Preclinical In Vivo Modeling of Cytokine Release Syndrome Induced by ErbB-Retargeted Human T Cells: Identifying a Window of Therapeutic Opportunity?


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References

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The ErbB network is dysregulated in many solid tumors. To exploit this, we have developed a chimeric Ag receptor (CAR) named T1E28z that targets several pathogenetically relevant ErbB dimers. T1E28z is coexpressed with a chimeric cytokine receptor named 4εβ (combination termed T4), enabling the selective expansion of engineered T cells using IL-4. Human T4+ T cells exhibit antitumor activity against several ErbB+ cancer types. However, ErbB receptors are also expressed in several healthy tissues, raising concerns about toxic potential. In this study, we have evaluated safety of T4 immunotherapy in vivo using a SCID beige mouse model. We show that the human T1E28z CAR efficiently recognizes mouse ErbB+ cells, rendering this species suitable to evaluate preclinical toxicity. Administration of T4+ T cells using the i.v. or intratumoral routes achieves partial tumor regression without clinical or histopathologic toxicity. In contrast, when delivered i.p., tumor reduction is accompanied by dose-dependent side effects. Toxicity mediated by T4+ T cells results from target recognition in both tumor and healthy tissues, leading to release of both human (IL-2/IFN-γ) and murine (IL-6) cytokines. In extreme cases, outcome is lethal. Both toxicity and IL-6 release can be ameliorated by prior macrophage depletion, consistent with clinical data that implicate IL-6 in this pathogenic event. These data demonstrate that CAR-induced cytokine release syndrome can be modeled in mice that express target Ag in an appropriate distribution. Furthermore, our findings argue that ErbB-retargeted T cells can achieve therapeutic benefit in the absence of unacceptable toxicity, providing that route of administration and dose are carefully optimized. The Journal of Immunology, 2013, 191: 4589–4598.
specificity against several pathogenetically relevant ErbB dimers (14). Target recognition is achieved using the T1E peptide, which is a promiscuous chimeric ErbB ligand derived from TGF-α and epidermal growth factor (EGF) (15). T cells engineered to express T1E28z are activated by target cells that express ErbB1- and ErbB4-based dimers and the potently mitogenic ErbB2/3 heterodimer. Expansion and enrichment of T1E28z+ T cells may be conveniently achieved ex vivo by coexpression with a chimeric cytokine receptor named 4β. In 4β, the ectodomain of the IL-4 receptor α subunit has been joined to the transmembrane and endodomain of the shared β-chain used by the IL-2 and IL-15 receptors. As a result, addition of IL-4 to T cells that coexpress 4β and T1E28z (combination T4) promotes the selective outgrowth of the gene-modified cells (16). Following expansion using IL-4, T4+ T cells mediate effective antitumor activity against head and neck squamous cell carcinoma (HNSCC), breast (14), or ovarian cancer (17).

In adulthood, all four ErbB receptors continue to be widely expressed and contribute to the function of several major organs (18–23). Consequently, a key challenge to the development of effective ErbB-targeted therapies is balancing efficacy against the risk of unacceptable toxicity (24, 25). In this study, we have undertaken in vivo safety testing of human T4+ T cell immunotherapy, selecting immune-compromised SCID beige mice for four reasons. First, regulatory authorities require that toxicity studies should involve the therapeutic product itself (e.g., human T cells). Second, we predicted and show in this study that the human T1E28z CAR, matched truncated control (Tr) (14), P28z CAR (targets prostate-specific membrane Ag) (28), and 4αβ chimeric cytokine receptor (16) have been described previously. T4 refers to the equimolar coexpression of 4αβ and T1E28z using a Thosea asigna 2A (T2A) peptide, downstream of a furin site (14). To construct P4, the 4αβ-furin-T2A–encoding Nco1 fragment was transferred from T4 into SFG P28z. SFG T4-luciferase (luc) was generated by ligation of 1) SFG T4 (Not1/Xho1, 8284 bp); 2) a 749-bp Not1/Nco1 fragment (encoding the remainder of T1E28z followed by furin and T2A sequences); GenScript, Piscataway, NJ); 3) a 1133-bp Nco1/Age 1 fragment from pBP luc (29); and 4) a 532-bp Age1/Xho1 fragment encoding the remainder of firefly luciferase followed by stop codon (GenScript). The second T2A sequence in SFG T4-luc was maximally codon wobbled to preserve vector stability.

