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Synergistic Chemoimmunotherapy of Epithelial Ovarian Cancer Using ErbB- Retargeted T Cells Combined with Carboplatin

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Epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy, underscoring the need for better therapies. Adoptive immunotherapy using genetically targeted T cells represents a promising new treatment for hematologic malignancies. However, solid tumors impose additional obstacles, including the lack of suitable targets for safe systemic therapy and the need to achieve effective T cell homing to sites of disease. Because EOC undergoes transcelomic metastasis, both of these challenges may be circumvented by T cell administration to the peritoneal cavity. In this study, we describe such an immunotherapeutic approach for EOC, in which human T cells were targeted against the extended ErbB family, using a chimeric Ag receptor named T1E28z. T1E28z was coexpressed with a chimeric cytokine receptor named 4εβ (combination termed T4), enabling the selective ex vivo expansion of engineered T cells using IL-4. Unlike control T cells, T4+ T cells from healthy donors and patients with EOC were activated by and destroyed ErbB+ EOC tumor cell lines and autologous tumor cultures. In vivo antitumor activity was demonstrated in mice bearing established luciferase-expressing SKOV-3 EOC xenografts. Tumor regression was accompanied by mild toxicity, manifested by weight loss. Although efficacy was transient, therapeutic response could be prolonged by repeated T cell administration. Furthermore, prior treatment with noncytotoxic doses of carboplatin sensitized SKOV-3 tumors to T4 immunotherapy, promoting enhanced disease regression using lower doses of T4+ T cells. By combining these approaches, we demonstrate that repeated administration of carboplatin followed by T4+ T cells achieved optimum therapeutic benefit in the absence of significant toxicity, even in mice with advanced tumor burdens. The Journal of Immunology, 2013, 191: 2437–2445.
of targeted or nontargeted ErbB receptor types (22–26) and/or increased ligand production or release (27). ErbB receptors may also be transactivated by other active receptor systems, including tyrosine kinases (e.g., MET) or G protein–coupled receptors (13, 28). The dysregulation of expression, pathogenetically relevant function, and flexibility of signaling by the ErbB network in EOC provides a rationale for targeting of multiple component dimers using a directly cytolytic approach. However, questions remain about how this may be achieved safely. We have previously developed a chimeric Ag receptor (CAR) named T1E28z that can target T cell specificity against the extended ErbB family (29). Target recognition by this CAR exploits the natural promiscuity of ErbB dimer binding by the T1E peptide—itself a chimera derived from TGF-α and epidermal growth factor (EGF) (30). T cells engineered to express the T1E28z CAR undergo activation by target cells that express ErbB1- and ErbB4-based dimers and the potentially 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 4αβ. In 4αβ, the ectodomain of the IL-4Rα subunit has been joined to the transmembrane and endodomain of the shared β-chain used by IL-2R and IL-15R. Consequently, IL-4 exerts dual effects upon T cells that express 4αβ. Signaling via the endogenous IL-4Rα is maintained, whereas signals delivered through 4αβ convert the weak mitogenic action of IL-4 into a potent growth signal, favoring selective growth of the gene-modified cells (31). Following expansion using IL-4, T cells that coexpress T1E28z and 4αβ exhibit polyfunctionality of cytokine production (with a Th1 bias) and mediate effective antitumor activity against head and neck or breast cancer, both in vitro and in vivo (29).

The hypothesis underlying the current study was that T4+ T cells could be harnessed to elicit antitumor immunity in EOC. In this study, we demonstrate proof of concept in support of this using patient-derived T cells, targeted against their own tumor. We then tested whether efficacy of T4 immunotherapy could be enhanced by two ancillary approaches, namely macrophage depletion [neutralizing the supportive role of macrophages in EOC (32–34)] and prior carboplatin chemotherapy. Optimum therapeutic benefit was achieved by exploiting a synergistic interaction between metronomically delivered carboplatin followed by ErbB-targeted T cells. Materials and Methods

Recombinant DNA constructs

The T1E28z (29) and P28z CARs (35) and 4αβ chimeric cytokine receptor (31) have been previously described. T4 refers to the equimolar coexpression of 4αβ and T1E28z using an intervening furin cleavage site, followed by Thosea Asixg A2A (T2A) peptide (29). To construct the P4 vector, the 4αβ-furin-T2A encoding Nco1 fragment was excised from T4 and ligated into the Nco1 site in the P28z plasmid. Recombinant transgenes were expressed using the SPG retrovector (vector 36).

