK cell cytotoxicity. GFP-NK effector cells were used in a 4 h ^{51}Cr release assay against the MHC class I$^+$ RMA-erbB2 cell line or class I deficient RMA-S-erbB2 tumor cell line. GFP-NK cells were able to more effectively kill the RMA-S-erbB2 cell line (open squares) compared with the RMA-erbB2 cell line (closed squares; *, $p < 0.05$, as determined by a Mann-Whitney *U* test). Results are representative of three independent experiments.

Ag-specific cytotoxicity mediated by anti-erbB2-NK cells

Although NK cells can mediate effective killing of target cells, they are often inhibited by recognition of MHC class I molecule. Indeed, this is supported by our data demonstrating low killing by GFP-NK effector cells of the MHC class I$^+$ lymphoma cell line RMA-erbB2 compared with the class I-deficient RMA-S-erbB2 cell line (Fig. 3). To determine whether our gene-modified NK cells expressing the α-erbB2 chimeric receptor could overcome MHC-class I inhibition we assessed their ability to kill RMA cells, either expressing the erbB2 TAA (RMA-erbB2) or not. Importantly, the level of MHC class I expression on RMA-erbB2 and RMA cells was equivalent (Fig. 4). We demonstrated at least a two fold increase in the level of killing of RMA-erbB2 cells by α-erbB2-NK cells compared with control GFP-NK cells (Fig. 5*A*). This enhanced killing was erbB2 Ag-specific because α-erbB2-NK and GFP-NK cells mediated comparable lysis of RMA parental cells (Fig. 5*B*). We next determined whether anti-erbB2-NK cells could increase killing of another erbB2$^+$ cell line. In this experiment we demonstrated enhanced killing by anti-erbB2-NK cells of a mouse melanoma cell line B16 expressing erbB2 Ag (B16-F10-erbB2) compared with control GFP-NK cells (Fig. 5*C*). Again this was erbB2-specific because equivalent killing of parental B16-F10 cells by anti-erbB2 or GFP-NK cells was observed (Fig. 5*D*). We also showed that expression of the scFv receptor or GFP had no impact on the endogenous cytotoxic ability of NK cells. We demonstrated comparable cytotoxicity of a NK cell-sensitive target cell

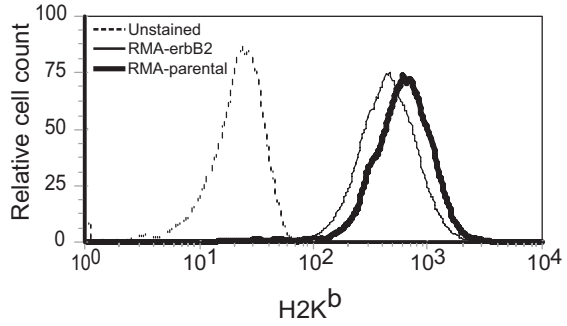

FIGURE 4. Expression of MHC class I on RMA tumor cells. MHC class I expression on RMA parental (thick line) and RMA-erbB2 (thin line) tumor cells was determined by staining cells with a PE-conjugated Ab specific for mouse H2k^b. Unstained RMA tumor cells (dotted line) were used as a control.

cells or gene-modified NK cells (Fig. 5*E*). These data demonstrated that expression of the scFv receptor targeting TAA could endow primary mouse NK cells with the ability to overcome MHC class I-mediated inhibition and kill NK cell-sensitive tumors.

Target cell lysis mediated by gene-modified NK cells was perforin dependent

It has been reported that NK cells lyse their targets predominantly via the granule exocytosis pathway involving perforin; however, they can also mediate apoptotic activity through FasL or TRAIL pathways (5, 32). To determine the mechanism of killing used by our gene-modified primary mouse NK cells, we genetically modified NK cells from C57BL/6 WT, perforin-deficient (pfp$^{-/-}$), and

