Tumor Ablation by Gene-Modified T Cells in the Absence of Autoimmunity

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Abstract
Adoptive immunotherapy involving genetic modification of T cells with antigen-specific, chimeric, single-chain receptors is a promising approach for the treatment of cancer. To determine whether gene-modified T cells could induce antitumor effects without associated autoimmune pathology, we assessed the ability of T cells expressing an anti-Her-2 chimeric receptor to eradicate tumor in Her-2 transgenic mice that express human Her-2 as a self-antigen in brain and mammary tissues. In adoptive transfer studies, we demonstrated significant improvement in the survival of mice bearing Her-2+ 24JK tumor following administration of anti-Her-2 T cells compared with control T cells. The incorporation of a lymphoablative step prior to adoptive transfer of anti-Her-2 T cells and administration of IL-2 were both found to further enhance survival. The reduction in tumor growth was also correlated with localization of transferred T cells at the tumor site. Furthermore, an antigen-specific recall response could be induced in long-term surviving mice following rechallenge with Her-2+ tumor. Importantly, antitumor effects were not associated with any autoimmune pathology in normal tissue expressing Her-2 antigen. This study highlights the therapeutic potential of using gene-engineered T cells as a safe and effective treatment of cancer.

Introduction
Strategies, including the use of cytokines, antibodies, and various vaccine regimens, aimed at harnessing the power of the immune system have long been investigated for the treatment of cancer (1–3). However, the generation of effective and long-lasting antitumor responses has remained a challenge. In recent studies, the genetic modification of T cells with scFv chimeric receptors that have the ability to target any characterized cell surface tumor antigen has been investigated as a new avenue of redirecting immune effector function (4–9). Adaptive transfer of scFv receptor gene–modified T cells in immunocompromised mouse models has shown great potential and demonstrated long-term protection against primary and secondary tumor challenge (8, 10).

Although the results achieved in the immunocompromised setting have been encouraging, these studies have been performed in models in which the targeted antigen has been expressed exclusively in tumor, which is rarely the case in a clinical setting. Consequently, this has not allowed investigation of whether host immunoregulatory factors may impact on the activity of transferred T cells or whether these cells may also damage normal tissue expressing the target antigen. The latter point is particularly important, given that adoptive cell therapies utilizing tumor-infiltrating lymphocytes (TILs) or gene-modified T cells have in some cases resulted in on-target toxicity, manifested as “autoimmunity” in patients including thyroiditis, uveitis, and respiratory and liver toxicity (11–14). The generation of human Her-2 transgenic mice represents an ideal model to investigate this issue. These mice have been reported to constitutively express human Her-2 as a self-antigen both in brain tissue and in the mammary gland during lactation under the control of the whey acidic protein promoter (15). Her-2+ tumor cells grow progressively when injected into these mice. Thus, Her-2 transgenic mice offer a physiologically relevant model that can be utilized for the preclinical evaluation of gene-modified T cells in a setting that more closely mimics the situation in patients.

In the current study, we utilized this Her-2 transgenic mouse model to evaluate the antitumor efficacy of Her-2-reactive, gene-modified T cells and assess whether these cells induce autoimmune pathology.
Materials and Methods

Cell culture and mice

The MHC class I–negative C57BL/6 mouse 24JK sarcoma cell line (kindly provided by Dr. Patrick Hwu, National Institutes of Health, Bethesda, MD), 24JK cells retrovirally transduced with the human Her-2 antigen (24JK-Her-2) and gp+e68 retroviral packaging cells were cultured as described previously. The 24JK tumor cell lines were routinely tested and authenticated in the last 6 months by flow cytometry and found to be MHC class I negative as per the original line acquired. C57BL/6 human Her-2 transgenic mice and congenic Thy1.1+ Her-2 mice were bred at the Peter MacCallum Cancer Centre, Victoria, Australia, and used for experimentation at 6 to 16 weeks of age. All animal experimentation was approved in advance by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

Generation of anti-Her-2–transduced T cells

The gp+e68 packaging line expressing the scFv-anti-Her-2 CD28– receptor that contained the extracellular scFv-anti-Her-2 monoclonal antibody (mAb) region (kindly provided by Dr. Winfried Wels) (16) was generated as previously described (17, 18). For transduction, splenocytes from C57BL/6 Her-2 transgenic mice were gene modified as previously described (19). Following transduction, splenocytes were cultured in RPMI, supplemented with 100 U/mL of IL-2 and 2 ng/mL of IL-7, and utilized for in vitro and in vivo experiments on days 7 and 8.

