Adoptive cellular immunotherapy of cancer has been limited to date mostly due to the poor immunogenicity of tumor cells and the difficulties in raising sufficient number of autologous tumor-specific T lymphocytes. To circumvent these limitations, new approaches have been designed to combine advantages of T cell–based and antibody-based immunotherapy by grafting cytotoxic T cells with chimeric antigen receptors composed of tumor-specific single-chain antibody (scFv) and a cellular activation motif (1–3). Chimeric antigen receptors combine the specificity of monoclonal antibodies (mAb) with the homing and killing potential of T cells. T cells engrafted with chimeric antigen receptors recognize a wide variety of tumor-associated antigens expressed on a broad range of tumors in a non–major histocompatibility complex (MHC)–restricted manner, thus circumventing potential tumor escape by MHC/peptide loss (4, 5). Chimeric antigen receptors were first constructed by fusing immunoglobulin variable genes to the constant domains of the α and β chains of T-cell receptors (6). Subsequently, other chimeric antigen receptors designs joined the coding sequence of an antibody-derived single-chain Fv (scFv) to the transmembrane and intracytoplasmic sequences of T cell–signaling molecules, ζ- or γ-signaling chains (1–3, 7). The engagement of ζ or γ chains, although sufficient to induce tumoricidal activity, may not suffice to elicit substantial lymphocyte activation in the absence of a concomitant costimulatory signal. Therefore, second-generation chimeric antigen receptors incorporate the cytoplasmic signaling domain of known T-cell costimulatory receptors, which mediate interleukin 2 secretion, including CD28 (8), ICOS (9), OX40 (10), 4-1BB (11), and DAP-10 (12, 13). The advantages of the design are that T cells receive primary and costimulatory signals for optimal activation after antigen ligation. Accumulating evidence has shown that human and mouse T cells modified with second-generation chimeric antigen receptors are much more active when tested in vitro and in murine models (5, 14–25). More recently, other investigators have published promising in vitro data describing “third-generation” chimeric
antigen receptors, which incorporate the cytoplasmic domains of other T-cell signaling ligands in CD28-containing second-generation chimeric antigen receptors, including OX-40 and Lck (21, 26). A later study showed that these constructs provided mouse T cells with stronger antitumor activity in vivo (27).

The erbB2 oncogene encodes an 185kDa transmembrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor (28). Overexpression of erbB2 is identified in ~30% of ovarian and breast cancers (29, 30). Several studies have shown that mouse T cells modified with the erbB2-specific chimeric antigen receptor composed of different signaling components could respond to and lyse the erbB2+ human tumor cells in vitro and significantly inhibit erbB2+ tumor growth in vivo in different tumor models (5, 22, 25, 31–33). Moeller et al. showed that administration of equivalent numbers of erbB2-reactive chimeric antigen receptor–modified CD8+ and CD4+ T cells leads to significant improvement in survival of SCID mice bearing established lung metastases compared with transfer of modified CD8+ T cells alone, highlighting the importance of the “help” from CD4+ T cells for the antitumor capacity of adoptively transferred gene-modified T cells (21). More recently, this group further showed that gene-modified CD4+ T helper 1 and CD8+ T cells could mediate a sustained antitumor response (24). In these studies, however, the in vivo efficacy of the adoptively transferred gene-modified T cells was generally checked in the immunocompromised animals (nude or SCID mice). Thus, the ability of erbB2-specific T cells to mediate an effective antitumor immunity against erbB2-expressing tumor cells in immunocompetent animal has not been fully evaluated.

In this study, we determined whether genetically targeted T cells increased survival in breast tumor–bearing syngeneic mice and whether the tumor-surviving mice developed a protective host immune response to breast tumor antigens. In addition, we determined which effector mechanisms genetically modified T cells required for therapeutic efficacy in vivo.

Materials and Methods

Mice. Female BALB/c mice to be used as wild-type (WT) mice and gene knockout (GKO) mice on the same background were purchased from the Jackson Laboratory. Animals were kept in a specific pathogen-free facility at the Animal Experimental Center of Second Military Medical University (Shanghai, China). Animal care and use were in compliance with institutional guidelines.