Retroviral transduction and culture of T4+ T cells

Blood samples were obtained under approval of the South East London Research Ethics Committee 1 (reference 09/H0804/92; healthy volunteers) and the West London Research Ethics Committee (references 08/H0707/188 and 09/H0707/86; patients with ovarian cancer and head and neck cancer, respectively). Activation of T cells was achieved prior to gene transfer using CD3/CD28-coated paramagnetic beads (1:1 bead/cell ratio; Life Technologies, Paisley, U.K.) or PHA (5 μg/ml; Sigma-Aldrich, Poole, U.K.). Retroviral transduction of activated T cells was performed using...
Binning was used for T cell imaging. Image software (PerkinElmer) as described (14, 29), except that large formed using an IVIS Lumina II or Spectrum (PerkinElmer) with Living similar signal intensity prior to T cell administration. Imaging was per-

Cells and cell culture

The firefly luciferase–expressing SKOV-3-luc-D3 cell line (SKOV-3 luc; PerkinElmer, Waltham, MA) and HN3-luc cell lines (14) were propagated in D10 medium, for example, DMEM (Lonza, Basel, Switzerland) sup-

Flow cytometry

Expression of CARs was performed as described (14, 16, 28). Alternatively, T1E28z was detected using biotinylated anti-EGF Ab (BAF236; R&D Systems, Oxon, U.K.) and streptavidin-PE (Life Technologies). To quantify ErbB expression in organs from SCID beige mice, dissected tissues were homogenized in PBS using a syringe plunger and filtered through a 100-µm cell strainer (30). After treatment with red blood lysis solution (Neoteny, Biotec, Bisley, U.K.), cells were fixed using 4% paraformaldehyde (37°C for 10 min), permeabilized using ice-cold methanol for 30 min, and washed with 40% D10/60% PBS. Next, cells were incubated with rabbit anti-ErbB1 (1005, sc03), ErbB2 (c-18), ErbB3 (c-17), ErbB4 (c-18; Santa Cruz Bio-

Cytokine analysis

Supernatants and sera were analyzed using ELISA kits (eBioscience, Hatfield, U.K.) or cytometric bead arrays (Th1/Th2/Th17; BD Biosciences, Oxford, U.K.), as described by the manufacturers.

Cytotoxicity assays

 Destruction of tumor cell monolayers by T cells was visualized by crystal violet staining (16). Tumor cell viability was quantified using an MTT assay (Life Technologies), as described by the manufacturer.

In vitro luciferase assay

A total of 0.5 × 10^6 transduced (and matched untransduced as control) were assayed using a luciferase assay system kit (Promega, Madison, WI), as described by the manufacturer. Assays were read using a FLUOStar Omega microplate reader with Omega software (version 1.20; all from BMG Labtech, Ortenberg, Germany).

In vivo studies

All in vivo experimentation adhered to U.K. Home Office guidelines, as specified in project license numbers 70/6832 and 70/6677. SKOV-3 luc (1 × 10^6) or HN3-luc (5 × 10^6) cells were inoculated into SCID beige mice (Charles River, Margate, U.K.) either i.p. in PBS or s.c. in 200 µl matrigel (BD Biosciences). Tumor engraftment was confirmed by bioluminescence imaging (BLI) and mice were sorted into groups with 

Statistical analysis

For comparison of two groups, datasets were analyzed using Excel with Microsoft Office for Mac 2008 (Microsoft, Redmond, WA) using a one-tailed Student t test. Additional statistical analysis was performed using Prism software (version 5; GraphPad Software, San Diego, CA).

Results

The human T1E28z CAR can engage murine ErbB receptors

Supplemental Fig. 1 shows that all four ErbB receptors are expressed in several healthy tissues in SCID beige mice. In light of this, we evaluated whether the mouse could provide a suitable preclinical model to study the potential toxicity of ErbB-retargeted human T^4^ T cells.