Culture and retroviral transduction of primary human T cells

Blood and tumor samples were obtained under approval of the West London Research Ethics Committee (reference 08/H0707/188; EOC patients) and South East London Research Ethics Committee 1 (reference 09/H0804/02; healthy volunteers). 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.). Gene transfer was performed using PG13 retroviral packaging cells, as described (29).

Tumor cell culture

The firefly luciferase (lac)-expressing SKOV-3 luc tumor cells (SKOV-3 luc) was purchased from Caliper (PerkinElmer, Waltham, MA). The IGROV-1 luc cell line was provided by I. McNeish (Institute of Cancer Sciences, University of Glasgow, U.K.) (37). Cell lines were grown in D10 medium (e.g., DMEM [Lonza, Basel, Switzerland] supplemented with 10% FBS (Sigma-Aldrich, Poole, U.K.)), GlutaMax, and antibiotic–antimycotic solution (Life Technologies).

Primary EOC tumor cells were isolated from ascites by gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Chalfont St. Giles, U.K.). Solid tumors were either mechanically disaggregated or separated into a single-cell suspension with the Human Tumor Dissociation Kit (Miltenyi Biotec, Bisley, U.K.), used with the Gentle MACS Dissociator (Miltenyi Biotec), according to manufacturer’s instructions. Tumor cells were placed in mammary epithelial basal medium (Lonza) containing 5 μg/ml insulin (Actrapid; Novo Nordisk, Crawley, U.K.), 20 ng/ml human EGF (Life Technologies), 10 ng/ml basic fibroblast growth factor (Life Technologies), and 0.4% FBS (Sigma-Aldrich) and cultured in Ultra Low Attachment plates (Corning, distributed by Sigma-Aldrich) for 15 d.

Flow cytometry analysis

Primary tumor material was prepared for flow cytometry, as above. Tumorspheres were prepared by trypsinization and vigorous pipetting to generate a single-cell suspension. During flow cytometric analysis of tumor material, forward scatter/side scatter gates were used to identify tumor cell populations wherever possible. Expression of ErbB1-4 was detected using ICR2, ICR12 (both from Institute of Cancer Research, Surrey, U.K.), clone H3.105.5, or clone H4.77.16 (both from Neomarkers, Fremont, CA), respectively, followed by appropriate secondary antibodies. Alternatively, detection was performed using anti-ErbB1 FITC (FAB10951F), anti-ErbB2 allophycocyanin (FAB1129A), anti-ErbB3 PerCP (FAB3480C), or anti-ErbB4 allophycocyanin (FAB11311A; all from R&D Systems, Minneapolis, MN). Expression of the T1E28z CAR was detected using mouse anti-human EGF mAb (clone 10825; R&D Systems), followed by PE-conjugated goat anti-mouse Ig (Dako, Ely, U.K.) or goat anti-EGF polyclonal antiserum (R&D Systems), followed by rabbit anti-goat IgG FITC-conjugated secondary Ab (Dako). Expression of P28z was detected, as described (34). Flow cytometry was performed using a FACSCalibur cytometer with CellQuest Pro software or a Coulter EPICS XL cytometer with Expt2 ADC software.

Protein analysis

Supernatants were analyzed using an human IFN-γ ELISA Ready-Set-Go kit (eBiosciences, Hatfield, U.K.), as described by the manufacturers.

Cytotoxicity assays

Destruction of tumor cell monolayers by T cells was quantified using a MTT (thiazolyl blue) assay. Confluent tumor cell monolayers (24-well plate) were incubated overnight with 1 × 10⁷ T cells and then washed with PBS to remove T cells. MTT (500 μg/ml) in D10 medium was added for 2–4 h at 37 C and 5% CO₂. After removal of the supernatant, formazan crystals were resuspended in 300 μl DMSO. Absorbance was measured at 570 nm. Tumor cell viability was calculated as follows: (absorbance of monolayer cultured with T cells/absorbance of untreated monolayer alone) × 100%. Alternatively, tumor monolayer destruction was visualized by non-quantitative crystal violet staining of residual monolayers, as described (31). In these assays, 1 × 10⁶ engineered T cells were cocultivated overnight with a confluent monolayer of tumor cells in a 24-well plate.

In a third approach, 1 × 10⁷ tumorspheres were cocultivated overnight with 1 × 10⁶ T cells. Residual tumorspheres were enumerated by trypsin exclusion.