Cell culture. The human breast tumor cells MCF-7 and SK-BR-3, mouse breast tumor cells D2F2 and 4T1, and the 293T and the fibroblast NIH3T3 were maintained in DMEM supplemented with 10% (volume for volume) FCS. D2F2/E2 stably expressing human WT erbB2 were maintained in medium containing 0.4 mg/mL G418 (Sigma). Mouse T cells were cultured in RPMI 1640 medium with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 mmol/L HEPES, 50 μmol/L L-mercaptoethanol, and 50 μmol/L recombinant human interleukin (IL) 2 (Chiron). All tissue culture reagents were purchased from Life Technologies unless described otherwise.

Chimeric antigen receptor construction. The heavy and light chain variable region genes of humanized anti-erbB2 mAb (4D5) were synthesized according to the sequences published previously (34). The heavy chain variable region gene was fused to the 5’ end of light chain variable region gene via a (Gly4Ser)3 linker sequence using the overlapping PCR method to generate anti-erbB2 scFv gene. For detection purposes, the scFv contained a human c-myc tag epitope at the C-terminus of the light chain variable region. The chimeric antigen receptor gene construct was composed of the anti-erbB2 scFv, c-myc tag, part of the extracellular membrane, transmembrane, and cytoplasmic regions of the mouse CD28 signaling chain fused to the cytoplasmic region of CD3ζ chain (Fig. 1A). The chimeric antigen receptor gene was cloned into a modified Moloney murine leukemia virus–based retroviral vector pCMMP-eGFP by place of eGFP gene to generate pCMMP-scFv–CD28-ζ plasmid. The pCMMP-Luc was served as control. The retroviral vector pCMMP-eGFP and the packaging plasmid pMD-gag-pol and pMD-G were gifts from Dr. Richard C. Mulligan (Children’s Hospital, Harvard Medical School, Boston, MA).

Virus production and gene transduction. Retroviral supernatants were generated by transient transfection of the 293T with above retroviral constructs and the packaging plasmids. Viruses were pseudotyped with vesicular stomatitis virus glycoprotein to increase transduction efficiency in the mouse primary T cells. High-titer retroviruses (>109/mL) were generated by ultracentrifugation of the supernatants (16.5 K for 2 h at 4°C). The concentrated supernatants were stored at -80°C. The viral titers were determined using mouse NIH3T3 cells.

Spleen cells from mice were initially depleted of RBCs by hypotonic lysis with NH4Cl and enriched by passing through a nylon wool syringe as described previously (34). Enriched T lymphocytes (1 × 107/mL) were stimulated in 24-well plates with 2 μg/mL immobilized CD3 (145.2C11) and 0.1 μg/mL soluble CD28 mAbs (37.51) for 2 d. Spin infection (1,200 × g at 32°C for 2 h) with high titer retrovirus at a multiplicity of infection of 5 to 10 was done on day 3 in the presence of 100 IU/mL recombinant human IL-2 and 8 μg/mL polybrene. After 24 h, the transduced cells were recovered and expanded in complete RPMI-1640 medium plus recombinant human IL-2 and 8 μg/mL polybrene. After 24 h, the transduced cells were recovered and expanded in complete RPMI-1640 medium plus recombinant human IL-2 in 6-well plates (1 × 105/mL) for 48 h before adoptive transfer. The expression of the scFv-CD28-ζ chimeric antigen receptor was subsequently determined by direct staining with a phycoerythrin-labeled c-myc mAb (Sigma). Background fluorescence was assessed using the control phycoerythrin-labeled anti–mouse immunoglobulin mAb. The phenotype of transduced cells was determined by direct staining with allophycocyanin-labeled anti–CD4 (RM4-5) and fluorescein isothiocyanate–labeled anti–CD8 (53-6.7) mAbs. All mAbs were purchased from BD Pharmingen unless described otherwise.

Antigen-specific cytotoxicity, cytokine secretion, and proliferation by modified T cells. The ability of modified T cells to specifically mediate
target cell lysis was assessed in a 6-h chromium-release assay, as described previously (22). The capacity of modified T cells to produce cytokines [interferon (IFN)-γ, IL-2, granulocyte macrophage colony-stimulating factor, IL-4] after erbB2 antigen ligation was determined by ELISA (R&D Systems), and the proliferative capacity of modified T cells was assessed in a [3H]-thymidine incorporation assay, as described previously (22). In the assay for the blocking effect of soluble erbB2, RPMI-1640 with recombinant erbB2 protein (2 μg/mL; R&D Systems) was used as the culture medium.