Flow cytometry

Expression of the chimeric anti-Her-2 receptor on the surface of tumor cells was determined by direct immunofluorescence with a c-myc tag Alexa Fluor 488–conjugated immunoglobulin (Cell Signaling Technology, Beverly, MA). Background immunofluorescence was assessed using an IgG2a Alexa Fluor 488–conjugated mouse isotype immunoglobulin (Invitrogen). Phenotypic characterization of transduced cells was determined using direct staining with fluorescein isothiocyanate–conjugated anti-CD4 (clone RM4-5; BD Pharmingen, San Jose, CA) and phycoerythrin-conjugated anti-CD8 (clone 53-6-7; BD Pharmingen).

Antigen-specific cytokine secretion, proliferation, and cytotoxicity by gene-modified T cells

The ability of transduced anti-Her-2 T cells to secrete IFN-γ in response to Her-2 antigen stimulation was analyzed by enzyme-linked immunosorbent assay (ELISA) following overnight coculture with 24JK-Her-2 or 24JK parental cells as previously described (20). The proliferative potential and capacity of transduced T cells to mediate antigen-specific tumor cell lysis was assessed by [3H]-thymidine incorporation and 5-hour chromium release assays, respectively, as described previously (21).

Adoptive transfer experiments

Groups of 5 to 10 Her-2 transgenic mice were intravenously injected with 1 × 10^6 24JK-Her-2 cells followed by transfer of scFv-anti-Her-2–transduced T cells or control LXSN–transduced T cells (1 × 10^7 per dose on days 0 and 1) or left untreated, and survival of mice was monitored. In some experiments, recipient mice received transduced donor T cells from congenic Thy1.1+ Her-2 mice. For experiments involving lymphodepletion, Her-2 transgenic mice were irradiated (5 Gy) followed by intravenous injection of 24JK-Her-2 tumor cells (1 × 10^6) and adoptive transfer of transduced T cells at 1 × 10^7 per dose at days 0 and 1, days 5 and 6, or days 10 and 11. Mice were also given twice-daily intraperitoneal injections of recombinant human IL-2 (Biological Resource Branch, National Cancer Institute, Frederick, MD) involving 9 doses of 50,000 IU/200 µL given subsequent to T-cell transfer. For all experiments, mice were monitored for survival. Mice displaying overt signs of distress were culled, and lungs were harvested and weighed to record tumor burden. To investigate the trafficking and persistence of adoptively transferred T cells in vivo, 1 × 10^7 donor T cells from congenic Thy1.1+ Her-2 mice were transduced and transferred into irradiated 24JK-Her-2 tumor bearing Her-2 recipient mice (Thy1.2+) on days 0 and 1 and received IL-2 as described previously. Flow cytometry was carried out to determine the frequency of all T cells and Thy1.1+ T cells in the organs.

Histology and immunohistochemistry

Lung, mammary (nonlactating female), and brain tissues (female and male) from Her-2 transgenic mice were removed, fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned. To examine tumor burden in lungs of mice (days 5 and 10 after 24JK-Her-2 tumor inoculation) and toxicity to mammary and brain tissues expressing Her-2 antigen following therapy, sections were stained with hematoxylin and eosin (H&E) and analyzed by a pathologist. To examine expression of the human Her-2 antigen in mammary and brain tissues from Her-2 transgenic mice and the 24JK-Her-2 and 24JK cell lines, sections were stained with a polyclonal rabbit anti-human Her-2 monoclonal antibody (A0485; DAKO Corp., Real Carpenteria, CA) following antigen retrieval in 10 mmol/L of citrate buffer (pH 6.0). Images were visualized with an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan), acquired using RT SE Diagnostics Instruments SPOT camera (Diagnostic Instruments, Sterling Heights, MI) in conjunction with SPOT Advanced Version 4.6 (Diagnostic Instruments), and compiled with Adobe Photoshop and Illustrator CS4 (Adobe systems, San Jose, CA).

Statistical analysis

Differences between mice survival distributions were analyzed using log-rank (Mantel–Cox) test to determine statistical significance. P < 0.05 was considered significant.