In vivo studies

All in vivo experimentation adhered to current United Kingdom Home Office guidelines, as specified in project license 70/6832. Firefly luciferase-expressing SKOV-3 luc tumor cells (1 × 10⁶ cells) were inoculated i.p. into SCID Beige mice. After d. 5, tumor burden was confirmed by bioluminescence imaging (BLI). In all experiments, mice were sorted into groups with similar mean BLI signal intensity.

To deplete macrophages, mice received a weekly i.p. dosage of 650 μg liposomal clodronate. Control mice received i.p. liposomal PBS (liposomes provided by N. van Rooijen, Foundation Clodronate Liposomes, Haarlem, The Netherlands). To confirm efficacy of macrophage depletion, 1 × 10³ i.p. Lavage was performed using 5 ml PBS. After treatment, blood was sampled from the tail, and blood lysis solution (Miltenyi Biotec), cells were pelleted, enumerated by trypan exclusion, and stained with F4/80 (clone BMS; Life Technologies) and CD45 (clone 30-F11; Life Technologies) prior to flow cytometry.

Engineered T cells were administered i.p. on the indicated days at doses of either 2.5 × 10⁷ or 1 × 10⁸ cells, as specified in individual experiments. Where indicated, carboplatin (Hospira, Lake Forest, IL) was administered i.p. at a dose of 45 mg/kg, which constitutes a moderate dose in mice, well below the maximum tolerated dose (38). BLI was performed using an IVIS Spectrum Imaging platform (PerkinElmer, Waltham, MA) with Living
Image software (PerkinElmer). Mice were injected i.p. with p-luciferin (150 mg/kg; PerkinElmer) and imaged under isoflurane anesthesia after 12 min. Image acquisition was conducted on a 15- or 25-cm field of view with small/medium binning and autoexposure. Animals were inspected daily and weighed weekly. Mice were culled when experimental endpoints had been achieved or, if symptomatic, as a result of tumor progression (whichever sooner).

Statistical analysis

For comparison of two groups, datasets were analyzed using Excel within Microsoft Office for Mac 2008 (Microsoft, Redmond, WA) using one-tailed Student t test.

Results

Expression of ErbB receptors in ovarian cancer and derived cell lines/tumorspheres

To test antitumor activity of ErbB-retargeted T cells against EOC, paired blood and tumor samples were obtained from patients who required debulking surgery. Expression of ErbB family members proved heterogeneous in freshly isolated tumor material, both in

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** ErbB receptor expression in epithelial ovarian cancer and derived cultures/cell lines. (A) Ovarian tumors were analyzed by flow cytometry for expression of the indicated ErbB receptors, either using two-step immunofluorescence staining (upper four samples) or using directly conjugated ErbB-specific Abs (lower six samples). Gating was performed using forward/side scatter dot plots on the tumor cell population, avoiding mononuclear leukocyte populations where present. Filled (control) histograms show staining with secondary Ab alone or isotype control as appropriate. ND, Not done. (B) Tumor cells were cultured for 2 wk in low-adherence serum-deprived conditions to generate tumorspheres. After pipetting in trypsin to generate a single-cell suspension, samples were stained with directly conjugated ErbB-specific Abs and analyzed by flow cytometry. Neither ErbB3 nor ErbB4 could be detected using this approach. (C) IGROV-1 and SKOV-3 luc cells were analyzed by flow cytometry for expression of the indicated ErbB receptors after two-step immunofluorescence staining. Filled (control) histograms show staining with secondary Ab alone.

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Coexpression of 4αβ and CAR in human T cells. (A) In 4αβ, the ectodomain of human IL-4Rα was fused to the transmembrane and endodomain of βc. IL-4–mediated pairing of 4αβ with γc is shown. The SFG retroviral vector was used to coexpress 4αβ with either T1E28z—a broadly reactive ErbB-specific CAR—or a matched control CAR named P28z, targeted against prostate-specific membrane Ag using the J591 scFv. Stoichiometric transgene coexpression using the resultant T4 or P4 vectors was achieved with an intervening Thosea Asigna 2A peptide, placed downstream of a furin cleavage site. (B) T cells from 17 preoperative patients with active EOC were transduced with the T4 vector and then cultured in IL-4 for 11 d. Expression of the T1E28z CAR was determined by flow cytometry after incubation with a monoclonal or polyclonal anti-EGF Ab. IL-4–driven enrichment of T4+ T cells within the cultures is indicated (**p < 0.01, comparing percentage of T1E28z+ T cells present on days 4 and 11). The arrowed sample indicates the single patient who had prior chemotherapy. Similar IL-4–mediated enrichment of P4+ T cells was also observed (data not shown). (C) A representative example of IL-4–mediated enrichment of T4+ and P4+ T cells. Flow cytometric analysis was performed 4 and 11 d after gene transfer.
terms of the pattern and intensity of receptor expression detected. Although this analysis does not discriminate between tumor and stromal cells, one or more ErbB receptors were nonetheless detectable in the majority of isolated samples (Fig. 1A). ErbB expression could also be demonstrated in cultured tumorsphere samples, although levels were low. Detection may have been compromised by the need for prior trypsinization of spheres to generate single cell suspensions (Fig. 1B).