In vivo antitumor activity. BALB/c mice received s.c. injection with 2 × 10^6 D2F2/E2 or D2F2 cells in the left flank on day 0. On day 8, tumor size reached ~20 to 40 mm^3. On days 8 and 10, mice were i.v. injected with mock or scFv-CD28-ζ-modified T cells (5 × 10^6 cells per injection) in a total volume of 100 μL of PBS. Mice treated with PBS served as a control. For mechanism studies, BALB/c mice were injected s.c. with 2 × 10^5 D2F2/E2 on day 0 and treated with 5 × 10^6 mock-modified T cells generated from WT or scFv-CD28-ζ-modified T cells derived from WT, IFN-γ− or perforin− mice on days 8 and 10. Tumor growth was monitored twice per week by caliper measurement of the largest diameter and its perpendicular. Tumor size was calculated as tumor volume (mm^3) = 0.5 × a × b^2, where a is the largest diameter and b is its perpendicular. If animals seemed moribund or the diameter of the tumors reached 15 mm, the mice were sacrificed and this was recorded as the date of death for survival studies. For challenging experiments, long-term surviving mice were implanted s.c. either with 2 × 10^5 D2F2/E2, D2F2, or 4T1 tumor cells. Naive BALB/c mice were used as controls.

PCR. The detection of gene-modified T cells following adoptive transfer in mice was assessed by PCR amplification of the chimeric antigen receptor gene. The splenocytes were harvested from naive or long-term surviving mice (200 after tumor inoculation). RBCs were depleted by hypotonic lysis with NH₄Cl and washed, and cells were suspended at 5 × 10⁶ cells/200 μL. Total DNA from splenocytes was subsequently purified for PCR using QIAamp DNA Blood MiniKit under the manufacturer’s instructions (Qiagen). Chimeric antigen receptor sense primer was 5’-TAGCTGTATTGGCCGATGT-3’ and antisense was 5’-AATCCACCTGTACCTCTT-3’; mouse β-actin sense primer was 5’-AGGCCGGTGGTCCTTGTTAT-3’ and antisense was 5’-GAAAGGCGGCTGGAAAGT-3’. The forward and reverse primers for chimeric antigen receptor and β-actin genes were designed to amplify fragments of ~400 and 150 bp using Taq polymerase (Promega).

**Cytokine production in secondary stimulation cultures.** Spleen cells (2.5 × 10^6) from naive or tumor-surviving mice were cultured with irradiated D2F2/E2, D2F2, or 4T1 tumor cells (2.5 × 10^6) for 72 h. Cell-free conditioned medium was assayed for IFN-γ by ELISA as described above.

**Intracellular cytokine staining.** Irradiated D2F2/E2, D2F2, or 4T1 cells (1.5 × 10^6) were cultured with spleen cells (3 × 10^6) from naive or tumor-surviving mice for 24 h. During the last 6 h of culture, 10 μg/mL brefeldin A was added to the wells (Sigma). Cells were then incubated with Fc-receptor block and cell surface stained with allophycocyanin-conjugated anti–CD4 and fluorescein isothiocyanate–labeled anti–CD8, and phycoerythrin-labeled anti–c–myc mAbs before flow cytometry analysis. The percentage of CD4+ or CD8+ T cells in transduced cells or the surface expression of the scFv-CD28-ζ chimeric antigen receptor in transduced cells is in the quadrants.