Because all human ErbB ligands are fully active on murine ErbB receptors (31–35), we hypothesized that human T1E28z T cells would engage and undergo efficient activation by mouse ErbB-expressing cells. To test this, T^4^ and control human T cells were cocultivated with B7E3, a mouse HNSCC cell line that coexpresses ErbB1–4 (Fig. 1A). Human T cells that expressed T4 (Fig. 1B) or T1E28z rapidly destroyed B7E3 tumor cell monolayers (Fig. 1C). Similar findings were not observed with control T cells that expressed a matched truncated CAR, either alone (Tr) or combined with 4eB (Tr4). We next compared activation of human T^4^ T cells by B7E3 or the human HNSCC tumor cell line HN3 (Fig. 1A). Comparable levels of tumor cell cytotoxicity were observed in both cases (Fig. 1D), accompanied by release of IL-2

FIGURE 2. Human T^4^ T cells are activated by cultured mouse ErbB^+^ LMECs. (A) Expression of ErbB1–4 was detected in LMECs by flow cytometry as described in Fig. 1 (open histograms). Filled histograms indicate nonimmune serum. (B) Human T^4^ T cells (1 × 10^6^ cells), control P4+ T cells (coexpress 4eB with P28z, a CAR of irrelevant Ag specificity), or untransduced T cells were cocultured overnight with a confluent monolayer of mouse LMECs (24-well plate). After washing, residual viable LMECs were quantified by MTT assay. (C) IFN-γ content in supernatants was measured by ELISA. All data show means ± SEM of triplicate analyses.
(Fig. 1E) and IFN-γ (Fig. 1F). Greater cytokine production was generally observed following stimulation by HN3, which may reflect the higher expression of ErbB receptors by HN3 than B7E3 cells (Fig. 1A).

Next, we explored whether nontransformed ErbB+ mouse cells could also activate human T4+ T cells. To test this, we selected mouse LMECs. These cells express moderate levels of ErbB1, 2, and 4 (Fig. 2A) and were destroyed by T4+ T cells (Fig. 2B), accompanied by production of IFN-γ (Fig. 2C). Similar findings were not observed with control untransduced or P4+ T cells, in which 4β was coexpressed with a CAR of irrelevant Ag specificity. These data demonstrate that the human T1E28z CAR is activated efficiently by murine ErbB-expressing cells.

Longevity of human T4+ T cells in SCID beige mice

Next, we sought advice from the United Kingdom drug regulatory body, the Medicines and Healthcare Products Regulatory Agency, on appropriate preclinical safety testing of T4 immunotherapy. Considering the data presented above, the Medicines and Healthcare Products Regulatory Agency agreed that suitably conducted murine studies would meet this need. However, it was indicated that timing of histologic analysis would need to be guided by in vivo longevity of human T4+ T cells in mice. The underlying rationale was that toxicity would be most apparent close to the point that T cells were no longer present.

To investigate this, we introduced a firefly luciferase gene into the T4 vector to create T4-luc (Fig. 3A). Coexpression of 4β and T1E28z was demonstrated in PG13 retroviral packaging cells (Fig. 3B) and transduced human T cells, albeit at lower levels (Fig. 3C and data not shown). Function of 4β was confirmed by IL-4-mediated expansion and enrichment of T4-luc- T cells (Fig. 3C). Function of luc was demonstrated in both PG13 and T4-luc- T cells by in vitro luciferase assay (Fig. 3D). Function of T1E28z was confirmed by the ability of T4-luc+ T cells to destroy ErbB-expressing human and mouse HNSCC monolayers while sparing ErbB2 human MDA-MB-435 tumor cells (Fig. 3E).

Longevity of T4-luc+ T cells was evaluated in tumor-bearing SCID beige mice by BLI. Animals with an established s.c. HN3 tumor received 2 × 10^7 T4-luc+ T cells, administered by the i.v. or intratumoral (i.t.) routes. In both cases, adoptively transferred T cells declined exponentially, reaching undetectable levels by 120 h (Fig. 3F). Following i.v. administration, the BLI signal was initially apparent in the lungs followed by the upper abdomen (presumably reflecting migration to liver and spleen). In contrast, the BLI signal was only apparent in the vicinity of the tumor following i.t. injection (Fig. 3G). No secondary rebound in T cell BLI signal was observed.