To provide positive control material for T cell/tumor cell cocultivation experiments, IGROV-1 and SKOV-3 luc EOC tumor cells were selected. As expected, coexpression of ErbB1 and ErbB2 was found in IGROV-1 and in SKOV-3 luc cells (which are known to harbor an amplified ErbB2 gene; Fig. 1C).

Efficient transduction and expansion of CAR-engineered T cells from patients with epithelial ovarian cancer
To target ErbB dimers expressed in EOC, T cells were engineered to express the T1E28z CAR (Fig. 2A). This fusion receptor engages several ErbB dimers that are commonly upregulated in EOC, notably ErbB1 homo- and heterodimers, ErbB2/3 heterodimers, and ErbB4-based dimers (29). The T1E28z CAR was coexpressed with a chimeric cytokine receptor named 4αβ (Fig. 2A), which binds the poorly mitogenic cytokine IL-4, converting this signal into a potent and selective growth signal in gene-modified T cells (31). The combination of T1E28z and 4αβ is termed T4. Control T cells were engineered to express P4, comprising 4αβ and the P28z CAR. P28z is specific for prostate-specific membrane Ag (35), a target that is not expressed on IGROV-1 or SKOV-3 tumor cells (data not shown). Successful transduction with the T4 vector was achieved in T cells from 16 of 17 patients with EOC, including one patient who had prior chemotherapy (arrowed; Fig. 2B). Gene-modified T cells were cultured in IL-4, leading to an increase in the proportion of transduced cells from 35.4 ± 19.7% (measured 4 d after gene transfer) to 55.2 ± 21.2% (measured 11 d after gene transfer, p = 0.0037; Fig. 2B). Ratio of CD4/CD8 cells in transduced cell products from patients was variable (CD4, 27.7 ± 29.8%; CD8, 65.0 ± 27.8%, mean ± SD, n = 3). A typical example of such

FIGURE 3. In vitro assessment of antitumor activity of patient-derived T4+ T cells against ovarian tumor cells. T cells from patients with EOC were transduced with the T4 or P4 vectors and cultured in IL-4 for 11 d. Untransduced (Untrans.) T cells were expanded using IL-2 in parallel. (A) A total of 1 × 10^6 of each T cell population was cocultivated in a 24-well plate with a confluent monolayer of autologous tumor, IGROV-1, or SKOV-3 luc cells. Supernatants were harvested after 24 h and analyzed in triplicate for IFN-γ content (mean ± SEM, n = 5). (B) T cells (1 × 10^6 engineered or untransduced cells) were assayed for cytotoxic activity against confluent IGROV-1 and SKOV-3 luc tumor monolayers (24-well plate) in an overnight assay (quantified using MTT). Data show mean ± SEM of residual tumor cell viability from two independent experiments using separate EOC patient samples. (C) A total of 1 × 10^6 of each T cell population was cocultivated overnight in a 24-well plate with a confluent monolayer of autologous tumor, IGROV-1, or SKOV-3 luc cells. T cells were removed by gentle rinsing, and residual monolayers were fixed and stained with crystal violet. Data are representative of five independent replicate experiments. (D) EpCAM+ tumorspheres were propagated from four separate EOC tumors, whereas, in parallel, autologous T cells were transduced with T4 or P4 vectors and expanded in IL-4. Overnight cocultures were then established in which 1 × 10^5 T cells were incubated with 1 × 10^4 tumorspheres (average size, 100 tumor cells). Supernatant was analyzed for IFN-γ content (mean ± SEM, n = 4). **p < 0.01; NS, comparing T4 versus other groups. (E) Images of cocultures after overnight incubation, representative of four experiments. (F) Residual tumorspheres were enumerated by trypan exclusion (mean ± SEM, n = 4). **p < 0.01 (comparing T4 versus other groups).
IL-4–mediated enrichment of T4+ and P4+ T cells is presented in Fig. 2C.