**Results.**

**Expression of the scFv-CD28-ζ chimeric antigen receptor in mouse primary T cells.** In preliminary experiments, stimulation of mouse T cells with CD3 and CD28 antibody for 48 hours, followed by infection with retroviral virus at 5 to 10 multiplicity of infection, consistently yielded a high percentage of chimeric antigen receptor expression: in 40 transduction experiments, 25% to 66% (median, 46%) of T cells expressed chimeric antigen receptor and β-actin genes were designed to amplify fragments of ~400 and 150 bp using Taq polymerase (Promega).
The capacity of gene-modified T cells to produce cytokines in response to erbB2-expressing tumor cells was examined by measuring cytokine levels in the culture supernatants. The scFv-CD28-ζ-modified T cells produced large quantities of T cytotoxic-1 cytokines IL-2, IFN-γ, and granulocyte macrophage colony-stimulating factor, and low levels of Tc2 cytokine IL-4 when they were cocultured with erbB2+ SK-BR-3 and D2F2/E2 tumor cells (Fig. 2B). The secretion of cytokines by scFv-CD28-ζ-modified T cells was antigen-specific because these T cells cocultured with erbB2- D2F2 parental tumor cells showed no significant cytokine secretion. In addition, mock-modified T cells cocultured with either cell line secreted no measurable cytokine.

We also observed antigen-specific proliferation by scFv-CD28-ζ-modified T cells (Supplementary Fig. S1). Collectively, these data showed that scFv-CD28-ζ-modified T cells could effectively mediate antigen-specific cytokine release, proliferation, and cytolytic activity.

Adoptive transfer of scFv-CD28-ζ-modified T cells to D2F2/E2 tumor-bearing mice leads to long-term tumor-free survival. We next evaluated the antitumor activity of gene-modified T cells against an established erbB2+ D2F2/E2 or erbB2- D2F2 tumor in syngeneic BALB/c mice. D2F2/E2 or D2F2 tumor cells (2 x 10^5) were implanted s.c. into groups of BALB/c mice at day 0. On days 8 and 10, mice were i.v. injected with mock or scFv-CD28-ζ-modified mouse T cells (5 x 10^6 cells per injection) in a total volume of 100 µL of PBS. The mice treated with PBS served as a control. Tumor developments were monitored, and animal survival was calculated. A, tumor volume of D2F2/E2 tumor. B, tumor volume of D2F2 tumor. C, survival of animals bearing D2F2/E2 tumor. D, survival of animals bearing D2F2 tumor. The data are from three experiments with comparable results. *, P < 0.05; **, P < 0.01. Tumor growth was significantly inhibited in the scFv-CD28-ζ-modified T-cell treatment group compared with the mock-modified T-cell or PBS treatment group.

Inhibition of tumor growth by scFv-CD28-ζ–modified T cells. D2F2/E2 or D2F2 tumor cells (2 x 10^6) were implanted s.c. into groups of BALB/c mice at day 0. On days 8 and 10, mice were i.v. injected with mock or scFv-CD28-ζ–modified mouse T cells (5 x 10^6 cells per injection) in a total volume of 100 µL of PBS. The mice treated with PBS served as a control. Tumor developments were monitored, and animal survival was calculated. A, tumor volume of D2F2/E2 tumor. B, tumor volume of D2F2 tumor. C, survival of animals bearing D2F2/E2 tumor. D, survival of animals bearing D2F2 tumor. The data are from three experiments with comparable results. *, P < 0.05; **, P < 0.01. Tumor growth was significantly inhibited in the scFv-CD28-ζ–modified T-cell treatment group compared with the mock-modified T-cell or PBS treatment group.

Adaptation of scFv-CD28-ζ–modified T cells to D2F2/E2 tumor-bearing mice leads to long-term tumor-free survival. We next evaluated the antitumor activity of gene-modified T cells against an established erbB2+ D2F2/E2 or erbB2- D2F2 tumor in syngeneic BALB/c mice. D2F2/E2 or D2F2 tumor cells (2 x 10^5) were implanted s.c. into syngeneic BALB/c mice. On day 8, animals with tumors sizing 20 to 40 mm³ were injected i.v. with 2 doses of 5 x 10^6 gene-modified T cells or PBS (days 8 and 10). Strikingly, treatment of D2F2/E2 tumor-bearing mice with scFv-CD28-ζ–modified T cells resulted in significant inhibition of tumor growth and prolonged survival of animals (Fig. 3A and C). Accumulating data from several experiments suggested that tumor regressed completely in 70% (D2F2/E2; 21 of 30) of the mice and those mice remained tumor-free for >200 days when treated by scFv-CD28-ζ–modified T cells. As controls, D2F2/E2 tumor-bearing mice receiving mock-modified T cell or PBS did not survive beyond 6 weeks. Furthermore, no antitumor effect was observed in D2F2 tumor-bearing mice regardless of either treatment (Fig. 3B and D).

