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Combination of Vaccination and Chimeric Receptor Expressing T Cells Provides Improved Active Therapy of Tumors

Hui-Rong Jiang,* David E. Gilham,† Kate Mulryan,* Natalia Kirillova,† Robert E. Hawkins,† and Peter L. Stern2*

We have generated murine T cells expressing chimeric immune receptors (CR) against human 5T4 oncofetal Ag (h5T4) and evaluated their tumor therapeutic efficacy alone and in combination with immunization using a replication-defective adenovirus encoding h5T4 (Rad.h5T4) and bone marrow-derived dendritic cells (BMDC). The h5T4-specific engineered T cells demonstrated Ag-specific, non-MHC-restricted cytolysis of h5T4-positive B16 and CT26 tumor cells in vitro by cytotoxicity assay and antitumor activity in vivo using a Winn assay. In the s.c. injected B16h5T4 melanoma model, early local but not systemic i.v. administration of syngeneic h5T4-specific CR T cells significantly increased mice survival. This improvement was further enhanced when combined with immunization with Rad.h5T4, followed by post-CR T cell treatment with BMDC in the active therapy model, possibly through mechanisms of enhancing Ag-specific cellular immune responses. This synergistic effect was lost without delivery of the BMDC. Our findings suggest that combining engineered T cells with specific vaccination strategies can improve the active tumor therapy. The Journal of Immunology, 2006, 177: 4288–4298.

Cell mediated tumor-specific immune responses in patients with advanced cancer are often found to be suppressed suggesting that immune control of tumor growth has been defeated by the range of mechanisms used by tumors (recently reviewed in Ref. 1). To counter the tumor in this setting, the adoptive transfer of immune effector cells into patients has been investigated. The administration of polyclonal populations of lymphokine-activated killer cells (2), lymphocytes derived from tumor biopsies (tumor-infiltrating lymphocytes (3)), or in vitro-sensitized lymphocytes (4) have been tested. Importantly, while these approaches have shown efficacy using in vivo model systems, the definitive potency of any such therapy in patients is yet to be clearly demonstrated. Nevertheless, the combination of tumor-specific tumor-infiltrating lymphocytes with nonmyeloablative chemotherapy regimens has been associated with some startling clinical responses in patients with advanced melanoma (5), encouraging the view that adoptive T cell therapies have significant potential.

While highly encouraging, the generation of clinically relevant numbers of Ag-specific T cells for the majority of cancer types is technically demanding due to the low relative frequency of tumor-specific T cell precursors found in peripheral blood or indeed the lack of defined tumor-associated Ags (TAA) with which to select the desired T cell effectors. Furthermore, tumors are known to avoid T cell surveillance by multiple mechanisms, including the down-regulation of MHC molecules that are required to present the TAA peptides (6–8). Polyclonal T cell populations expressing chimeric immune receptors (CR) is one approach that can circumvent such tumor escape mechanisms (9–11). CR usually contain Ag-binding domains (most often single-chain variable fragments (scFv)) fused with the signal-transducing domains of cellular receptors, such as CD3ζ or γ chains (11, 12). Consequently, T cells expressing CR can effectively respond to target cells through the binding of the Ab moiety of the CR to intact tumor-associated protein Ags on the surface of the target cell, thus avoiding MHC restriction and resulting in the elimination of tumor cells (13–15). Efficient gene transfer to T cells is accomplished predominantly using retroviral vectors and identifying many suitable TAA; so far, an expanding range of Ags, including erbB-2, Muc1, TAG-72, LewisY, carcinoembryonic Ag, CD19, and neural cell adhesion molecule, have been successfully targeted (recently reviewed in Refs. 16 and 17).