In vitro testing of T4 immunotherapy of ovarian cancer

To evaluate antitumor activity in vitro, T4+, control P4+, or untransduced T cells from EOC patients were cocultivated with autologous tumor monolayers, IGROV-1 cells, SKOV-3 luc cells (Fig. 3A–C), or autologous tumorspheres (Fig. 3D–F). Selective activation of T4+ but not control T cells was observed in all cases. This was indicated by target cell–dependent production of IFN-γ (Fig. 3A, 3D) and killing of autologous or immortalized EOC tumor monolayers (Fig. 3B, 3C) and autologous tumorspheres (Fig. 3E, 3F).

In vivo testing of T4 immunotherapy of ovarian cancer

Ovarian cancer tends to remain confined to the peritoneal cavity, providing a rationale for regional delivery of therapeutic agents. To model established EOC in vivo, SCID Beige mice were inoculated i.p. with SKOV-3 luc cells. Tumor formation was typified by the growth of three or more i.p. solid tumors, accompanied by ascites containing large numbers of F4/80+ macrophages (Supplemental Fig. 1A). Tumors were allowed to progress until day 18, by which time discrete foci could be visualized by BLI. In light of the supportive role of macrophage infiltration in EOC progression (32–34), T4 immunotherapy was first tested alone or following macrophage depletion, achieved using liposomal clodronate. Experimental design is summarized in Fig. 4A. In pilot studies, we showed that weekly treatment with liposomal clodronate had no effect on the growth of SKOV-3 luc xenografts (data not shown). Engineered T cells were expanded using IL-4 (Supplemental Fig. 1C), and CAR expression was confirmed by flow cytometry (Fig. 4B) prior to i.p. injection of 1 × 10⁷ T cells into tumor-bearing mice. At the time of adoptive transfer, T4+ but not control P4+ T cells demonstrated antitumor activity in vitro in monolayer destruction assays (Supplemental Fig. 1D). All mice treated with T4+ T cells exhibited rapid tumor regression, followed by progression of disease (Fig. 4C, 4D). Efficiency of macrophage depletion was confirmed by flow cytometry (Supplemental Fig. 1A, 1B). However, this intervention did not improve efficacy of T4 immunotherapy. Indeed, a trend toward poorer tumor control was observed in macrophage-depleted mice, although the study was underpowered to detect significance (note day 32 images in Fig. 4D).

Repeated treatment improves tumor control using T4 immunotherapy

Next, we tested whether repeated administration of 1 × 10⁷ T4+ T cells would improve therapeutic efficacy. Mice with 18-d–established

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**FIGURE 4.** In vivo testing of T4 immunotherapy in mice with SKOV-3 luc xenografts, alone or combined with macrophage depletion. (A) Summary of experimental design. SKOV-3 luc tumors were established for 18 d before administration of 1 × 10⁷ T4+ or P4+ T cells or PBS to six mice each. Half of the mice in each group received weekly liposomal clodronate (LC) to deplete macrophages, whereas the remaining three mice received liposomal PBS (LP) as control. BLI was performed at the indicated time points. (B) CAR expression (open histograms) and CD4/CD8 ratio of T4+ and control P4+ transduced T cells at the time of treatment of mice. Closed histograms show staining of untransduced T cells with the same CAR detection reagents. (C) Serial BLI of mice before and after T cell administration. Data show mean ± SEM of three mice in each of six groups. ***p < 0.001 comparing LP + T4+ T cells versus LP + P4+ T cells on day 25; **p < 0.01 comparing LC + T4+ T cells versus LC plus P4+ T cells on day 25. (D) BLI images of all mice before and after T cell injection, shown on the same scale.
SKOV-3 luc EOC xenografts received T4⁺, control P4⁺ T cells, or PBS. A similar dose of T cells (derived from the same culture) was administered after a further week (Fig. 5A). As before, engineered T cells had been expanded using IL-4 (Supplemental Fig. 2A), and CAR expression was confirmed by flow cytometry (Fig. 5B) prior to adoptive transfer. Functional competence of T4⁺ T cells was confirmed at the time of administration using in vitro ovarian tumor monolayer destruction (Supplemental Fig. 2B) and IFN-γ production assays (Supplemental Fig. 2C).

Fig. 5C demonstrates that two sequential treatments with T4⁺ T cells achieve superior tumor control, compared with a single treatment. Differences in tumor burden between single- and double-treated mice reached statistical significance on day 46. Images of individual mice before and after single and double treatment with T4⁺ T cells are shown in Fig. 5D.

