

Gene-Engineered T Cells as a Superior Adjuvant Therapy for Metastatic Cancer¹

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The major limiting factor in the successful application of adjuvant therapy for metastatic disease is the lack of adjuvant specificity that leads to severe side effects. Reasoning that T cells of the immune system are highly specific, we generated tumor-specific T cells by genetic modification of mouse primary T cells with a chimeric receptor reactive with the human breast cancer-associated Ag erbB-2. These T cells killed breast cancer cells and secreted IFN- γ in an Ag-specific manner in vitro. We investigated their use against metastatic breast cancer in mice in an adjuvant setting, and compared their effectiveness with the commonly applied adjuvants doxorubicin, 5-fluorouracil, and herceptin. Mice were inoculated orthotopically with the human erbB-2-expressing spontaneously metastatic mouse breast cancer 4T1.2 in mammary tissue, and the primary tumor was surgically removed 8 days later. Significant metastatic disease was demonstrated in lung and liver at the time of surgery on day 8 with increased tumor burden at later time points. T cell adjuvant treatment of day 8 metastatic disease resulted in dramatic increases in survival of mice, and this survival was significantly greater than that afforded by either doxorubicin, 5-fluorouracil, or herceptin. *The Journal of Immunology*, 2004, 173: 2143–2150.

Adjuvant therapy of cancer following primary resection is often used in an attempt to eradicate metastases, and can lead to improved outcomes for patients. Agents used as adjuvants include chemotherapeutics such as doxorubicin and 5-fluorouracil (5-FU).⁴ Improvements in time to progression and survival in patients receiving surgery and adjuvant therapy have been demonstrated (1, 2). Nevertheless, many tumors are refractory to current therapies, and a large percentage of patients receiving adjuvant therapy following removal of primary tumors go on to die of breast cancer. The major limiting factor in the successful application of adjuvant therapies is the lack of specificity of the therapeutic agent. Doses necessary to eliminate occult or minimal residual disease are often associated with high toxicity to normal tissue.

In contrast to chemotherapy, immunotherapy is potentially highly specific. Several tumor-associated Ags have been identified, thereby affording us the opportunity to target tumor with reasonable specificity. Included among these Ags are erbB-2, Muc1,

Lewis^X, carcinoembryonic Ag, and TAG-72 (3). ErbB-2 is a member of the epidermal growth factor receptor family of cell surface proteins whose ligand has yet to be defined (4). Overexpression of erbB-2 can be found on 25–30% of breast cancers, in addition to a proportion of carcinomas of ovary, uterus, lung, kidney, stomach, and pancreas (5). Lower levels of erbB-2 are expressed on a range of normal tissues, including epithelial cells of the gastrointestinal tract, respiratory, reproductive, urinary, skin, breast, and placenta (4).

Immunotherapy using a humanized mAb, herceptin (trastuzumab), specific for erbB-2, is gaining acceptance as an adjuvant following the surgical excision of erbB-2-expressing primary tumors. Herceptin is well tolerated in the majority of patients, with a minor proportion of patients developing cardiac toxicity that can be managed satisfactorily (6). However, although encouraging, only a modest rate of long-term responses has been achieved using herceptin (7, 8).

An alternative immunotherapeutic approach for the treatment of breast cancer involves the generation of tumor-reactive T cells from PBLs by in vitro genetic modification. The gene used in this process codes for a chimeric cell surface receptor made up of an extracellular single-chain mAb (single-chain variable fragment (scFv)) specific for erbB-2, linked to the transmembrane and cytoplasmic domains of T cell trigger molecules such as the ζ -chain of the TCR complex (TCR- ζ) or the γ -chain of the Fc receptor complex (γ). Genes are introduced into T cells using retroviral vectors. Gene constructs incorporating one of a range of Ab specificities have been used to direct T cell activity against a variety of tumor or viral Ags (9–16). T cells armed with chimeric anti-erbB-2 have been demonstrated to react with tumor cells expressing erbB-2, resulting in cytokine secretion from T cells and cytotoxicity of tumor cells (17–20). In addition, intratumoral injection of anti-erbB-2-transduced T cells can inhibit tumor growth in mice (21).

More recently, we and others conceived a strategy involving the design of an improved chimeric receptor that incorporates both

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⁴ Abbreviations used in this paper: 5-FU, 5-fluorouracil; MSCV, murine stem cell vector; MTD, maximum tolerated dose; scFv, single-chain variable fragment.

activation and costimulatory domains in a single cytoplasmic region (22–25). We achieved this using the cytoplasmic regions of TCR- ζ and CD28 to generate the chimeric receptor anti-erbB-2-CD28- ζ . PBMC can be genetically modified with this gene encoded in a retroviral vector, to produce T cells able to respond against erbB-2. We have demonstrated in previous investigations that mouse T cells gene modified to express this improved receptor have an enhanced ability to inhibit experimental lung metastases and early s.c. tumors of the human breast cancer cell line, MDA-MB-435 (11).

However, to date, gene-modified T cells have not been demonstrated to impact on widespread metastatic breast cancer in mice. Reasoning that T cells armed with the improved chimeric receptor may be effective in a physiologically relevant model of metastatic breast cancer, we decided to determine the effect of adoptive transfer in an adjuvant setting following surgical removal of large primary breast tumor in a spontaneous metastatic model of breast cancer in BALB/c mice.

The model we used was the well-characterized 4T1.2 mouse mammary carcinoma model in BALB/c mice. The 4T1.2 is derived from the mouse breast cancer cell line 4T1 (26) that is highly tumorigenic and spontaneously metastatic to distant sites, including lung, liver, lymph node, heart, brain, bone, and thoracic cavity (27, 28). In this respect, 4T1.2 closely approximates the situation with metastatic breast cancer in patients.