Our studies have focused on 5T4, a 72-kDa cell surface oncofetal Ag defined by a mAb raised against human trophoblast glycoproteins (18). 5T4 is expressed by a wide spectrum of cancers but is not detected on most normal adult tissues (19, 20). In colorectal, gastric, and ovarian carcinomas, h5T4 expression is associated with poorer clinical outcome (21–23). The restricted expression of 5T4 on tumor tissues, as well as its association with tumor progression, makes it a promising new candidate for immunotherapy. Several approaches to developing immunotherapies against this target are under development. Our work has established the use of Modified Vaccinia Ankara-based 5T4 immunization for the therapy

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Abbreviations used in this paper: TAA, tumor-associated Ag; BMDC, bone marrow-derived dendritic cell; CR, chimeric immune receptor; DC, dendritic cell; h5T4, human 5T4 oncofetal Ag; LN, lymph node; Rad.GFP, first generation E1/E3 deleted adenovirus expressing GFP; Rad.h5T4, first generation E1/E3 deleted adenovirus expressing human 5T4; scFv, single-chain variable fragment.
of tumors in preclinical models (24). This and the establishment of a repertoire of human CD8 T cell responses (25) has supported ongoing clinical trials in colorectal cancer patients. Furthermore, mouse models using the highly aggressive B16.h5T4 model system have demonstrated that vaccination using an adenoviral vector encoding h5T4 induces specific immune responses. However, in an active therapy model, vaccination with recombinant adenovirus expressing h5T4 before a boost vaccination with a dendritic cell (DC) line expressing h5T4 resulted in a significant improvement in median survival (26 days for control compared with 19 days for the vaccinated animals), while other combinations of vaccines failed to provide an equal degree of antitumor response (26). We are also harnessing the tumor cell surface expression of h5T4 by using Abs to targeted drugs and/or activation of effectors (27, 28).

Previously, we have shown that human T cells expressing a 5T4-specific CR can specifically lyse 5T4-expressing target cells in vitro (29, 30). In this study, we sought to determine whether mouse T cells transduced to express the 5T4-specific CR could challenge tumor growth in vitro and in vivo. Furthermore, this study investigated whether vaccination schedules involving adenovirus and BMDC could enhance the overall antitumor activity when combined with 5T4-specific CR T cells in stringent tumor model systems.

Materials and Methods

Abs and reagents

Except were stated, all Abs were purchased from BD Pharmingen, and all cell culture medium was purchased from Invitrogen Life Technologies.

Cell lines

Cell lines B16F10 and CT26 were from a spontaneous C57BL/6 melanoma (31) and a chemically induced BALB/c colon carcinoma (32) respectively and have both been used in previous chimeric receptor targeting studies (33). We transfected both CT26 and B16 tumor cell lines with PCMVA plasmid under neomycin selection to express h5T4 (B16/h5T4 and CT26/h5T4). Control cell lines were made by transfection with empty vector (B16neo and CT26neo). The expression of h5T4 was confirmed by FACS analysis using h5T4 mAb. All cell lines were maintained in DMEM supplemented with 2 mM t-glutamine, 10% FCS (Sigma-Aldrich) and G-418 (PAA Laboratories). The GP+e producer cell line (34) was provided by Dr. L. Fairbairn (Paterson Institute for Cancer Research, University of Manchester, Manchester, U.K.) and was maintained in DMEM supplemented with 1% newborn calf serum (NBCS), t-glutamine, and streptavidin/penicillin.

Mice

Inbred C57BL/6 and BALB/c mice were purchased from Harlan Sprague Dawley. Mice of 6–10 wk of age were used in all experiments. The procedures adopted conformed to the regulations of the Animal License Act (U.K.).

Production of ectropic producer cells

The h5T4-specific CR used in these studies consisted of the h5T4-specific scFv fused to the human CD3ζ receptor via an extracellular spacer consisting of the hinge CH2 and CH3 regions of human IgG. The rKat.h5T4-scFv-CD3ζ internal ribosome entry site GP vector used was that as documented in Refs. 30 and 35. Amphotropic retroviral particles were generated from 293T cells as previously described (30) and used to transduce the GP+e ectropic packaging cell line. Cell sorting using a FACSVantage (BD Biosciences) was performed to isolate the GP+ e producer cells, which produced a retroviral titer of sufficient quantity for further studies.