Sensitizing effect of carboplatin chemotherapy upon T4 immunotherapy

In an alternative approach, we explored whether pretreatment with carboplatin would enhance antitumor activity of IL-4–expanded T4⁺ T cells against SKOV-3 luc tumor xenografts (Fig. 6A). Prior to adoptive transfer, CAR expression (Fig. 6B) and function of T4⁺ T cells were validated in vitro by measurement of tumor cell killing (Supplemental Fig. 3A) and target-dependent IFN-γ production (Supplemental Fig. 3B). Eighteen days after tumor cell implantation, carboplatin was administered to mice in relevant groups. Two days later (day 20), mice received a lowered dose of 2.5 × 10⁶ T4⁺ or P4⁺ T cells i.p. (or PBS as additional control). Fig. 6C shows that carboplatin alone exerted no effect on tumor growth, whereas administration of low doses of T4⁺ T cells caused tumor regression, followed by progression. Notably, however, the sequential use of carboplatin, followed by T4⁺ T cells, led to a significant enhancement of antitumor activity, compared with T4⁺ T cells alone or carboplatin followed by P4⁺ T cells. BLI of mice before and after T cell injections is shown in Fig. 6D.

Combination therapy of EOC with metronomic carboplatin followed by T4 immunotherapy

Next, we evaluated whether repeated (metronomic) administration of carboplatin followed by T4 immunotherapy would lead to improved tumor control in this model (Fig. 7A). To provide a more stringent test of efficacy, tumors were allowed to progress to day

FIGURE 5. In vivo testing of repeat administration of T4 immunotherapy to mice with SKOV-3 luc xenografts. (A) Summary of experimental design. SKOV-3 luc tumors were established for 18 d before treatments were initiated. Mice were divided into five groups of four mice each, which were treated with 1 × 10⁷ T4⁺ T cells, 1 × 10⁷ P4⁺ T cells, or PBS. The final two groups received two sequential doses of 1 × 10⁷ T4⁺ T cells or P4⁺ T cells on days 18 and 25. (B) CAR expression (open histograms) and CD4/CD8 ratio of T4⁺ and control P4⁺ transduced T cells at the time of treatment of mice on day 18. Closed histograms show staining of untransduced T cells with the same CAR detection reagents. (C) Serial BLI of mice before and after T cell administration (arrowed). Data show mean ± SEM of four mice in each of five groups. ****p = 0.00013, comparing mice that received one dose of T4⁺ T cells or P4⁺ T cells on day 25 (n = 8 mice each); *p = 0.012, comparing mice that received a single versus two sequential treatments with T4⁺ T cells on day 46 (n = 4 mice each). (D) BLI images of all mice before and after T cell injections, shown on the same scale.
FIGURE 6. Carboplatin sensitizes SKOV-3 luc tumor xenografts to T4 immunotherapy. (A) Summary of experimental design. SKOV-3 luc tumors were established for 18 d before i.p. treatments were initiated, as follows. Mice were divided into five groups of four to five mice each, which were treated with the following: 1) carboplatin (45 mg/kg) followed on day 20 by 2.5 × 10⁶ T4⁺ T cells; 2) carboplatin followed by 2.5 × 10⁶ P4⁺ T cells; 3) carboplatin followed by PBS; 4) PBS alone on day 20; or 5) 2.5 × 10⁶ T4⁺ T cells on day 20. (B) Expression of T1E28z and P28z in T4⁺ and control P4⁺ T cells at the time of treatment of mice on day 20 (open histograms). Closed histograms show staining of untransduced T cells. (C) Serial BLI of mice before and after T cell administration (arrowed), making comparison with background light emission detected from a tumor-free mouse. Data show mean ± SEM of four to five mice in each of five groups. When comparing carboplatin followed by T4⁺ T cells versus T4⁺ T cells alone, *p = 0.026 (day 25), ***p = 0.002 (day 32), **p = 0.004 (day 39). (D) BLI images of all mice before and after T cell injection, shown on the same scale.