In this study, 4T1.2 cells expressing human erbB-2 were inoculated into the mammary fat-pad of BALB/c SCID mice and allowed to metastasize. The primary mammary lesion was then resected 8 days later, and the effect of adoptive immunotherapy using mouse T cells genetically modified to react against erbB-2 was studied, and compared with the effect of treatment by either doxorubicin, 5-FU, or herceptin.

Materials and Methods

Cells and mice

The 4T1.2-erbB-2 was generated by transduction of 4T1.2 with a retroviral vector (murine stem cell vector (MSCV)) encoding the cDNA for human erbB-2. The MSCV vector also contained the cDNA for GFP. Fluorescence-activated cell sorting was used to enrich the GFP-transduced population for cells expressing vector-encoded protein. A431 is a human epidermoid carcinoma cell line, and MCF-7 is a human breast cancer cell line. Complete medium used for tumor cell culture consisted of DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from Commonwealth Serum Laboratories, Parkville, Australia). All experiments involving mice were performed according to Animal Experimentation Ethics Committee guidelines.

Abs and flow cytometry

Abs used in this study were a mouse monoclonal anti-human erbB-2 (clone 9G6.10; NeoMarkers, Fremont, CA), and a mouse mAb specific for a 9-aa epitope of human *c-myc* that was incorporated into the chimeric receptor design (clone 9B11; Cell Signaling Technology, Beverly, MA). For the detection of surface-expressed erbB-2 or *c-myc*, cells were stained with specific Ab ($\sim 1 \mu$ g/ 10^6 cells) in 50 μ l of PBS/10% FCS for 25 min/4°C, followed by two washes in PBS/10% FCS. Bound Ab was then detected by staining with a fluorochrome-conjugated secondary Ab, PE sheep anti-mouse Ig (Chemicon International, Melbourne, Australia). Cells were then washed twice and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA).

Generation of erbB-2-reactive T cells

Reactivity against erbB-2 is provided by scFv anti-erbB-2 (29) encoded in the retroviral vector, anti-erbB-2-CD28- ζ . Mouse T cells reactive with erbB-2 were generated, as described previously (11). Briefly, BALB/c splenocytes were incubated at 1×10^6 cells/ml in 10 ml of complete medium in 100-mm tissue culture dishes in the presence of 5×10^5 GP+E86 retroviral producer cells. PHA (2 μ g/ml) and IL-2 (100 IU/ml) were added to activate T cells, and polybrene (4 μ g/ml) was added to

facilitate transduction. T cells were separated from the adherent producer cell line after 3 days of culture at 37°C/10% CO₂, and used for in vitro or in vivo experiments.

Assays for cytokine production and cell lysis

For cytokine release, 5×10^5 4T1.2-erbB-2 tumor cells or control 4T1.2-MSCV tumor cells were plated in 12-well tissue culture plates and allowed to adhere (2–4 h). T cells (5×10^5), either anti-erbB-2 transduced or control LXS vector transduced, were then added in a final volume of 1 ml, and plates were incubated overnight at 37°C/10% CO₂. Supernatant was harvested and assayed for IFN- γ using ELISA, according to manufacturer's instructions (BD Pharmingen, San Diego, CA). A positive control for each experiment consisted of a well containing anti-mouse CD3 and anti-mouse CD28 (1 μ g each), which triggered T cells through their endogenous TCR.

For cell lysis assays, T cells were incubated with 1×10^5 ⁵¹Cr-labeled target cells (in 200 μ l of complete medium) in triplicate wells of a 96-well flat-bottom plate at various E:T ratios, and incubated for 4 h at 37°C/10% CO₂. The percentage of specific release of ⁵¹Cr into the supernatant was determined, as described previously (11).

Intracellular cytokine assays and immunohistochemistry

T cell cultures were incubated alone or in the presence of immobilized anti-CD3/CD28 (1 μ g/well of 12-well tissue culture plates) or immobilized anti-tag for 8 h at 37°C/10% CO₂. GolgiStop was then added for 4 h, and cells were harvested and processed, according to manufacturer's instructions (BD Biosciences; Cytofix/Cytoperm) using monoclonal 2.4G2 to block Fc receptor binding, followed by rat anti-mouse IFN- γ conjugated to allophycocyanin or allophycocyanin rat IgG2a isotype control, and either PE rat IgG2a isotype control or PE rat anti-mouse CD4 (all from BD Biosciences) or rat anti-mouse CD8a PE Cy5.5 (Caltag Laboratories, Burlingame, CA). Cells were then analyzed on a BD Biosciences LSR flow cytometer.

H&E staining and immunohistochemistry were performed on frozen sections. Abs used were FITC rat anti-mouse CD4, biotin rat anti-mouse CD8b.2, allophycocyanin rat anti-mouse CD11b, and isotype controls (all from BD Biosciences), and streptavidin goat anti-mouse Ig (Molecular Probes, Eugene, OR).

Tumor clonogenic assays

At various time points after tumor inoculation, tissues from groups of four mice were dissociated using collagenase type IV (2 mg/ml) and elastase (30 U/ml) (Sigma-Aldrich, St. Louis, MO) in HBSS at 4°C for 75 min with rocking. Dissociated tissues were then passed through a 70- μ m strainer and plated at various concentrations in six-well plates in complete medium containing 60 μ M 2-amino-6-mercaptopurine (Sigma-Aldrich). After incubation at 37°C/10% CO₂ for 10 days, plates were fixed (methanol) and stained (methylene blue), and colonies were counted. In this way, the absolute number of viable tumor cells per tissue was determined.

Mouse tumor model

Fifty thousand tumor cells in 20 μ l of DMEM were inoculated into the fourth mammary fat-pad of BALB/c SCID mice. Tumor was allowed to grow for 8 days, by which time it measured ~ 7 mm in diameter. On day 8, mice were anesthetized with methoxyflurane (Medical Developments, Melbourne, Australia); the primary tumor was surgically removed; and the wound was closed with surgical clips. The following treatments commenced 24 h later.