Retroviral gene transfer of mouse spleen and lymph nodes (LN) T lymphocytes

The GP+e producer cell line at a concentration of 80% was cultured with a minimal but sufficient volume of freshly 10% newborn calf serum supplemented with complete RPMI 1640 medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 50 μM 2-ME, 2 mM t-glutamine, and streptavidin/penicillin) overnight, and supernatant was collected and filtered through a 0.45-μm filter before adding to lymphocytes. Meanwhile, mouse lymphocytes from spleen (depleted of RBC using ammonium chloride lysing reagent from BD Pharmingen) and LN (inguinal, mesenteric, and cervical LN) were activated in complete RPMI 1640 medium supplemented with soluble anti-CD3 and anti-CD28 mAbs (clones 2C11 and 37.51, both at 100 ng/ml) for 30 h in flasks. Cells were then collected and recultured in the cell-free retroviral supernatant in 6-well nonadhesive culture plates, which had been precoated with 5 μg/ml retinectin (Takara) and 1 μg/ml anti-CD3 mAb (2C11) for 4 h. After an overnight incubation, the medium was replaced with fresh complete RPMI 1640 during the day and then replaced once more with a fresh batch of virus supernatant over the second night. After a third cycle of transduction, T cells were analyzed by fluorescence microscopy and flow cytometry to determine the level of transduction as assessed by the expression of the GFP marker gene and then used for future experiments. Mock T cells were transduced with supernatant from nontransduced (empty) GP+e cells.

Flow cytometry

The relative frequency of GFP+, CD4+, and CD8+ cells was determined by flow cytometry as described previously (30).

Generation of recombinant adenovirus expressing h5T4

As previously described by Hardy et al. (36), recombinant replication-defective E1/E3-deleted φ5 viruses were constructed by transfecting Cre8 cells with φ5 viral DNA and φ5f-digested pAdlox.h5T4. Then first generation E1/E3-deleted adenovirus expressing human 5T4 (Rad.h5T4) was expanded in Cre8 cells and purified by cesium chloride gradient centrifugation. The virus titer was determined by end point dilution, and stocks of Rad.h5T4 had titers of 3.7 × 1010 PFU/ml. Control first generation E1/ E3-deleted adenovirus expressing GFP (Rad.GFP) vectors were generated as described previously (37). Expression of h5T4 by Rad.h5T4 was confirmed by FACS analysis of BHK cells infected with Rad.h5T4 mAb.

Generation of BMDC

BMDC were prepared by a modification (38) of the procedure described by Inaba et al. (39). In brief, a single-cell suspension of bone marrow cells from femurs and tibias of normal C57BL/6 mice was depleted of B cells and T cells by using rat anti-mouse mAbs to MHC-II (clone p77; Serotec), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD45R/B220 (clone RA3-6B2), and Dynabeads coated with sheep anti-rat IgG (Dynabeads; Dynal Biotech). Then, cells were cultured at 7.5 × 106 cells/ml in 12-well plates in complete RPMI 1640 medium supplemented with 7.5% GM-CSF supernatant. The GM-CSF-producing cell line was a gift from Prof. Forrester (Aberdeen University, Aberdeen, U.K.). From day 2, the cultures were refed with fresh medium. On day 6 of the culture, immature DC were harvested and used after depleting Gr-1+ cells with anti-mouse Gr-1 mAb (clone RB6-8C5) and Dynabeads (Dynal Biotech). BMDC were then cultured overnight, and LPS was added for 2 h before the cells were collected for in vivo injection.

Winn assay

Winn assay was performed by the s.c. injection of tumor cells (2 × 105 cells/animal) mixed with either saline control or T cells (104 cells/animal). Mixing of cells was performed immediately before injection to minimize T cell and target interaction within the syringe. Mice were observed on a daily basis, and tumor development was determined by caliper measurements of perpendicular tumor dimensions as described previously (24). Animals were culled when tumor size reached a maximum of 1.24 cm3 or at defined experimental time points; n = 5–9 mice in each group.