Induction of a protective host antitumor immune response in long-term surviving mice. It has been shown in other tumor
models that survival of adoptively transferred T cells is correlated with antitumor in vivo efficacy (1, 17, 18). To determine whether scFv-CD28-ζ-targeted T cells are being maintained long-term in vivo, PCR assay specific for chimeric antigen receptor was done. The spleens of long-term surviving mice from Fig. 3A did not contain any scFv-CD28-ζ-modified T cells 200 days after tumor injection (Fig. 4A). This indicated that the scFv-CD28-ζ-modified T cells were not maintained long term in vivo, although the mice remained long-term survivors. To determine whether long-term surviving mice have developed host immune responses to D2F2/E2 cells, spleen cells from surviving mice or from naive BALB/c mice were cultured with the irradiated D2F2/E2 cells, D2F2 cells, or syngeneic 4T1 cells. Spleen cells from surviving mice secreted IFN-γ when cultured with D2F2/E2 cells, but not with 4T1 cells (Fig. 4B). Importantly, IFN-γ production also was detected in supernatants from coculture of spleen cells from surviving mice and parental D2F2 cells. Naive spleen cells did not secrete IFN-γ when cultured in any of the conditions. These data indicate the development of a host immune response to D2F2/E2 tumor antigens in long-term survivors.

To further characterize the development of a host T-cell memory response to tumor antigens, intracellular cytokine analysis was done on spleen cells from naive B6 or long-term survivors. IFN-γ and TNF-α production was analyzed in spleen cells cultured with the irradiated D2F2/E2 cells, D2F2 cells, or 4T1 cells. A significant population of CD8+ and CD4+ T cells from long-term survivors produced IFN-γ and TNF-α after...
cytokines in vivo. However, T cells in vivo for or FasL-/- mice. Naive T cells from naive mice did not produce these cytokines. 

T cells (5 experiments with comparable results. *, P < 0.01, compared with mock-modified WT T-cell treatment. **, P < 0.01, compared with scFv-CD28-ζ-modified WT T-cell treatment.

stimulation with D2F2/E2 and D2F2 cells, but not 4T1 cells (Fig. 4C). The percentage of CD8+ T cells secreting these cytokines was higher than that of CD4+ T cells. However, T cells from naive mice did not produce these cytokines.

To test whether the memory response was protective, naïve and long-term surviving mice were challenged with D2F2/E2 cells. The parental D2F2 cells or syngeneic 4T1 cells were used as controls. The survivors rejected subsequent challenges with the D2F2/E2 cells and remained tumor-free until the end of the experiment, whereas the mice could not reject syngeneic unrelated 4T1 tumor (Fig. 4D). Interestingly, 7 of 10 tumor-surviving mice completely rejected the rechallenge with the parental D2F2 cells, and 3 mice had a slow tumor growth, which was in line with the results of intracellular cytokine analysis. All naïve mice died of progressive tumor growth, irrespective of challenge with either tumor cells. Taken together, these data show that treatment with the scFv-CD28-ζ-modified T cells led to the establishment of host antitumor immunity, with generation of CD8+ and CD4+ D2F2/E2-specific T cells and protection against these tumors challenge.

scFv-CD28-ζ-modified T cells require cytotoxicity and cytokines for in vivo efficacy. We have shown that scFv-CD28-ζ-modified T cells can secrete cytokines and lyse erbB2+ tumor cells in vitro; however, the molecular mechanisms scFv-CD28-ζ-modified T cells require to decrease tumor burden in vivo are unknown. Previous studies have shown that genetically modified T cells were able to use multiple cytotoxic pathways to directly kill tumor cells, including perforin and Fas-FasL. (17, 22, 25). To determine the in vitro mechanisms that scFv-CD28-ζ—modified T cells use to kill D2F2/E2 cells, scFv-CD28-ζ—modified T cells derived from WT BALB/c mice, perforin−/−, or FasL−/− mice were used as effectors against 51Cr-labeled D2F2/E2 cells (Fig. 5A). Similar to T cells from WT mice, FasL−/−scFv-CD28-ζ-modified T cells were able to lyse D2F2/E2 cells. However, scFv-CD28-ζ—modified T cells deficient in perforin were unable to kill D2F2/E2 cells, indicating that scFv-CD28-ζ—modified T cells use perforin but not FasL to lyse D2F2/E2 cells.