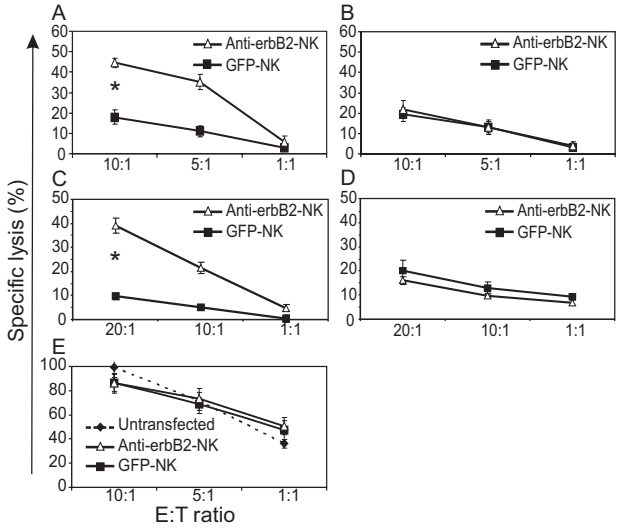

FIGURE 5. Enhanced cytotoxicity of erbB2$^+$ tumor cells by anti-erbB2 NK cells. *A*, Gene modification of primary mouse NK cells with the scFv chimeric anti-erbB2 receptor enhanced killing of RMA-erbB2 target cells compared with GFP-NK cells. *B*, Anti-erbB2 NK and GFP-NK cells equivalently killed RMA parental cells. *C*, Gene modification of primary mouse NK cells with the scFv chimeric anti-erbB2 receptor enhanced killing of B16-F10-erbB2 target cells compared with GFP-NK cells. *D*, Anti-erbB2 NK and GFP-NK cells equivalently killed B16-F10 parental cells. *E*, Anti-erbB2 NK, GFP-NK, or untransfected cells equivalently killed the NK cell sensitive target YAC-1. (*, $p < 0.05$, as determined by a Mann-Whitney *U* test). Results are expressed as average ± SEM of triplicates from three

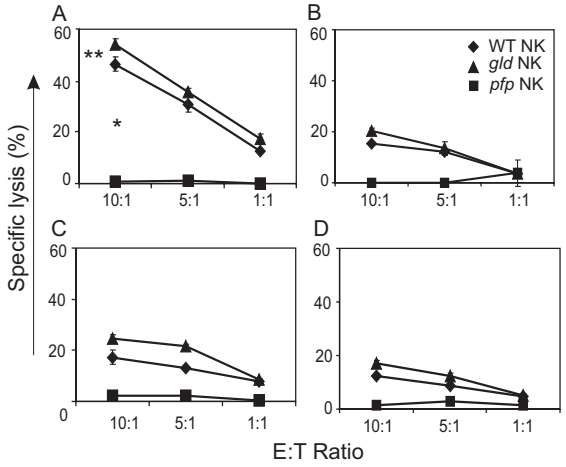

FIGURE 6. Cytotoxicity of erbB2$^+$ tumor cells by anti-erbB2 NK cells is perforin-dependent. Gene-modified NK cells derived from WT, perforin-deficient (pfp$^{-/-}$), or *gld* (Fas ligand mutant) mice were used in a 4 h ^{51}Cr release assay. *A*, Killing of RMA-erbB2 target cells by anti-erbB2 NK cells derived from pfp$^{-/-}$, but not WT or *gld* mice, was completely abrogated. Equivalent background killing of RMA-erbB2 tumor cells (*B*) and RMA parental cells (*C*) by GFP-NK cells or RMA-parental cells by α-erbB2 NK cells (*D*) derived from either WT or *gld* mice. (*, $p < 0.05$, **, $p > 0.05$ as determined by a Mann-Whitney *U* test). Results shown are representative of two independent experiments.

gld (mutant FasL) mice. Importantly, the level of expression of the scFv chimeric receptor was comparable in NK cells derived from WT and gene-targeted mice (data not shown). It is also important to note that previous studies have shown that other functional pathways of NK cells from perforin-deficient mice (i.e., FasL-mediated killing) are intact (33). In cytotoxicity assays, we demonstrated no killing of RMA-erbB2 target cells by α-erbB2 NK cells derived from pfp$^{-/-}$ mice (Fig. 6*A*). In contrast, the sensitivity of RMA-erbB2 cells to α-erbB2 NK cells derived from *gld* mice or WT mice was similar (Fig. 6*A*). As further specificity controls we observed comparable background killing of RMA-erbB2 by GFP-NK cells and RMA-parental cells by either α-erbB2 or GFP-NK cells derived from WT and *gld* mice (Fig. 6, *B–D*). These data demonstrated that gene-modified primary mouse NK cells mediated Ag-specific cytotoxicity through a perforin-dependent mechanism.