Adaptive transfer model

Because of the aggressive nature of the tumor model used in our study, we reduced the inoculation tumor cell number to 3 × 103 in the active therapy
experiments, which was still able to induce 100% of disease incidence but with a slower tumor growth rate. This increase in time frame permitted the subsequent manipulations to be performed without the danger of the tumor growing to maximum size before completion of the procedure. B16hST4 tumor cells (3 x 10^5) s.c. on day 0, then at defined time points (days 1 and 3, 3 and 7, or days 7 and 9) 10^7 CR T cells were injected either locally (s.c. injection at the approximately the same site as tumor cell injection) or systemically (i.v.) through the tail vein. Control PBS or mock-transduced injection at the approximately the same site as tumor cell injection) or mock T cells, or Rad.GFP + CR T cells, or Rad.h5T4 + mock T cells as compared with mice injected with Rad.h5T4 + CR T cells; n = 7–11 mice in each group. At day 21 after tumor challenge, the mice were culled, and the sera and spleens collected for humoral and cellular immune response analysis.

To further investigate the role of BMDC in the combined therapy after Rad.h5T4 injection at day 7, in some experiments, mice received four doses of CR T cells but without BMDC on day 13. Tumor growth was monitored as above; n = 7–9 in each group.

**Ab response to h5T4 Ag**

Human 5T4-Fc fusion protein (40) was used in ELISA to detect anti-h5T4 serological responses. ELISA plates were coated with 0.5 μg/ml h5T4-IgG Fc fusion protein overnight at 4°C. Human IgG was used to test for serological responses against the hFc spacer region present on CR T cells. Plates were washed and then blocked with 2% Marvel milk for 1 h and followed by 2-h incubation with dilutions of sera (1/200–1/409,600). Finally, plates were incubated with HRP-labeled anti-mouse IgG and developed with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich). ELISA was conducted on sera of individual animal, and results were averaged.

**Spleen cell proliferation**

Spleens were collected and pooled within each group. Then, 1 x 10^7 splenocytes/well were cultured in 96-well U-bottom plates at 37°C in 5% CO₂ for 72 h in complete RPMI 1640 medium alone or supplemented with 10 μg/ml h5T4-Fc protein. A total of 0.5 μCi of [3H]thymidine (PerkinElmer Life Sciences) per well was added in the last 18 h of the culture, and the incorporation of 3H was measured using a scintillation counter (Topcount; Canberra).

**Statistical analysis**

Experiments were repeated at least twice, and some were repeated for more than five times. Figures show data from one representative experiment.
Survival data were illustrated with Kaplan-Meier plots and analyzed by log-rank using Statsdirect (Microsoft). Analysis of lymphocyte proliferation responses was performed by independent t test. Probability values of <0.05 were considered statistically significant.

Results

Phenotype of transduced murine T lymphocytes

To efficiently transduce mouse T cells, ecotropic retroviral producer cell lines were generated with the 5T4-H9256 vector, and cell-free supernatants were used to transduce primary mouse T cells of either spleen or LN origin. After three rounds of transduction, murine T lymphocytes were activated and expanded in clusters (Fig. 1A) with the GFP marker gene expression obvious by fluorescent microscopy (Fig. 1B). Flow cytometric analysis of the cells collected immediately after three rounds of transduction confirmed that ~24.8 ± 3.1% (n = 10) of the total cells were transduced. Thus, all in vitro and in vivo assays used CR T cells transduced to a level of not <20% of the total population.

Phenotypically, the transduced populations consisted of 20% CD4+ and approaching 70% CD8+ cells (data not shown). The preferential proliferation of CD8+ T cells probably results from using anti-CD3 and anti-CD28 Abs for activation as reported by others (12, 41).

Ag-specific, MHC-independent cytolytic activity of murine CR T cells

Transduced mouse CR T cells were assessed for their ability to lyse tumor cells expressing h5T4 using a standard 4-h chromium release assay. While mock T cells failed to show any specific lysis of control B16neo or B16h5T4 tumor cells, mouse T cells armed with the ST4-ζ CR efficiently lysed B16h5T4 melanoma but not B16neo cells (Fig. 2, A and B). The specificity of this interaction was apparent from the absence of lysis of B16neo cells by 5T4-H9256 CR T cells. Furthermore, this Ag-specific killing was not MHC restricted because 5T4-H9256-expressing T lymphocytes of C57BL/6 origin (H-2b) were able to specific kill h5T4-expressing CT26 colorectal tumor cells (BALB/c, H-2d background) but not CT26neo cells; mock-transduced T cells failed to lyse both these targets (Fig. 2, C and D).