To explore the in vitro effector mechanisms required for scFv-CD28-ζ—modified T-cell antitumor activity, scFv-CD28-ζ—modified T cells derived from WT, perforin−/−, or IFN-γ—deficient mice were used to treat D2F2/E2 tumor-bearing mice (Fig. 5B). The scFv-CD28-ζ—modified T cells deficient in perforin were able to significantly inhibit tumor growth compared with mock-modified T-cell–treated mice. Strikingly, scFv-CD28-ζ—modified T cells deficient in IFN-γ were unable to suppress tumor growth, pinpointing the requirement of scFv-CD28-ζ—modified T cell–derived IFN-γ for tumor inhibition. The loss of efficacy by the knockout-derived scFv-CD28-ζ—modified T cells was not due to a deficiency in other effector functions because the IFN-γ−/− scFv-CD28-ζ—modified T cells were able to lyse D2F2/E2 cells as well as gene-modified T cells from WT mice (Fig. 6A). Perforin−/− scFv-CD28-ζ—modified T cells secreted similar amounts of IFN-γ as WT-derived scFv-CD28-ζ—modified T cells when cultured with tumor cells
(Fig. 6B). Taken together, scFv-CD28-ζ–modified T cells require cytotoxic function and cytokine secretion to eliminate an established D2F2/E2 tumor.

Discussion

Adoptive immunotherapy strategies involving the genetic modification of autologous T cells with scFv chimeric antigen receptor or T-cell receptor genes is gaining wider acceptance as a promising treatment for cancer. The strategy to modified T cells with chimeric antigen receptor is considered to have several advantages for tumor immunotherapy. First, chimeric antigen receptor–modified T cells can be induced easily from nonspecifically activated polyclonal T cells. Therefore, it can overcome the difficulties in inducing and expanding natural tumor-specific CD8+ and CD4+ T cells (35, 36). Second, chimeric antigen receptor–modified T cells can recognize the target cells to exert their T-cell function in an MHC-independent manner. Therefore, chimeric antigen receptor–modified T cells are applicable to cancer patients without the restriction of human leukocyte antigen types. Furthermore, chimeric antigen receptor–modified T cells can exhibit their function against tumor cells that do not express MHC and costimulatory molecules, which is often observed in a variety of tumor tissues (37–39). Indeed, recent advances in the design of scFv chimeric antigen receptor comprising primary T-cell receptor-ζ and CD28 costimulatory signaling domains have shown striking results against early s.c. tumor growth and tumor metastases in mice after adoptive transfer (5, 14–25).

However, most of, if not all, studies exploited immunodeficient SCID mice to investigate the in vivo efficacy of genetically targeted T cells. Although lymphopenia environment in SCID mice can be mimicked in patients by a nonmyeloablative regimen, such as cyclophosphamide administration, it remains interesting for us to determine whether gene-modified T cells can induce potent recall responses in an immune-competent host. In this study, we have shown a dramatic improvement in survival of mice bearing erbB2+ D2F2/E2 breast tumor following adoptive transfer of erbB2-specific scFv-CD28-ζ–modified T cells. Importantly, tumor eradication in mice correlated with induction of an active antitumor immunity in vivo, which protected long-term surviving mice from subsequent rechallenge with erbB2+ D2F2/E2 also parental erbB2+ D2F2 tumor cells.