T cell treatment involved the injection of (5×10^6) – (1×10^7) T cells in 400 μ l of DMEM i.v. on days 9, 11, and 15 after tumor inoculation.

Doxorubicin (Pharmacia, Milan, Italy) was administered at a dose of 2 mg/kg i.v. weekly. We previously determined this dose to be the maximum tolerated dose (MTD) in BALB/c SCID mice. The MTD was calculated as that dose that resulted in $\sim 10\%$ loss of body weight.

The 5-FU (Commonwealth Serum Laboratories, Melbourne, Australia) was administered at a dose of 40 mg/kg i.p. daily for 5 consecutive days beginning day 9 after tumor inoculation. This dose was the MTD, as defined previously.

Herceptin (Roche, Sydney, Australia) was administered at an initial dose of 8 mg/kg i.v. on day 9 after tumor inoculation. Subsequent doses of 2 mg/kg were administered i.v. weekly. This regimen has been described as optimal by investigators in other tumor models (5).

Mouse survival was monitored daily. Survival was defined as the period with no overt signs of distress (lethargy, labored breathing, ruffled or huddled appearance), as assessed by two independent observers. The metastatic burden was assessed visually and by clonogenic assay, as described above.

Results

In vitro characterization of anti-erbB-2 T cells and 4T1.2-erbB-2 tumor cells

Following their isolation from the spleens of BALB/c mice, T cells were transduced with a retroviral vector encoding chimeric anti-erbB-2. Cell cultures (both anti-erbB-2 and control LXSN transduced) were found to be greater than 80% CD8⁺ T cells and ~10% CD4⁺ T cells, with 10% being CD4⁺CD8⁻ (data not shown). Transduced T cells were demonstrated to express the chimeric receptor by flow cytometry following staining for a *c-myc* tag that was incorporated into the chimeric construct (Fig. 1A). Control empty vector-transduced T cells did not express chimeric anti-erbB-2 (Fig. 1B).

The 4T1.2 tumor cells were analyzed for expression levels of erbB-2 by flow cytometry following staining with an erbB-2-specific mAb. The 4T1.2-erbB-2 cell line expressed high levels of erbB-2 when stained with the erbB-2-specific Ab compared with staining with secondary reagent alone (Fig. 1C). The parental 4T1.2 cell line did not express erbB-2 (Fig. 1D).

Ag-specific cytokine secretion and cytolytic activity by anti-erbB-2-transduced T cells

Transduced mouse T cells were next assessed for their ability to respond to erbB-2 Ag expressed on tumor target cells using ELISA for secreted cytokine following overnight coculture with target cells. T cells transduced with chimeric anti-erbB-2 secreted high

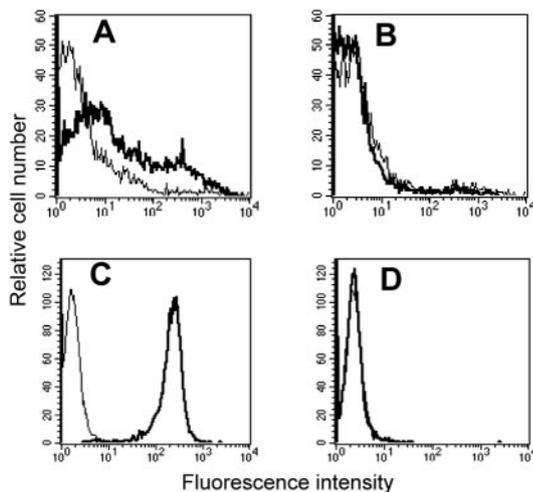


FIGURE 1. Expression of chimeric anti-erbB-2 on T cells, and erbB-2 Ag on tumor cells. *A*, T cells transduced with a retroviral vector encoding chimeric anti-erbB-2 expressed the chimeric receptor as demonstrated by flow cytometry following staining with a mAb specific for the *c-myc* tag that was included in the chimeric receptor design (thick line), compared with control Ab (thin line). Similar results were obtained in five experiments. *B*, The requirement for transduction with chimeric anti-erbB-2 for receptor expression is evident from the lack of staining of empty vector-transduced T cells using anti-*c-myc* tag (thick line), compared with control (thin line). *C*, The erbB-2-transduced 4T1.2 tumor cell line expressed erbB-2 as determined by staining with specific anti-erbB-2 mAb (thick line), compared with control (thin line). Similar results were obtained in two experiments. *D*, The parental 4T1.2 cell line did not stain with anti-erbB-2 (thick line) when compared with control (thin line).