Ag-specific inhibition of tumor growth mediated by anti-erbB2-NK cells

We next assessed the ability of gene-modified mouse NK cells expressing the α-erbB2 chimeric receptor to mediate Ag-specific inhibition of tumor growth in vivo. Tumor cells (RMA-parental or RMA-erbB2) were injected i.p. into RAG-1$^{-/-}$ mice that then received early transfer (days 0, 1) or delayed transfer (days 3, 4) of 2×10^6 gene-modified NK cells (α-erbB2-NK or GFP-NK cells). In these experiments, we demonstrated significantly increased survival of mice with RMA-erbB2 tumor that received α-erbB2 gene-modified NK cells delivered at early or at later time points (Fig. 7, *A* and *B*). This effect was Ag-specific because there was no significant increase in survival of mice with RMA-erbB2 that received control GFP-NK cells. Furthermore, α-erbB2 NK cells had no anti-tumor effect in mice injected with RMA parental tumor. In another experiment we demonstrated that coadministration of high dose IL-2 (200,000 IU/ml) with gene-modified NK cells did not improve the anti-tumor effect in mice (data not shown). We also investigated the persistence of our adoptively transferred gene-

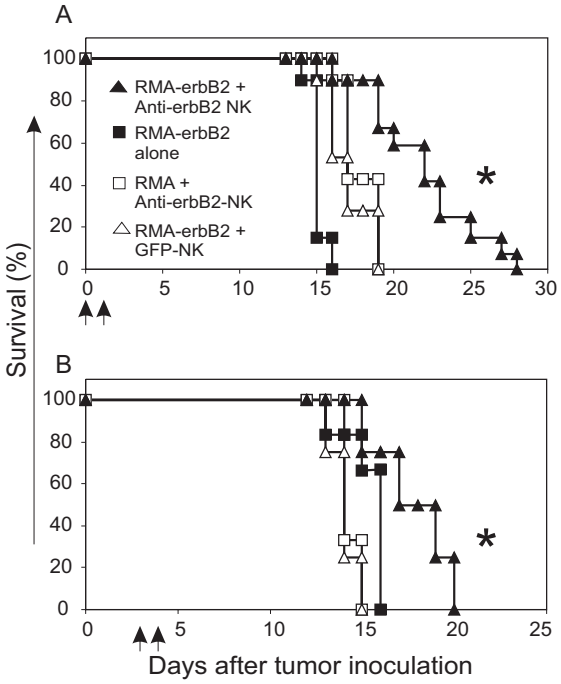

FIGURE 7. Enhanced survival of tumor-bearing mice after adoptive transfer of α-erbB2 NK cells. Groups of 5–10 mice were injected i.p. with 2×10^5 RMA-erbB2 tumor alone (closed squares) or injected with RMA-erbB2 tumor and treated with two doses of 2×10^6 α-erbB2-NK cells (closed triangles) or GFP-NK cells (open triangles) on days 0 and 1 (*A*) or days 3 and 4 (*B*). Mice bearing RMA tumor were treated with 2×10^6 α-erbB2-NK cells (open squares). Mice bearing RMA-erbB2 tumor and treated with α-erbB2 NK cells showed significantly increased survival compared with mice treated with control GFP NK cells (*, $p < 0.05$, as determined by a Mann-Whitney *U* test). Results shown are representative of two experiments performed. Arrows indicated days of NK cell transfer.

6-*PTPRC*^{a} mice. In these experiments we could not detect significant persistence of these cells 7 days post transfer in recipient mice (data not shown). To determine whether increasing the number of doses of anti-erbB2-NK cells could enhance the anti-tumor response, RAG-1$^{-/-}$ recipient mice bearing RMA-erbB2 tumor were injected i.p with 2×10^6 anti-erbB2-NK or GFP-NK cells on days 0, 1, 2, 3. We demonstrated that increasing the number of doses of anti-erbB2-NK significantly enhanced the survival of mice (~35% mice tumor-free) compared with previous experiments involving two injections of gene-modified NK cells (Fig. 8). Mice that received control GFP-NK cells or RMA-erbB2 tumor alone rapidly succumbed to disease. Collectively, this data demonstrated for the first time that adoptive transfer of gene-modified primary mouse NK cells could mediate an effective Ag-specific tumor response in vivo.