Previous study showed that gene-modified T cells could persist at least 100 days even after tumor eradication in mice (22, 25). Similarly, we also detected the trace of gene-modified T cells in vivo 90 days after tumor inoculation (data not shown); however, the gene-modified T cells did not persist beyond 200 days, as indicated by PCR analysis of chimeric antigen receptor gene in long-term surviving mice (Fig. 4A). Although scFv-CD28-ζ–modified T cells did not persist long-term in the tumor-surviving mice, this treatment did lead to the induction of host T-cell memory responses to tumor antigens. The memory response that developed in these mice was protective against tumor rechallenge, as tumor-free mice were able to reject the tumor rechallenge, illustrating the development of a functional memory response to breast tumor antigens. One indication that the development of host memory responses was specific is that CD8+ and CD4+ T cells specifically produced IFN-γ and TNF-α when cultured with D2F2/E2 cells, but not with unrelated syngeneic 4T1 cells. Interestingly, host T-cell memory responses were also directed to the parental erbB2+ D2F2 tumor cells. In line with intracellular cytokine staining, the long-term surviving mice rejected subsequent rechallenge with the parental tumor cells. Presumably, the development of long-term immunologic memory was not only dependent on the erbB2 antigen but also on other unidentified antigens of D2F2/E2 tumors. The development of host memory responses to other tumor antigens may be important for tumor immunity because immune responses to additional antigens would be beneficial if the tumor cells down-regulate erbB2 expression or if not all of the tumor cells express erbB2.

Although T cells are able to kill tumor cells through multiple mechanisms, we showed that scFv-CD28-ζ–modified T cells depend solely on perforin to kill D2F2/E2 cells in vitro as scFv-CD28-ζ–modified T cells deficient in perforin expression were not able to lyse D2F2/E2 cells. Despite inability to kill in vitro, perforin−/−scFv-CD28-ζ–modified cells were still able to significantly, but not completely, reduce the tumor burden in vivo. However, T cell–derived IFN-γ was essential for reducing the tumor burden because T cells deficient in this cytokine could not reduce the tumor burden. Although the exact role of IFN-γ in T cell–mediated tumor immunity still remains unclear, a number of studies have shown that IFN-γ can mediate its effect directly by enhancing antigen presentation on tumor cells through MHC class I and II pathways (40, 41), increasing tumor cell susceptibility to apoptosis by up-regulation of Fas expression (41) and/or by inducing cell cycle arrest (42, 43). Thus, direct cytotoxicity and cytokine secretion by scFv-CD28-ζ–modified T cells are involved in complete antitumor efficacy. It is possible that scFv-CD28-ζ–modified T cells not only directly kill tumor cells, thereby decreasing the tumor burden and releasing tumor antigens, but also activate and recruit antigen-presenting cells (such as dendritic cells and macrophages), helping to initiate host immune response to additional tumor antigens, and lead to the development of a host immune response that is involved in eliminating tumor cells and results in the development of T-cell memory responses.

Soluble antigen, which can be detected frequently in cancer patients in high serum concentrations, is considered to block the receptor of grafted effector cells and thus prevent the recognition of target cells and effector function (2, 44). In particular, patients with erbB2 expression are frequently found to have high serum concentrations of erbB2 (45). For example, in a study with erbB2-specific antibody therapy in patients bearing erbB2-expressed tumors, the higher soluble erbB2 level was found to be correlated with a decreased therapeutic efficacy of administrated erbB2-specific antibody (46). Therefore, adverse effects of serum erbB2 should be tested for the application of erbB2-specific gene-modified T cells. We did not find any blocking effect or nonspecific activating effect of soluble erbB2 on the gene-modified T cells specific to erbB2 (Supplementary Fig. S2), which was consistent with a recent study showing that soluble carcinoembryonic antigen did not inhibit the function of carcinoembryonic antigen–specific gene-modified human T cells (47). The exact mechanism to the absence of inhibitory effects was unclear, although the membrane-bound and immobilized erbB2 were speculated to preferentially trigger chimeric antigen receptors. However, it is also possible that the chimeric antigen receptors on the surface of modified T cells have a certain turnover rate; thus, the
soluble erbB2 may not be able to completely block the signaling delivered by newly synthesized chimeric antigen receptors. In addition, the chimeric antigen receptor–modified T cells can function properly in vivo because the mice with D2F2/E2 tumors are not detected to have high concentration of soluble erbB2. Certainly, further study is needed to elucidate the mechanisms underlying this observation, and it will be better to validate this effect in clinical trials.

In conclusion, the experiments described in this study show for the first time that the provision of gene-modified antigen-specific T cells can give increase to long-term tumor-free survival, induce protective host antitumor immune responses, and mediate a potent recall response in a clinically relevant syngeneic mouse tumor model. This finding may lay a solid foundation on clinical application of chimeric antigen receptor–modified T-cell therapy of malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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