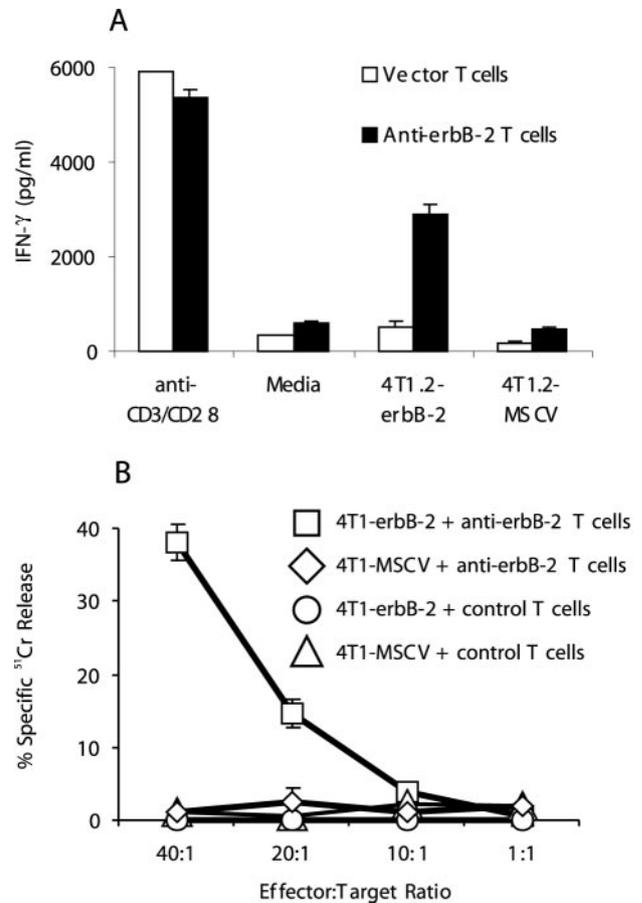


FIGURE 2. Transduced T cells respond to erbB-2 Ag. *A*, T cells were incubated with target cells, and supernatant was harvested 18 h later and analyzed for the presence of IFN- γ by ELISA. Chimeric anti-erbB-2-transduced T cells secreted IFN- γ specifically in response to incubation with 4T1.2 breast cancer cells expressing erbB-2 (4T1.2-erbB-2), but not in response to 4T1.2 cells lacking expression of erbB-2 (4T1.2-MSCV). The requirement for expression of chimeric anti-erbB-2 is supported by the absence of IFN- γ secretion in response to 4T1.2-erbB-2 cells by T cells lacking expression of chimeric anti-erbB-2 (control T cells). The lack of response by control-transduced T cells was not due to an inherent lack of activity because both groups of T cells responded equally to stimulation through the native TCR following incubation with immobilized anti-CD3 and anti-CD28. Results are representative of two experiments. *B*, Chimeric anti-erbB-2-transduced T cells specifically lyse erbB-2-expressing 4T1.2 cells, but not control 4T1.2-MSCV cells. Control-transduced T cells were unable to lyse either 4T1.2-erbB-2 or 4T1.2-MSCV cells.

amounts of IFN- γ in response to target cells expressing erbB-2, but not in response to erbB-2-negative target cells (Fig. 2A). Empty vector-transduced T cells did not secrete IFN- γ in response to erbB-2-positive tumor cells, despite their ability to secrete IFN- γ in response to immobilized anti-CD3 and anti-CD28 to a degree equivalent to chimeric anti-erbB-2-transduced T cells.

Chimeric anti-erbB-2-transduced T cells were also able to lyse erbB-2-expressing target cells in ⁵¹Cr release assays (Fig. 2B). The specificity of the interaction was apparent from the absence of lysis of erbB-2-positive tumor cells by control-transduced T cells and the lack of lysis of erbB-2-negative tumor cells by anti-erbB-2 T cells.

The ability of individual CD4⁺ and CD8⁺ T cell subsets to secrete IFN- γ was assessed using intracellular cytokine staining following ligation of the chimeric receptor or endogenous TCR. The majority of cells producing IFN- γ in response to anti-CD3/

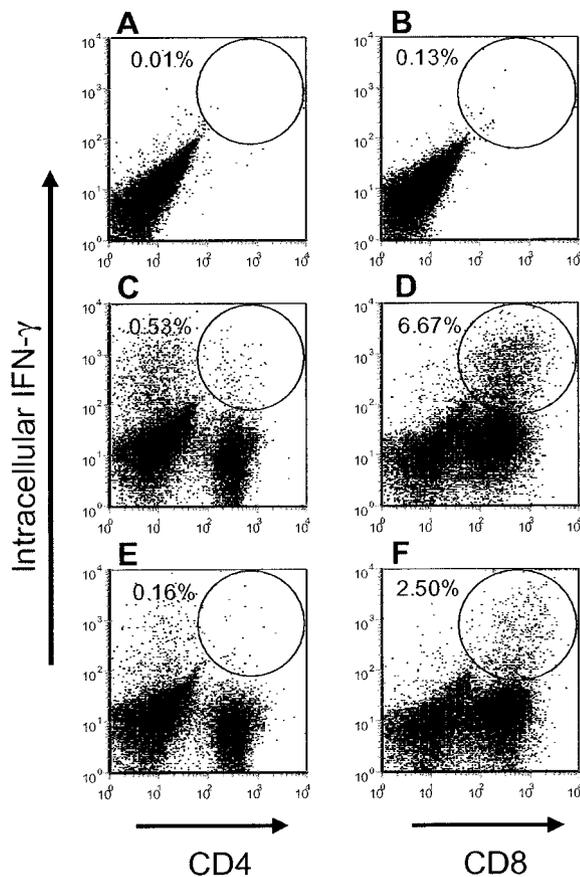


FIGURE 3. Intracellular IFN- γ in CD8⁺ and CD4⁺ T cells. Anti-erbB-2 T cell cultures were incubated in the presence of immobilized anti-CD3/CD28 (C and D) or anti-tag (E and F) and assayed for intracellular IFN- γ . The majority of T cells responding to receptor ligation were CD8⁺ cells (D and F) with a lesser proportion of CD4⁺ cells producing IFN- γ (C and E). Cells stained with isotype control Abs are shown (A and B). This experiment was performed twice with similar results.

CD28 was demonstrated to be CD8⁺, 6.67% of total cells, compared with CD4⁺ at only 0.53% of total cells (Fig. 3, C and D). The percentage of CD8⁺ cells responding to anti-CD3/CD28 was 16.5% compared with only 2.1% of CD4⁺ cells. This result was also reflected in the T cell response to chimeric receptor ligation through anti-tag, with IFN- γ -producing CD8⁺ cells comprising 2.5% of total cells compared with CD4⁺ cells at 0.16% (Fig. 3, E and F). The proportion of CD8⁺ cells that responded to anti-tag was 5.4%, compared with 0.6% of CD4⁺ cells. This result demonstrated that the majority of IFN- γ was produced by CD8⁺ cells with a smaller contribution from CD4⁺ cells.