Toxicity testing of T4 immunotherapy administered using the i.t. or i.v. routes

It was apparent that imaging of T4-luc+ T cells was relatively insensitive, owing to poor transgene expression from the tricistronic T4-luc vector. To allow for this, we evaluated toxicity of T4 immunotherapy 7 d after T cells were administered to mice bearing a 2-wk established s.c. HN3-luc tumor. Healthy donor T cells were transduced using clinical-grade SFG T4 retroviral vector. As expected, T4+ T cells underwent IL-4-mediated en-
richment (Fig. 4A) and an exponential increase in number during GMP manufacture (Fig. 4B). On day 15, a total of $2 \times 10^7$ T4+ T cells were administered to tumor-bearing mice using either the i.v. or i.t. routes. In both cases, significant albeit partial tumor regression was observed during the ensuing week, indicated by reduced BLI signal intensity (Fig. 4C, 4E). Animals exhibited normal behavior and weight gain (Fig. 4D). Seven days after T cell administration (day 22 after tumor implantation), animals were culled and independent histopathologic analysis of major organs was undertaken. No significant pathology was detected nor were residual T cells observed in any organ (Supplemental Table I). Similarly, when T cells that expressed T1E28z alone were injected i.v. into tumor-free mice, no alteration in behavior or weight gain was observed, even following preactivation of these T cells ex vivo (Supplemental Fig. 2).

**Induction of toxicity by i.p. administration of T4 immunotherapy**

The failure of i.v. injected T4+ T cells to induce any detectable pathology raised concerns that the SCID beige model was not suitable to detect toxicity. Evidence to refute this emerged from studies in which i.p. injected T4+ T cells were tested for antitumor activity against SKOV-3 luc ovarian cancer xenografts (17). Tumor formation occurs throughout the peritoneal cavity, accompanied by a copious influx of macrophages (Supplemental Fig. 3). In one such experiment, 12 mice with very advanced i.p. tumor burdens were treated with T cells that were homogeneously positive for the T1E28z CAR (Fig. 5A). Within 72 h, 11 of these mice had died or needed to be culled, whereas control animals treated with PBS remained clinically unaffected. The single surviving T4-treated mouse (n = 1 of 12; Fig. 5B) exhibited an almost 3 log reduction in tumor-derived BLI signal, whereas tumor had progressed in control animals (Fig. 5B, 5C). At postmortem examination, no cause of death was apparent and no residual tumor was detected (n = 5).

These results suggested that T4+ T cells had the potential to elicit severe toxicity when delivered i.p., perhaps owing to uncontrolled tumor-mediated activation. Consistent with this, we observed that animals with less advanced SKOV luc tumors exhibited reversible weight loss (Fig. 5D), accompanied by a 1–2 log tumor regression (data not shown). Weight loss was dependent on the T1E28z CAR because it was not observed with the P4 control (Fig. 5D).

To assess the generality of these findings, we administered T4+ T cells i.p. to mice that were tumor-free or in which there was a moderately advanced i.p. burden of HN3-luc tumor. As before, T4+ T cells induced regression of HN3 tumors (14), a finding that was not observed with untransduced T cells (Fig. 6A). Serial testing revealed that T4 immunotherapy resulted in a rapid, transient, and dose-dependent loss of weight (Fig. 6B, 6C). Notably, weight loss was not dependent on the presence of tumor, indicating that human T4+ T cells can also elicit toxicity in tumor-free mice. Similarly, when T1E28z+ T cells were preactivated in vitro and then transferred i.p. (but not i.v.) to tumor-free mice, transient and modest weight loss was observed in the absence of other behavioral alteration (Supplemental Fig. 2).

**Induction of cytokine release syndrome by i.p. T4 immunotherapy**

The rapid onset of death without associated major organ pathology observed in some T4+ T cell–treated mice raised the possibility that cytokine release syndrome was responsible for this toxicity. To investigate further, three groups of tumor-free mice were treated with escalating doses of T4 immunotherapy, administered i.p. Because there was a risk of severe toxicity induction, only two mice were included in each group for ethical reasons (and as specified in the Project License governing this work). Animals that received 3 or 10 million cells exhibited no alteration in behavior or weight during the ensuing 48 h. In contrast, animals that received 30 million cells demonstrated subdued behavior, piloerection, and...
reduced mobility within 24 h, accompanied by rapid weight loss (Fig. 7A). Both animals died within 48 h. Serial blood samples revealed that human IFN-γ, human IL-2, and mouse IL-6 (Fig. 7B) were all detectable in the circulation of mice that had received the lethal dose of T4+ T cells. Significant dose-dependent elevation of other human or mouse cytokines were not detected (from IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TNF-α; data not shown). Animals that received 30 million P4+ or untransduced T cells exhibited no weight or behavioral alteration (data not shown).