Results

Expression of the anti-Her-2 chimeric receptor in Her-2 transgenic mouse T cells

Using flow cytometry, we observed reproducibly high-level receptor expression on transduced T cells (Fig. 1A) (46 ± 3.2% SEM, n = 16). There was no detectable anti-tag staining on the
surface of T cells transduced with an empty LXSN retroviral vector (Fig. 1B). These transduced T cells were predominantly CD8\(^+\) (79.5 ± 4.4% SEM, \(n = 8\)), with only a small percentage of CD4\(^+\) T cells present in the culture (7.3 ± 2.7% SEM, \(n = 8\)) (Fig. 1C and D).

Antigen-specific cytokine secretion, tumor lysis, and proliferation by transduced Her-2 transgenic mouse T cells

The functional capacity of transduced transgenic T cells was tested in several in vitro assays. Following stimulation by overnight coculture with the C57BL/6 sarcoma cell line 24JK expressing human Her-2 antigen (24JK-Her-2), transgenic anti-Her-2 T cells secreted high levels of IFN-\(\gamma\) as determined by ELISA (Fig. 2A), whereas coculture with the Her-2-24JK parental tumor cell line demonstrated no significant release of IFN-\(\gamma\). Anti-Her-2 T cells could also significantly lyse 24JK-Her-2 target cells compared with Her-2-24JK tumor cells (Fig. 2B). The proliferative capacity of transduced transgenic T cells was measured by a \([^{3}H]\)-thymidine incorporation assay following stimulation with plate-bound anti-c-myc antibody. Anti-Her-2 T cells could specifically proliferate following stimulation with anti-c-myc antibody but not with isotype control antibody (Fig. 2C). Taken together, these results showed that transgenic T cells could be effectively transduced with the scFv-anti-Her-2 receptor and mediate antigen-specific cytokine secretion, killing, and proliferation.

Adoptive transfer of anti-Her-2 T cells can mediate enhanced survival of tumor-bearing mice and localize to the tumor site

We have previously shown that adoptive transfer of T cells gene-modified with the scFv-anti-Her-2 receptor could specifically eradicate 5-day established lung metastases in immunocompromised SCID mice (10). Thus, we wanted to assess whether transduced T cells could impact on Her-2\(^+\) tumor in an immunocompetent setting in a similar way. In this experiment, we compared the survival of Her-2 transgenic mice bearing 24JK-Her-2 lung metastases treated with anti-Her-2 or control LXSN–transduced T cells administered intravenously on days 0 and 1. A significant increase in
survival of mice (~20% long-term, tumor-free survivors) was observed following transfer of anti-Her-2 T cells compared with mice that received control T cells or no T cells, which all succumbed to lung disease \((P < 0.05; \text{Fig. 3A})\). It has been reported both in mouse studies and in patients that the use of lymphodepletion as a preconditioning treatment prior to adoptive T-cell transfer can enhance their therapeutic efficacy \((22–25)\). To test whether this was the case in our model using gene-engineered T cells, Her-2 transgenic mice were irradiated prior to tumor challenge and transfer of transduced T cells and administered IL-2 between days 0 and 4. In this experiment, a striking increase in survival was observed in recipient mice treated with anti-Her-2 T cells in combination with irradiation and IL-2 \((70\% \text{ long-term, tumor-free survivors}; \text{Fig. 3B})\) compared with mice given anti-Her-2 T cells with no preconditioning \((20\% \text{ survivors}; \text{Fig. 3A})\). Interestingly, the combination of both irradiation and IL-2 along with anti-Her-2 T cells was critical for this therapeutic effect, given the decreased survival that was observed in groups of mice that received anti-Her-2 T cells with either IL-2 or irradiation. There was no survival of mice that received control T cells with the combined regimen (Fig. 3B). Importantly, treatment of mice with irradiation or IL-2 alone did not significantly alter the growth kinetics of the 24JK-Her-2 tumor in recipient mice and no overt toxicity was observed in mice following therapy (Fig. 3B). To determine whether transduced T cells could be detected following transfer, mice were challenged with 24JK-Her-2 tumor and irradiated before receiving transduced congenic Thy1.1+ T cells and IL-2 was investigated by flow cytometry and expressed as a percentage of total viable cells. A, a significant increase in the percentage of anti-Her-2 T cells could be detected in the lungs of mice compared with mice treated with control LXSN T cells at day 18 following transfer \((*, P < 0.05)\). There was no significant difference \((\text{ns})\) in the percentage of transferred T cells in the spleens of mice treated with control or anti-Her-2 T cells or in the total numbers of T cells analyzed from both organs. B, adoptively transferred anti-Her-2 T cells could also be detected in the lungs and spleens of mice at day 53, indicating persistence of these cells. Data shown are average \pm SEM from 5 mice analyzed.
mice and that combined treatment with irradiation and IL-2 could further increase the therapeutic effect similar to that observed in a patient setting receiving TIL and lymphodepletion with IL-2.