Adoptively transferred T cells increase survival of mice with metastatic cancer

Having demonstrated that mouse T cells could be genetically modified to react vigorously against mouse tumor cells *in vitro*, we next wished to assess their potency against erbB-2-expressing tumor in mice.

Previous experiments in our laboratory have found adoptive transfer of antitumor T cells to be ineffective against s.c. tumor larger than 5 mm in diameter. However, considering that many primary breast tumors can be surgically removed in humans and that undetected micrometastases are often present that eventually lead to disease progression, we decided to test the efficacy of adoptively transferred anti-erbB-2 T cells in an adjuvant setting against

mouse breast cancer metastases following surgical excision of the primary lesion.

In preliminary experiments, we determined that a considerable metastatic tumor burden was present at day 8, and we therefore decided to investigate the efficacy of anti-erbB-2 gene-modified T cells against day 8 metastatic disease. The most frequent treatment for breast cancer is surgical removal of the primary tumor, followed by adjuvant treatment if metastases are detected or suspected. Adjuvants in common use include doxorubicin, 5-FU, and herceptin. Therefore, we set out to determine the effect of gene-modified T cells as an adjuvant therapy for day 8 metastatic breast cancer, compared with other adjuvants.

Primary tumors were removed on day 8 after tumor inoculation, at which time they measured ~ 7 mm in diameter. Treatment of the mice (five to nine per group) was commenced on day 9, and involved adoptive transfer of anti-erbB-2-modified T cells or control empty vector-modified T cells on days 9, 11, and 15. Other groups received the alternate adjuvants, doxorubicin, 5-FU, or herceptin, according to their previous optimally defined regimen, as described in *Materials and Methods*.

Mice undergoing surgery, but no further treatment, did not survive beyond day 30 (Fig. 4). Treatment of mice with control T cells, doxorubicin, 5-FU, or herceptin did not result in any increased survival, with all mice in these groups dying due to metastatic disease. This was despite clear activity of doxorubicin and 5-FU preparations because some toxicity (lethargy and $\sim 10\%$ weight loss) was observed in mice. Similarly, preparations of herceptin were demonstrated to bind to erbB-2-expressing 4T1.2 cells (data not shown). Mice treated with anti-erbB-2-modified T cells survived significantly longer than all other groups ($p = 0.001$, Kruskal-Wallis test). Two of these mice continued to survive long-term (>100 days) following treatment with no sign of further tumor growth.

Interestingly, of the seven mice that died in the group receiving anti-erbB-2 T cells, only two succumbed to metastatic disease. Five of the seven developed primary tumor regrowth and had to be euthanized when tumor size reached 15 mm diameter. Metastases were not visible in any of these five mice at postmortem. Therefore, T cells were able to inhibit metastases, but were less effective against residual primary tumor regrowth. Gene-engineered anti-erbB-2 T cells did not affect the metastases of parental 4T1.2 cells (data not shown).

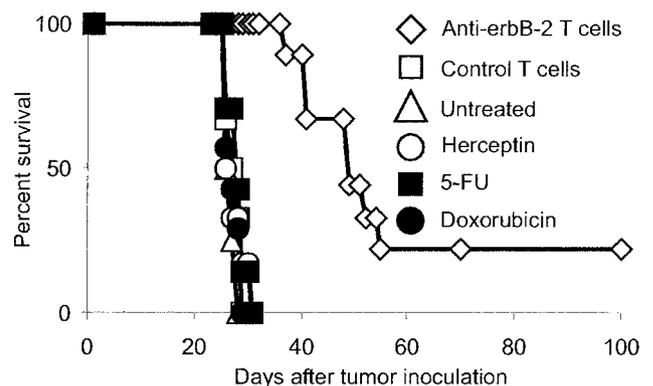


FIGURE 4. Impact of various adjuvants on survival of tumor-bearing mice. Mice were treated with the adjuvants listed after surgical removal of 8-day primary mammary tumor. Doxorubicin, 5-FU, or herceptin was ineffective at prolonging survival of mice. Mice treated with anti-erbB-2 gene-modified T cells survived significantly longer than all other groups ($p = 0.001$, Kruskal-Wallis test).

A role for T cells in the inhibition of further metastases in these mice is supported by the persistence of adoptively transferred T cells in the circulation. A total of 3–4% of circulating leukocytes was derived from transferred cells for at least 57 days after tumor inoculation.

Metastatic burden in tumor-bearing mice

To quantitate the effect of various treatments on 4T1.2-erbB-2 metastases, lung and liver were taken at day 8, 16, 24, 28, or 36 after tumor inoculation, and clonogenic assays were performed.

All mice had a considerable burden of viable tumor cells in both lung and liver at the time of primary removal on day 8 (Fig. 5). Lung burden (Fig. 5A) was considerably higher than that of liver (Fig. 5B) at this and subsequent time points. The extent of lung and liver metastasis increased in all groups by day 16, but was less in mice receiving anti-erbB-2 T cells than in all other groups ($p = 0.083$). The metastatic burden continued to increase from day 16 to 24 in all groups of mice, except in anti-erbB-2 T cell-treated mice in which metastatic progression had ceased ($p = 0.034$).