25. Animals then received carboplatin followed, on day 27, by low-dose immunotherapy with 2.5 × 10⁶ T4⁺ T cells. The same treatments were repeated on day 32 (carboplatin) and day 34 (T4⁺ T cells, from the same culture). Control mice received either PBS or two treatments with T4⁺ T cells, administered on days 27 and 34. Following expansion in IL-4, CAR expression by T4⁺ T cells is confirmed in vitro using assays of tumor monolayer destruction (MTT assay) and cytokine production (data not shown). BLI of mice before and after T cell injections is shown in Fig. 7C. Fig. 7D shows that mice that received two treatments with metronomic carboplatin followed by T4 immunotherapy had significantly improved tumor control compared with mice that received T4 immunotherapy alone. When analyzed on day 39 (1 wk after the second T cell treatment), three of five mice that received carboplatin and T4 immunotherapy had BLI signal emission levels below that of a tumor-free mouse (Supplemental Fig. 4). Mild and reversible weight loss was the only toxicity observed in mice treated with T4 immunotherapy alone (4.8 ± 7.1%, mean ± SD, n = 21), or combined with carboplatin (12.7 ± 4.1%, mean ± SD, n = 9).

Discussion

Epithelial ovarian cancer represents an attractive model for adoptive T cell immunotherapy owing to the compartmentalized nature of the disease within the peritoneal cavity. In this study, we describe a regionally delivered experimental therapy for EOC that exploits the prevalence of ErbB dysfunction in this disease. T cells were engineered using the T4 retroviral vector to coexpress an IL-4–regulated chimeric cytokine receptor (4αβ), together with a CAR (T1E28z) targeted against several ErbB dimers. After ex vivo expansion using IL-4, T4⁺ T cells from healthy donors and patients with operable EOC exhibited robust antitumor activity against immortalized ovarian tumor cell lines and autologous tumor cell cultures. Therapeutic activity was further confirmed using a series of in vivo experiments in which T4⁺ T cells were administered to mice bearing advanced SKOV-3 luc ovarian tumor xenografts.

An important advantage of the system described in this work is that it targets a repertoire of Ags comprising multiple pathogenetically relevant signaling units. This differs from the traditional strategy in which CARs are targeted against a single molecular species that often plays a marginal role in disease evolution. A limitation of the latter approach is the risk of immune evasion through selection for Ag loss, as has recently been demonstrated when CD19-targeted CAR T cells were used to treat acute lymphocytic leukemia (39).

Although brisk and substantial tumor regressions were achieved using this approach, complete tumor elimination was not observed. We have described similar findings previously in models of head and neck or breast cancer (29). Rapid tumor shrinkage followed by progressive regrowth raises the possibility that poor T cell persistence was responsible for this finding. Alternative possibilities include viral promoter silencing or effects of Ag downregulation, internalization, or shedding. In keeping with the former possibility, we have found that T cells coexpressing T1E28z and renilla luciferase undergo exponential loss following i.p. injection in mice bearing SKOV-3 luc xenografts (L. Whilding, A.C. Parente-Pereira, S. Ghaem-Maghami, and J. Maher, unpublished observations). To address this limitation, repeated administration of T4⁺ T cells was performed and achieved greater and more sustained tumor control. We have not observed Ag loss in vivo to account for limited therapeutic activity of ErbB-retargeted T cells.

Although the human T1E28z CAR can engage murine ErbB receptors, immunotherapy using T4⁺ T cells was well tolerated. We have recently undertaken a comprehensive assessment of
toxicity of human T4+ T cells in SCID Beige mice. These studies demonstrate that administration of therapeutic doses of these cells is well tolerated. However, if larger doses are administered i.p. to tumor-bearing or tumor-free mice, profound toxicity is induced owing to cytokine release syndrome (S.J.C. van der Stegen, D.M. Davies, S. Wilkie, J. Foster, J.K. Sosabowski, J. Burnet, L.M. Whilding, R.M. Petrovic, S. Ghaem-Maghami, S. Mather, J.P. Jeannon, A.C. Parente-Pereira, and J. Maher, submitted for publication). Previously, we have shown that i.p. injected T cells remain largely restricted to the peritoneal cavity for at least 48 h (40). Consequently, such T4+ T cell–induced toxicity is most likely an on-target effect induced by activation of these cells in the peritoneal cavity, followed by systemic cytokine absorption.

The findings described above indicate that there is a therapeutic window that may be exploited clinically using ErbB-retargeted T cells. To expand this, we explored a number of strategies that would allow us to use a smaller dose of T4+ T cells. Despite the species barrier, murine macrophages are known to promote the sustained growth of human ovarian tumor cells (32). Furthermore, murine macrophages facilitate engraftment and metastasis of SKOV-3 and other ovarian tumors in SCID Beige and other immune compromised mice, owing to production of matrix metalloproteinases and enhanced angiogenesis (33, 34). However, depletion of macrophages by repeated administration of liposomal clodronate did not exert antitumor activity in this model nor did it lead to improved efficacy of T4 immunotherapy.