**Adoptive transfer of anti-Her-2 T cells can mediate regression of established tumor**

Given that the majority of patients will present with established disease, we next assessed whether our therapy could impact on advanced lung metastases in mice. In 2 separate experiments, the antitumor ability of anti-Her-2 T cells in combination with irradiation and IL-2 was examined against day 5 and day 10 established disease. Immunohistochemical staining using H&E demonstrated increased 24JK-Her-2 metastases in lung sections of mice at days 5 and 10 compared with untreated mice (Fig. 5A). In the first experiment, mice were irradiated and challenged with 24JK-Her-2 tumor on day 0 followed by transfer of transduced T cells on days 5 and 6 and administered IL-2 between days 5 and 9. The majority of mice that received anti-Her-2 T cells combined with irradiation and IL-2 survived (~80% long-term, tumor-free survivors; Fig. 5B). This effect was dependent on T cells modified with the scFv anti-Her-2 receptor, as mice treated with control LXSN-transduced T cells succumbed to lung metastases similar to mice challenged with 24JK-Her-2 tumor alone (Fig. 5B). In the next experiment, mice were irradiated on day 10 and challenged with 24JK-Her-2 tumor followed by transfer of transduced T cells on days 10 and 11 and administered IL-2 between days 10 and 14. Strikingly, a significant increase in survival of mice was observed in mice with day 10 established disease that received anti-Her-2 T cells in combination with irradiation and IL-2 compared with control treated mice (Fig. 5C). Again, no overt toxicity was observed in mice following therapy. These experiments showed that gene-modified T cells could significantly impact on established disease in immunocompetent Her-2 transgenic mice, which has important implications for development of this approach for the treatment of patients with advanced cancer.

**Long-term surviving mice can respond to tumor rechallenge in an antigen-specific manner**

Given that tumor relapse can often occur in patients following treatment, the ability for cancer immunotherapies to induce a strong recall response is an important issue. To test whether this was the case for our therapy, long-term surviving mice (120 days after primary 24JK-Her-2 tumor inoculation) that were irradiated and treated with anti-Her-2 T cells and IL-2 were rechallenged with either 24JK-Her-2 or parental 24JK tumor cells. A significant increase in survival of mice was observed following rechallenge with 24JK-Her-2 compared with 24JK-Her-2 tumor cells (Fig. 6). These experiments demonstrated for the first time in an immunocompetent setting that adoptively transferred, gene-engineered T cells could induce a strong recall response in an antigen-specific manner.

**Adoptive transfer of transduced T cells does not cause autoimmunity in mice**

An important issue with regard to adoptive immunotherapy using gene-engineered T cells is whether these cells may...
cause severe autoimmune effects against normal tissue expressing the target antigen. In Her-2 transgenic mice, the human Her-2 antigen has been shown to be expressed as a self-antigen in parts of the brain and mammary tissue under lactating conditions (15). We confirmed these results and showed with a specific antibody recognizing Her-2 positive staining for this antigen in the cerebellum of brain sections from transgenic mice but not in nontransgenic littermates. Sections are representative of 5 mice analyzed.