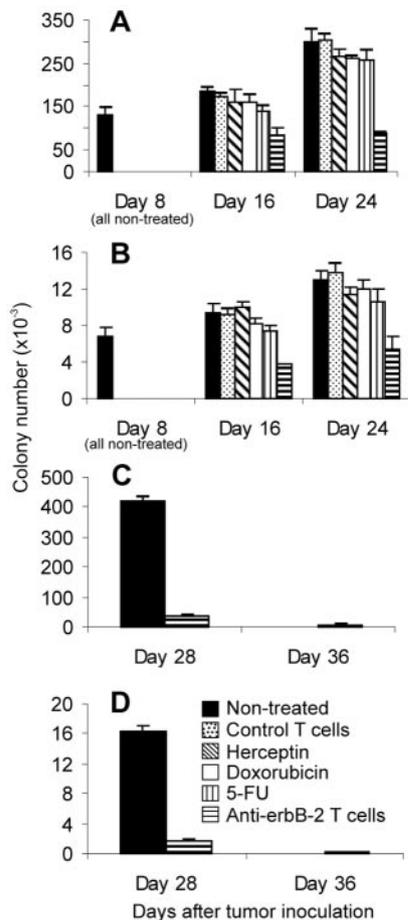


FIGURE 5. Metastatic burden in mice receiving various adjuvants. Primary 4T1.2-erbB-2 mammary tumors were surgically removed 8 days after inoculation, and mice treated with the adjuvants were listed. Lungs and liver were removed on day 8, 16, or 24 after inoculation, and the number of viable tumor cells was determined using a clonogenic assay. Large numbers of tumor cells were present in all mice at day 8 before removal of primary tumor. The tumor burden increased by day 24 in both lungs (A) and liver (B), in all groups of mice, except those treated with anti-erbB-2 gene-modified T cells. In these experiments, nontreated mice survived until day 36. Low numbers of tumor cells were still detectable on days 28 and 36 in mice treated with anti-erbB-2 T cells after tumor inoculation in both lung (C) and liver (D). Results are expressed \pm SEM with three mice per group. This experiment was performed a second time with similar results.

Metastases were also quantitated in mice treated with anti-erbB-2 T cells at later time points beyond the survival range of other groups. The metastatic burden in treated mice decreased further in lung and liver to day 36, which was the latest time point evaluated (Fig. 5, C and D). Two of the four mice assayed at day 36 had no metastases in lung or liver.

A further appreciation of the effect of anti-erbB-2 T cells on the metastatic burden can be gained from photographs of the thoracic cavity of mice 27 days after tumor inoculation (Fig. 6). The normal appearance of lungs, heart, liver, and thoracic cavity is seen in Fig. 6A. Mice receiving no treatment or control T cells developed extensive visible metastases of the lungs, heart, and chest wall (Fig. 6, B and C). Normal lung tissue was reduced to <25% of that of normal SCID mice. Mice receiving either doxorubicin, 5-FU, or herceptin developed obvious thoracic and other metastases to a similar degree as seen in nontreated mice (data not shown). In contrast, mice receiving anti-erbB-2 T cells had no visible signs of metastases (Fig. 6D), and tissues of the thoracic cavity appeared normal.

CD8⁺ T cells are present in the lungs of responding mice

H&E staining of mouse lungs 28 days after tumor inoculation demonstrated extensive metastatic tumor formation in nontreated mice (Fig. 7B) and in mice receiving control (empty vector)-transduced T cells (Fig. 7C). In contrast, the lung architecture of mice

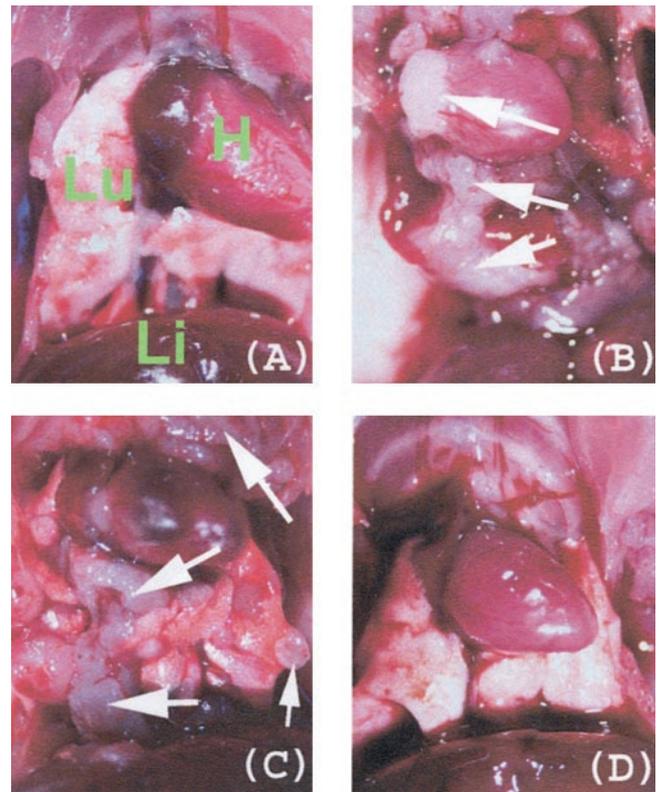


FIGURE 6. Anti-erbB-2 gene-modified T cells inhibit metastases. A, Thoracic cavity of normal, nontumor-bearing SCID mouse. H, heart; Lu, lung; Li, liver. B–D, Primary 4T1.2-erbB-2 mammary tumors were removed on day 8 after inoculation, and photographs were taken on day 28 after inoculation. B, Metastases to the heart, lung, and thoracic cavity (arrows) in a mouse that received no treatment. C, Extensive metastases in a mouse that received control T cells. D, Metastases are absent from the tissues of the thoracic cavity in a mouse that received anti-erbB-2 T cells on days 9 and 15 after tumor inoculation. These pictures are representative of three experiments with 5–10 mice per treatment group.