To confirm and extend these results, repeat studies were performed in tumor-free mice using different batches of T4+ and control T cells. In light of the emerging clinical evidence implicating IL-6 in CAR T cell–induced cytokine release syndrome, liposomal clodronate was administered first to some mice to deplete macrophages, an important source of this cytokine. In total, five tumor-free mice received 30,000,000 T4+ T cells without prior macrophage depletion. All five animals exhibited rapid weight loss and died or were culled within 48 h (Fig. 7C). Human IFN-γ and mouse IL-6 were detectable in serum of all animals antemortem (Fig. 7D), accompanied by IL-2 in four of five cases. In contrast, mice treated with control P4+ T cells maintained weight, did not exhibit detectable cytokinemia, and did not require culling owing to toxicity. Although macrophage depletion was incomplete (magnitude and validation shown in Supplemental Fig. 3), this was nonetheless sufficient to protect against lethal outcome and weight loss in two of two mice that were treated with 30,000,000 T4+ T cells. Furthermore, serum levels of both human IFN-γ and mouse IL-6 were markedly reduced in these mice, when compared with macrophage-replete SCID beige mice that received an identical dose of T4+ T cells (Fig. 7D).

FIGURE 5. Lethal toxicity induced by T4+ T cells in mice bearing SKOV-3 luc xenografts. Twelve mice with advanced (day 34) i.p. SKOV-3 luc tumors were treated with 10^6 T4+ T cells that had been enriched to purity by culture in IL-4 for 23 d. Control mice received PBS (n = 9). (A) Cell surface expression of T1E28z was demonstrated by flow cytometry (open histogram). Filled histogram indicates untransduced T cells. (B) Serial BLI was performed at the indicated intervals to monitor tumor status (means ± SEM). Timing of T cell administration is indicated by the arrow. Lethal toxicity ensued in 11 of the 12 T4+ T cell–treated mice within 72 h. (C) Imaging on day 42 in the T4 group was performed on the single surviving mouse. Comparison is made with one representative control mouse, imaged before and after administration of T4+ T cells or PBS (all shown on the same scale). (D) Measurement of weight of SKOV-3 luc tumor-bearing mice following T4 immunotherapy. Animals had 18 d established i.p. SKOV-3 luc tumors at the time of treatment with 10^6 T4+ T cells (arrow). Control mice received P4+ T cells or PBS. ***p = 0.007 comparing T4+ T cell–treated mice on day 18 versus day 25.

FIGURE 6. Toxicity induced by i.p. T4 immunotherapy also occurs in tumor-free mice. HN3-luc tumor cells were inoculated i.p. into six SCID beige mice whereas four mice served as tumor-free controls. Two weeks after tumor inoculation, T4+ T cells (either 5 × 10^6 or 10^6 cells) or untransduced T cells (10^6 cells) were administered i.p. (A) Tumor-derived light emission was determined by BLI (means ± SEM). Serial weight (means ± SEM) was measured in tumor-bearing or tumor-free mice treated i.p. with T4+ T cells [5 × 10^6 cells (B) or 10^6 cells (C)] or untransduced T cells (10^6 cells, shown in both panels). Weights of tumor-bearing and tumor-free mice following treatment with either dose of T4+ T cells are not significantly different. *p < 0.05, **p < 0.01 comparing T4+ T cells with untransduced T cells; p < 0.05 comparing the effect of 5 × 10^6 with 10^6 T4+ T cells in tumor-free mice. The latter difference is not significant in tumor-bearing mice.
dose of T4+ T cells (Fig. 7D). Macrophage activation did not result from CAR-mediated recognition because cocultures of T4+ T cells with either human or mouse macrophages did not result in specific induction of human IL-2 or IFN-γ production (data not shown).