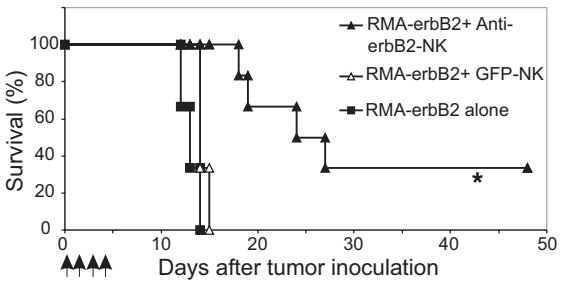

FIGURE 8. Increased transfer of α-erbB2 NK cells leads to tumor free survival of mice. Groups of six mice were injected i.p. with 2×10^5 RMA-erbB2 tumor alone (closed squares) or injected with RMA-erbB2 tumor and treated with four doses of 2×10^6 α-erbB2-NK cells (closed triangles) or GFP-NK cells (open triangles) on days 0, 1, 2, and 3. Mice bearing RMA-erbB2 tumor and treated with four doses of α-erbB2 NK cells showed significantly increased survival compared with mice treated with control GFP NK cells (*, $p < 0.05$, as determined by a Mann-Whitney

Discussion

The use of NK cells for cancer immunotherapy is gaining much attention. The most promising developments have come from the transfer of allogeneic NK cells in the allogeneic transplant stem cell setting (34). Recent results have demonstrated that treatment of acute myeloid leukemia patients with alloreactive NK cells could substantially increase their survival without associated graft-vs-host effects (14). Nevertheless, improvements in the use of alloreactive NK cells are required given that these cells had no apparent effect in patients with acute lymphoid leukemia (14). Other therapies involving the transfer of IL-2-activated LAK cells have shown only modest anti-tumor effects in patients. This is due in part to their nonspecific nature and to HLA-mediated inhibitory signals induced by interaction with NK cell inhibitory receptors (9). A novel way to overcome these problems and enhance NK cell anti-tumor activity involves their genetic modification with scFv chimeric receptors that can specifically recognize TAA. To test this we used the Amaxa transfection system to genetically engineer primary mouse NK cells with a chimeric scFv receptor with specific recognition for the erbB2 TAA. Importantly, the expression of the chimeric receptor in mouse NK cells did not interfere with their natural cytotoxic capability against NK-sensitive target cells. We demonstrated that NK cells engineered with the scFv anti-erbB2 receptor could significantly enhance killing of an essentially NK-insensitive lymphoma cell line in an erbB2$^+$ Ag-specific manner. Furthermore, for the first time, we demonstrated that adoptive transfer of receptor-modified primary mouse NK cells could specifically enhance the survival of tumor-bearing mice.

A number of studies have shown that gene modification of various mouse and human NK cell lines with scFv chimeric receptors could specifically enhance their anti-tumor activity in vitro (25, 27, 35, 36). Another report demonstrated that human primary NK cells expressing an anti-CD19 scFv receptor could specifically kill CD19$^+$ leukemic cells (28). Nevertheless, the ability of primary NK cells to mediate Ag-specific anti-tumor effects in vivo has not been formally tested. This has been due to difficulties in using retroviral-based approaches to efficiently express chimeric scFv receptors in NK cells, which is particularly the case for primary mouse NK cells. In our study, we were able to demonstrate proof of principle that adoptively transferred gene-modified primary mouse NK cells could specifically mediate anti-tumor inhibition in vivo. Indeed, mice treated with four doses of anti-erbB2-NK cells resulted in ~35% long term survivors. Nevertheless, these experiments were performed in RAG-1$^{-/-}$ mice where the presence of endogenous NK cells may have competed for important growth factors and cytokines limiting both the persistence and activity of gene-modified NK cells. Persistence could potentially be improved by a non-myeloablative conditioning regimen before adoptive NK cell transfer to produce a conducive cytokine environment. This type of approach has been demonstrated to enhance the therapeutic efficacy of adoptively transferred T cells in both mouse models and in patients (37–41). Alternatively, in future experiments, the use of RAG$\gamma^{-/-}$ recipient mice (that also lack NK cells) may overcome

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