Discussion

The above-described model closely approximates the clinical setting in which the Her-2 antigen is overexpressed in ~30% of breast cancer patients as well as several other cancers including ovarian, lung, and head and neck cancer (28–30) while also present on normal tissue including those of epithelial, mesenchymal, and neuronal origin (26, 27). In this study, we showed that anti-Her-2 T cells could significantly impact on 24JK-Her-2 lung metastases in Her-2 transgenic mice compared with control T cells.
An important consideration for the therapeutic use of adoptively transferred, gene-engineered T cells is whether they may induce extensive autoimmune damage to normal tissues expressing the target antigen (31). In this study, we investigated whether the transfer of gene-modified T cells in combination with lymphoablation and IL-2 into tumor-bearing Her-2 transgenic mice caused any autoimmune toxicity to brain and/or mammary tissues expressing the Her-2 target antigen. In these experiments, no autoimmune damage to these tissues was observed by engineered T cells at early and late time points analyzed. The fact that we did not observe any autoimmune may be in part due to differences in Her-2 antigen levels between tumor and normal brain and mammary tissues. Our immunohistochemical analysis indicated comparably stronger Her-2 antigen staining on 24JK-Her-2 tumor cells relative to brain and mammary tissues, which is generally the case seen in the patient setting. Our results correlate well with a recent report showing no overt toxicity in mice following adoptive transfer of scFv-transduced T cells targeting mouse CD19 antigen, although in this study, scFv-transduced T cells did not persist long term in mice. This may have been due to lack of costimulation with the use of a chimeric receptor that contained only the CD3-ζ signaling domain (19). In the present study, we used an scFv receptor containing both the CD28 costimulatory and CD3-ζ domains. Our results are also consistent with another study that showed that nonmyeloablative preconditioning and adoptive transfer of carcinoembryonic antigen–specific, MHC-restricted, T cells mediated antitumor activity without associated autoimmune (32). However, autoimmune was observed in this model following increased lymphoablation at a myeloablative dose (9.5 Gy) or the addition of anti-IL-10 receptor blocking antibody, indicating that the type of immunomodulation employed must be treated with caution.

Despite these reports, several studies have reported mild to severe autoimmune toxicity following transfer of tumor reactive T cells (33–35) including liver toxicity in patients involving transfer of T cells gene-modified with an anti-CAIX scFv receptor (13). Toxicity, manifested by increased levels of transaminase in serum, was thought to be due to CAIX expression on bile duct epithelium. Resolution of toxicity was achieved by cessation of therapy and administration of corticosteroids.

A recent trial involving adoptive transfer of gene-modified T cells targeting the Her-2 antigen resulted in the death of a patient 5 days after treatment; this might have been due to a cytokine storm resulting from recognition of Her-2 on normal tissue (14). Pulmonary edema occurred within 1 hour of cell infusion requiring intubation. Hypotension, bradycardia, and gastrointestinal bleeding led to cardiac arrest and death. The herceptin-based scFv receptor utilized in this trial incorporated the CD28, 4-1BB, and CD3-ζ domains, which may have led to increased secretion of cytokines from T cells following antigen recognition and subsequent toxicity (14). Interestingly, in another study, no toxicity was reported in patients following transfer of autologous anti-Her-2 CTL clones targeting the same antigen (36).

In another phase 1 clinical study, a serious adverse event involving death of a patient was observed following adoptive transfer of T cells gene-modified to respond against CD19 (37). The patient, with chronic lymphocytic leukemia, received (1.2–3.0) × 10^7 T cells/kg following cyclophosphamide conditioning. Hypotension and acute renal failure led to respiratory arrest. However, a definitive role for the infused T cells was not indicated in this case and death was thought to be as a result of sepsis due to infection leading to hypotension.

Collectively, studies both in preclinical mouse models and in patients have indicated that the location and levels of antigen expressed on normal tissue, the number of T cells transferred, the type of signaling domains incorporated into chimeric receptors, and the level of immune preconditioning used have to be carefully considered for adoptively transferred T-cell therapy. Given the potential severity of autoimmunity that has been reported in some studies, it would be prudent in the future to use scFv chimeric receptors targeting carefully selected antigens that are either tumor-specific or expressed at relatively low levels on normal tissue compared with tumor.

In support of this idea, we have found that T cells engineered with an scFv-anti-Lewis^y^ chimeric receptor could strongly react with tumor cells expressing high Lewis^y^ antigen but did not react against endogenous neutrophils expressing low levels of Lewis^y^ (8). The possibility of redirecting T cells to multiple tumor-associated antigens expressed on tumor cells may further enhance their specificity for tumor while minimizing their activity against healthy tissue. Another alternative may be the inclusion of a regulated “suicide” gene function such as hsv-tk or the cytoplasmic domain of Fas or an inducible caspase incorporated into genetically engineered T cells to abort any aberrant T-cell responses (38–40).

In summary, this study has demonstrated that transfer of gene-engineered T cells combined with lymphoablation and IL-2 could mediate significant antitumor effects in a self-antigen setting without causing damaging autoimmune effects. These results suggest that redirected T-cell therapy has great potential as a safe and viable option for the treatment of cancer patients in the future.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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