In an alternative approach, we examined whether carboplatin could be used to enhance the sensitivity of ovarian tumor cells to T4 immunotherapy. Carboplatin is a chemotherapeutic agent that is intrinsically resistant to the cytotoxic action of this agent (41). Consistent with this, we observed no tumor regression when carboplatin alone was administered to tumor-bearing mice. By contrast, pretreatment with a moderate dose of this agent (45 mg/kg) enhanced tumor regression induced by a relatively modest therapeutic dose of 2.5 million T4+ T cells. When two weekly cycles of carboplatin followed by T4 immunotherapy were administered, three of five mice achieved an undetectable tumor burden, having initiated therapy with advanced (25-d–established) tumors. We are currently exploring the mechanisms underlying this effect and whether further cycles of therapy can achieve tumor eradication in this model. Studies of this approach in immune competent models are also planned, in light of the known immunomodulatory effects of carboplatin (42, 43). In addition, recent data suggest that CAR-engineered T cells may enhance endogenous antitumor immunity against EOC, further emphasizing the need for such studies (44).

In conclusion, the data presented in this work provide support for an immunotherapeutic strategy for EOC in which ErbB-targeted T cells are administered to the peritoneal cavity. Repeated administration of low doses of T cells following carboplatin sensitization offers an opportunity to maximize efficacy and safety of this approach.

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Disclosures
The authors have no financial conflicts of interest.
Supplemental Figure 1. Additional data pertaining to in-vivo experiment 1.
In this experiment, efficacy of T4 immunotherapy was evaluated in mice that underwent macrophage depletion with weekly injections of liposomal clodronate, making comparison with mice that received liposomal PBS as control. (A) F4/80⁺ macrophage number was determined in mice bearing 46 day established SKOV-3 xenografts, following weekly treatment from day 4 with either liposomal clodronate or liposomal PBS. Average number present in tumor-free mice is indicated by the dotted line. (B) Gating strategy used to identify F4/80⁺ macrophages. (C) Ex-vivo expansion of T-cells following transduction with T4 and P4 vectors and culture in IL-4. (D) Overnight monolayer destruction assays using 1 × 10⁶ T4⁺ and indicated control T-cell populations. Assays were conducted overnight in 24 well plates containing confluent monolayers of the indicated EOC tumor cell lines. Residual monolayers were washed, fixed and stained with crystal violet. Untrans. - untransduced.
Supplemental Figure 2. Additional data pertaining to in-vivo experiment 2.

In this experiment, efficacy of repeated T4 immunotherapy was evaluated. (A) Ex-vivo expansion of T-cells following transduction with T4 and P4 vectors and culture in IL-4. (B) Overnight monolayer destruction assays using $1 \times 10^6$ T4+ T-cells and indicated control T-cell populations. Assays were conducted overnight in 24 well plates containing confluent monolayers of the indicated EOC tumor cell lines. Residual monolayers were washed, fixed and stained with crystal violet. (C) Production of IFN-γ by T4+ and control T-cells following activation on monolayers, as described in B. Untrans. - untransduced.
Supplemental Figure 3. Additional data pertaining to *in-vivo* experiment 3.

In this experiment, efficacy of T4 immunotherapy was evaluated following pre-administration of carboplatin. (A) An overnight monolayer destruction assay was performed in which using $1 \times 10^6$ T4⁺ and indicated control T-cell populations were incubated with a confluent SKOV-3 luc monolayer in a 24 well plate. After gentle washing to remove T-cells, residual monolayers were treated with MTT and residual tumor cell viability determined. A viability setting of 100% corresponds to a confluent SKOV-3 luc tumor cell monolayer. (B) Production of IFN-γ by T4⁺ and control T-cells cultured on SKOV-3 luc monolayers (as described in A) or in medium alone. Untrans. - untransduced.
Supplemental Figure 4. Additional data pertaining to in-vivo experiment 4.
In this experiment, efficacy of repeated T4 immunotherapy combined with carboplatin was evaluated. This figure shows the total flux emitted from each of 5 mice per group on day 39 following i.p. inoculation of SKOV-3 luc tumor cells. Mice had been earlier treated with 2 cycles of carboplatin (45mg/kg on days 25 and 32) plus T4+ T-cells (2.5 x 10^6 cells, injected on days 27 and 34). Control mice received 2 cycles of T4+ T-cells on the same days or PBS alone. The dotted line indicated background light emission, measured from a tumor-free mouse.