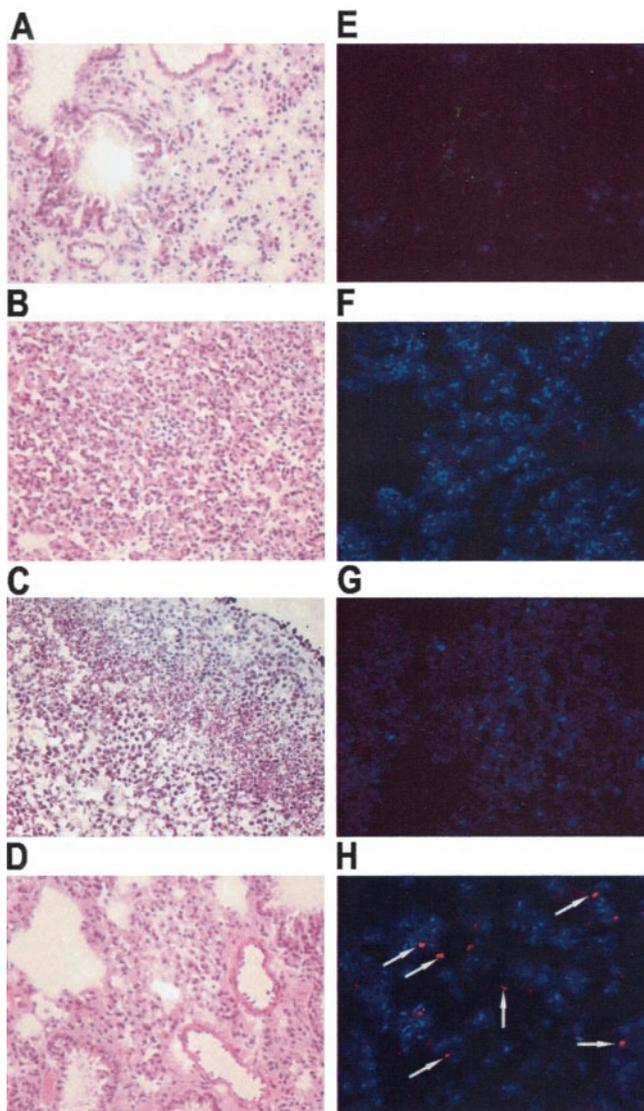


FIGURE 7. CD8⁺ T cells are present in the lungs of responding mice. H&E staining (A–D) and immunohistochemical staining (E–H) of mouse lungs at day 28 from the following groups: A and E, normal nontumor-bearing SCID mouse that received no T cells; B and F, nontreated tumor-bearing mouse; C and G, tumor-bearing mice receiving control T cells; D and H, tumor-bearing mouse that received anti-erbB-2 T cells. Macrophages (blue) are present in lungs of all tumor-bearing mice, but only CD8⁺ T cells were detected in mice that received anti-erbB-2 T cells (stained red and highlighted by arrows). Representative fields of multiple sections are shown. Magnification, $\times 200$.

receiving anti-erbB-2-transduced T cells (Fig. 7D) was comparative to that of normal nontumor-bearing SCID mice (Fig. 7A). When examined by confocal microscopy following immunohistochemical staining, macrophages were found to be present in the lungs of nontreated, control T cell-treated, and anti-erbB-2 T cell-treated mice (Fig. 7, F–H). Interestingly, CD8⁺, but not CD4⁺ T cells were detected in the lungs of mice receiving anti-erbB-2 T cells (Fig. 7H). No T cells were detected in lungs from control mice (Fig. 7, E–G). As a positive control, both T cell subsets were detected in spleens of BALB/c mice using the same immunohistochemical staining method (data not shown).

Discussion

Survival of patients following the initial diagnosis of cancer correlates directly with the extent of metastases. The early detection

and removal of primary tumors can result in long-term survival if achieved before metastases arise. Improved screening procedures and an increased awareness of the value of regular screening have led to a decrease in the mortality rates of some cancers, including cancers of the breast, in the U.S. and elsewhere. Nevertheless, a large percentage of breast cancers has already metastasized before the removal of the primary lesion. Such metastases are often present as microdeposits and are undetectable for many months, but eventually progress, which can lead to death. There is a clear need for a safe, specific, and effective means of adjuvant therapy aimed at eradicating metastases following excision of primary lesions.

The use of immunotherapeutics for cancer is attracting much interest from scientists and clinicians due to a low, but significant rate of dramatic responses to immunotherapy (30). One of the chief attractions of using immune system elements to target cancer is the potential specificity. In this study, we describe the use of tumor-specific T cells that were generated by genetic modification. Significant increases in survival of mice were afforded by treatment with tumor-specific T cells. T cells were superior to three of the most commonly used therapies of doxorubicin, 5-FU, or herceptin.

The 4T1.2 mouse breast cancer model was specifically chosen for this study because of its relevant spontaneous metastatic pattern and its inherent resistance to chemotherapy, which closely resembles the disease of many patients.

The study demonstrates for the first time that T cells engineered with an enhanced antitumor gene (incorporating both CD28 and TCR- ζ signaling motifs) can effectively eradicate widespread spontaneous metastases.

Interestingly, gene-modified T cells were more effective than herceptin, which also targets erbB-2. The reason for this may lie in the different mechanisms used by T cells compared with NK cells and macrophages that may be used by herceptin (31, 32). NK cells and macrophages require efficient and stable loading of Fc receptors with specific Ab to exert their antitumor effects. It is possible that tumor reactivity generated by this loading is less efficient than that afforded to T cells by genetic modification. It is also possible that T cells armed with the anti-erbB-2-CD28- ζ receptor use more efficacious mechanisms than Fc receptor-bearing leukocytes.

T cells can use a variety of different effector mechanisms. T cells can be directly cytolytic against tumor cells through exocytosis of granules containing the apoptosis-inducing molecules perforin and granzymes (33). Other mechanisms of cell killing used by T cells against target cells are mediated by T cell-expressed Fas ligand or TRAIL that interact with Fas or TRAIL receptor on the surface of target cells.