Cytokine release is induced by i.p. but not i.t. T4 immunotherapy

Finally, we examined whether route of administration influenced the risk of cytokine release in response to T4 immunotherapy. Equal numbers of T4+ T cells were either administered by i.p. or i.t. injection in mice with established HN3-luc xenografts, growing respectively within the peritoneal cavity or s.c. As before, i.t. injection of T4+ T cells resulted in tumor regression (Fig. 8A, 8B) without induction of weight loss (Fig. 8C). Human cytokines were not detected in the circulation of these mice (Fig. 8D and data not shown). In contrast, i.p. administration of the same number and batch of T4+ T cells led to weight loss (Fig. 6C) accompanied by detectable circulating IFN-γ, peaking 24 h after T cell administration (Fig. 8D). These findings demonstrate that cytokine release syndrome is not required for efficacy of T4 immunotherapy when administered using the i.t. route.
Discussion
The extended ErbB family has attracted great interest as a target for pharmacologic intervention in several tumor types. However, therapeutic resistance to cytostatic ErbB-targeted drugs occurs frequently. A direct approach to achieve cytolysis involves the use of T cells engineered to express an ErbB-specific CAR. We have engineered such a molecule, named T1E28z, which targets several ErbB dimer species that contribute to tumorigenesis (14). When coexpressed with a chimeric cytokine receptor, 4β, T1E28z T cells can be selectively expanded during manufacture using IL-4 (16). The resultant combination, named T4 immunotherapy, exerts antitumor activity in models of several ErbB tumor types (14) (17).

The key concern with potentiated targeting of the ErbB network is the risk of on-target toxicity, owing to the widespread expression of these receptors in healthy tissues. Exemplifying this, mAbs targeted against ErbB1 and ErbB2 have a propensity to cause cutaneous (36) or cardiac (37) toxicity, respectively. Furthermore, i.v. infusion of large numbers of ErbB2-specific CAR+ T cells in a lymphodepleted recipient resulted in a lethal adverse reaction (38) owing to recognition of low levels of target in pulmonary endothelium (39). In that case, death resulted from adult respiratory distress syndrome, multiorgan failure, and cytokine storm. These considerations emphasize the substantial challenge posed by achieving effective and safe targeting of this pivotal receptor family in cancer.

In this study, we have undertaken preclinical safety testing of ErbB-targeted human T4 immunotherapy in the mouse. Critical to the validity of this approach was the demonstration that the human T1E28z CAR could efficiently retarget human T cells against mouse ErbB-expressing cell types. This finding is consistent with several reports indicating that all human ligands exert potent activity on orthologous mouse ErbB receptors. First, human and mouse EGF exert an equimolar capacity to promote eyelid opening in newborn mice (31), augment cytokine production by mouse splenocytes (34), stimulate proliferation of mouse BALB/c 3T3 cells, and inhibit binding of radiolabeled mouse EGF to mouse ErbB1 (34). Second, human TGF-α is highly active in promoting bone resorption in mice (35) and is as active as mouse EGF in accelerating eyelid opening in newborn mice (31). Third, human heregulin (neuregulin)-1B exerts potent therapeutic activity in murine models of Parkinson’s disease (32) and viral myocarditis (33). Furthermore, human CAR+ T cells undergo equally efficient activation by mouse and human target cells that express comparable levels of target Ag (28). In light of these findings, we predicted and show in the present study that the human T1E peptide (a chimera of human EGF and TGF-α) effectively retargets human CAR+ T cells against mouse cells that naturally express ErbB receptors.

Despite these considerations, i.v. injection of large numbers of T4+ T cells caused partial tumor regression without detectable

![Figure 8](http://www.jimmunol.org/DownloadedFrom)
toxicity. One factor that may have contributed to this finding is upregulated expression of EGF receptor by tumor-associated endothelium (40), as this is directly accessible to circulating T4+ T cells. Similarly, i.t. injected T4+ T cells elicited tumor shrinkage without toxicity. In contrast, when T4 immunotherapy was administered i.p., toxicity was reproducibly observed. With modest T cell dosing, tumor regression was accompanied by mild and reversible weight loss. Weight loss was also observed in tumor-free mice, demonstrating the capacity of the human T1E28z CAR to recognize murine ErbB receptors in vivo. However, administration of larger numbers of T4+ T cells to mice with advanced tumor burdens resulted in lethal cytokine release syndrome. This event was triggered within hours of T cell administration, indicated by the presence of both human and murine cytokines in the circulation. We think that this reflects the magnitude of target recognition in tumor deposits and peritumoral cells within the peritoneal cavity, followed by systemic cytokine absorption. Three points are consistent with this hypothesis. First, when human CAR+ T cells are administered i.p., cells remain within the peritoneal cavity, without detectable relocation to other anatomic sites (27). Second, ErbB1 is expressed by normal mesothelial cells (41), providing a potential source of stimulation to T4+ T cells in the peritoneal cavity. Third, we have obtained preliminary evidence that both efficacy and toxicity of T4 immunotherapy are increased when cells are delivered in larger volumes to the peritoneal cavity (data not shown). The resultant increase in i.p. pressure may increase absorption of small molecules such as cytokines, and it exerts proinflammatory effects leading to increased production of IL-6 and other cytokines (42).