T cells can also secrete an array of cytokines, including IL-2, IL-4, IL-10, IFN- γ , GM-CSF, and TNF- α . These cytokines can have a range of functions. For example, TNF- α can be directly cytolytic. IL-2 can aid in the activation and survival of lymphocytes, IFN- γ can sensitize target cells to apoptosis, while GM-CSF can stimulate other leukocytes. Chemokines can also be secreted by T cells that can attract further leukocytes to the tumor site (34).

Interestingly, the predominant responding lymphocyte population was the CD8⁺ subset, as demonstrated by intracellular IFN- γ staining following chimeric receptor ligation. This suggests that the antitumor reactivity was due largely to CD8⁺ cells, although a role for CD4⁺ cells cannot be fully excluded. The major role of CD8⁺ cells is also supported by the presence of CD8⁺ rather than CD4⁺ T cells within the lungs of responding tumor-bearing mice. Macrophages were present in the lungs of tumor-bearing mice, but eosinophilia was not observed.

In this study, adoptively transferred tumor-specific T cells were demonstrated to impact on the extent of metastases. Metastatic disease in the lungs and liver of T cell-treated mice was inhibited at least until day 36 (the latest time assayed). It will now be of interest, upon accrual of sufficient long-term survivors, to determine the absolute duration of tumor and T cell persistence. In future studies, we will also determine the role of Ag expression levels in tumor and T cell persistence.

Although treatment with anti-erbB-2 T cells impacted on widespread metastatic disease, it was less effective against tumors that regrew at the original site of inoculation despite the continued expression of erbB-2 (data not shown). We consider tumor regrowth at this site to originate from residual deposits of disease that remained after surgery rather than reseeding of metastases from other sites, because breast disease only ever recurred at the site of injection and not in other mammary glands. Indeed, tumor regrowth appeared to be s.c. rather than mammary gland associated, and most likely resulted from invasion of the s.c. region from the original tumor implanted in mammary tissue. All visible primary tumor was removed from mammary tissue, along with a reasonable margin of 2–3 mm. However, the large area of skin associated with the fourth mammary gland used could not be removed entirely for ethical reasons. The reason for the relative resistance to T cell treatment of primary regrowth compared with metastases is not clear, although size and/or site considerations could play a role. The complete removal of primary tumors in patients is more successful, and complications due to primary regrowth in mice should not detract from the potential of this therapy in patients.

SCID mice used in this study are deficient in the lymphoid compartment. Therefore, adoptively transferred T cells in this setting are likely to expand and persist to a greater degree than in immunocompetent mice due to altered lymphoid homeostasis in SCID mice. It will be of interest to try this approach in immunocompetent mice, although this will require a system with a scFv-CD28- ζ chimeric receptor specific for a mouse self Ag. A satisfactory system does not exist at present, although we are in the process of establishing a relevant system. Nevertheless, it is possible in patients to deliver proportionally greater numbers of T cells more frequently to provide sufficient cells. This may involve the delivery of up to 1×10^{12} T cells, which is entirely possible using current technologies (35). The possibility also exists in patients to mimic the immunodeficient state using nonmyeloablative conditioning. This semiablative procedure has been used in melanoma patients receiving nongene-modified peptide-specific T cells, and dramatic expansion and persistence of adoptively transferred T cells have been demonstrated (36). Thus, our approach, which uses gene-modified T cells programmed with both Ag-specific activation and costimulatory abilities, may be directly applicable in patients, with maximum benefit for those patients with early or occult metastases.

The tumor Ag used in this study was human erbB-2, whose expression was entirely limited to tumor. Therefore, it is not possible to determine the extent of toxicity that erbB-2-specific T cells might have if the Ag was expressed on normal tissues as it is in the human situation. However, because tumor-associated Ags are often greatly overexpressed on tumor cells, and chimeric receptor-directed T cell activity is dependent on Ag density (37), it is possible that gene-modified T cells will be able to target tumors bearing tumor-associated Ags with reasonable specificity and correspondingly low toxicity. In support of this, no significant toxicity attributable to cells alone was encountered in a phase I clinical trial using gene-modified T cells specific for the ovarian cancer-associated Ag, folate-binding protein (P. Hwu, unpublished observations). Although folate-binding protein is overexpressed on a large percentage of ovarian tumors, it is also expressed to a lesser degree

on some normal tissue, including alveolar epithelium, pancreas, and kidney tubules (38). Nevertheless, it may be prudent to engineer a self-destruct or suicide capability into the gene modification strategy, which will enable the elimination of rogue T cells should they induce autoimmunity or become transformed themselves. A suitable suicide strategy involves the incorporation of the HSV-thymidine kinase gene into the retroviral construct. This strategy has been demonstrated to enable control of gene-modified T cells through the administration of gancyclovir (39), and may be a suitable safeguard despite the generation of an anti-HSV-thymidine kinase response in 25% of humans (40).

Although erbB-2 is expressed on only 25–30% of breast cancers, the approach is potentially more widely applicable due to the availability of single-chain Abs against other commonly expressed breast cancer Ags, Muc1, Lewis^Y, carcinoembryonic Ag, and TAG72. Targeting these other, often coexpressed Ags also renders tumor escape by Ag loss less likely.

Interestingly, the use of tumor-reactive/costimulated T cells in our application did not require the use of exogenously supplied IL-2 that many adoptive immunotherapy strategies find necessary. This removes another possible source of treatment-related toxicity, namely IL-2 (41).

The results of the studies presented in this work support the use of tumor-reactive T cells against metastatic breast cancer. T cells reactive with most tumor types can be generated from all individuals by genetic modification with appropriate chimeric receptors. These T cells may be of low toxicity in tumor-bearing recipients. In particular, the demonstration of their enhanced potency, when compared with doxorubicin, 5-FU, or herceptin, against early disseminated disease supports their use as a viable alternative adjuvant in patients following surgical removal of primary tumor.

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