Studies in humans using CAR-engineered T cells have implicated IL-6 as a key effector of toxicity, a finding that may be reversed with the anti–IL-6 receptor Ab tocilizumab (43). Because tocilizumab has no effect on the mouse IL-6 receptor, we are reversed with the anti–IL-6 receptor Ab tocilizumab (43). Because tocilizumab has no effect on the mouse IL-6 receptor, we are trying to source a suitable blocking Ab to evaluate the role played by this cytokine in the toxicity described in this study. The role of mouse IL-6 in mediating this toxicity is also supported by the mitigating effect of prior macrophage depletion on cytokine release syndrome induced by T4+ T cells. Importantly, note in this regard that macrophage function is normal in SCID beige mice (27). To test this, we are about to undertake a phase 1 study in which autologous T4+ T cells are administered by this route in patients with locally advanced or recurrent HNSCC (ClinicalTrials.gov no. NCT01813823).

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Disclosures

The authors have no financial conflicts of interest.

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SAFETY TESTING OF ErbB-RETARGETED T CELLS

In press.
Supplemental Figure 1. ErbB receptor expression in healthy organs in adult SCID Beige mice.
A representative flow cytometric analysis is shown in which ErbB expression is indicated by the open histograms. Closed histograms indicate non-specific staining obtained using control serum from non-immunized rabbits. Data show the mean ± SD of percentage positivity (n=4 independent analyses, except intestine where n=1).
Supplemental Figure 2. Effects of T1E28z-transduced T-cells upon weight of SCID Beige mice.

(A) Flow cytometric analysis to demonstrate expression of T1E28z in retrovirus-transduced human T-cells (open histogram). Non-specific staining of untransduced T-cells is indicated by the filled histogram. Analysis was performed immediately prior to (B) adoptive transfer into SCID Beige mice. T-cell dose and route of administration are indicated. Phosphate buffered saline (PBS) served as negative control. T1E28z+ T-cells were propagated in IL-2 alone and, where indicated, were pre-activated by stimulation with HN3 HNSCC tumor cells followed by expansion for 1 further week with IL-2. After T-cell infusion, animals were monitored for behavior and weight at the indicated intervals.
Supplemental Figure 3. Peritoneal macrophages in healthy and tumor engrafted mice and their depletion using liposomal clodronate.

(A) SCID Beige mice with 18 day established i.p. SKOV-3 luc tumors were compared to tumor-free control mice for total number of peritoneal macrophages. Peritoneal macrophages (shown on a log scale) were enumerated as the product of total viable cell number multiplied by percentage F4/80+CD45+ cells, as determined by flow cytometry (mean ± SEM, n=3). (B) To deplete peritoneal macrophages, two healthy control mice received liposomal clodronate, administered in divided doses separated by 3 days. One day later, peritoneal macrophages were enumerated as above (n=2, mean ± SEM). One control mouse received liposomal PBS, indicating that background number of peritoneal macrophages is not altered by empty liposomes. (C) Liposomal clodronate was further validated for its ability to deplete peritoneal macrophages in mice with advanced SKOV-3 luc tumors. Control mice received liposomal PBS. Peritoneal macrophages were enumerated as above one day after completion of treatment (n=3, mean ± SEM).
**Supplemental Table 1.** Histologic assessment of mice treated with T4 immunotherapy. Two groups of six mice (each comprising 3 male and 3 female mice) with 15 day established HN3-luc tumors received $2 \times 10^7$ T4$^+$ T-cells, administered using either the i.v. or intratumoral (IT) routes. After 7 days, animals were culled and all major organs were fixed and transported for independent histopathologic analysis. Similar findings were observed in all animals. Pulmonary collapse was considered to be an artifact since cadavers were not pre-fixed by perfusion with formalin.

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