Efficacy of redirected T cells in vivo in B16 and CT26 Winn assay tumor models

In vivo activity was assessed in a Winn assay by coinjection s.c. into C57BL/6 mice of 1 × 10^7 total mock or ST4-ζ CR T cells with either 2 × 10^5 B16neo or B16h5T4 tumor cells. No difference was observed in the growth rates of either B16neo or B16h5T4 tumors in mice treated with either saline or mock T cells. In these control groups, animals started to develop a tumor at approximately day 5 after inoculation with all animals bearing tumor by day 9 (Fig. 3A and B). By contrast, mice injected with 5T4-ζ-bearing T cells showed a significant delay of B16h5T4 tumor growth (~10 days over control; Fig. 3B) but not of B16neo tumor cells (Fig. 3A). No difference was observed in the appearance rates of B16neo tumors in mice treated with either CR T cells or mock T cells or saline.
C57BL/6 mice were inoculated with 3 × 10⁶ B16h5T4 cells at day 0, then 1 × 10⁷ CR T cells were injected either locally (s.c.) (A) or systemically (i.v.) (B) at days 1 and 3, or days 3 and 7, or days 7 and 9. Early (days 1 and 3) i.v. administration of CR T cells only induced slight inhibition of tumor growth, but early local injection of T cells induced significant delay of tumor growth and increased mice survival (p < 0.05 vs control groups). Delayed treatment did not have marked change of mice survival. Mice treated with PBS or mock T cells at days 1 and 3 were regarded as control groups.

The growth in tumor volume of B16h5T4 tumors also clearly demonstrated the specificity of the 5T4-ζ T cell antitumor activity (Fig. 3C). However once tumors were apparent, their growth curves were parallel to those in the control mice, which might indicate that CR T cells alone could not induce sufficient long-term antitumor immune responses in vivo. Consequently, the delay in the onset of tumor was mirrored by an improvement of ~10 days in overall survival rates (defined as the time to achieve a maximal tumor volume) in animals treated with B16h5T4/5T4-ζ CR T cells as against controls (Fig. 3D).

H-2b-restricted 5T4-ζ CR T cells were also able to significantly delay the onset of H-2b-restricted CT26h5T4 but not CT26neo tumors in BALB/c mice with 60% of the mice remaining tumor free after 8 wk of observation (Fig. 3, E and F). Once again, mock-transduced T cells showed no effect on the time of tumor onset compared with that of the saline control.

These experiments were performed with either spleen-derived or LN-derived T cells, and there appeared to be no significant functional difference between LN- or spleen-transduced 5T4-ζ CR T cells. In subsequent experiments, a mix of splenic- and LN-derived T cells was used. Overall, Ag-specific CR T cells were effective in delaying the growth of B16h5T4 cells in a Winn assay model.

**Early local but not systemic injection of CR T cells improves antitumor efficacy in unconditioned mice**

Encouraged by the Ag-specific antitumor activity of CR T cells in both aggressive tumor models of B16 and CT26 using a basic in vivo assay, next, we questioned whether the engineered T cells were effective in an established tumor model (active therapy). C57BL/6 mice were inoculated with 3 × 10⁶ B16h5T4 tumor cells s.c. at day 0, followed by 10⁷ T cells (5T4-ζ CR or mock transduced) either systemically (i.v.) or locally at the same site of tumor injection (s.c.). To investigate the timing of CR T cell administration, two doses of T cells were given on days 1 and 3, or days 3 and 7, or days 7 and 9. In this more stringent model, the local injection of 5T4-ζ CR T cells at the earliest time points (days 1 and 3) induced a small but significant delay of tumor growth and subsequent prolonged survival rates when compared with the PBS-treated group or mock T cell-treated group (p < 0.05; Fig. 4A). Later administration (days 3 and 7, or days 7 and 9) of the engineered T cells locally had no obvious effect on tumor growth. Moreover, 5T4-ζ T cells administrated systemically failed to induce any significant effect on tumor growth even when given at early time points (Fig. 4B).

These observations using a highly aggressive melanoma model system suggested that engineered T cells appeared to be effective only when meeting tumor at an early stage of establishment either through a coinjection scheme (Winn assay) or when introduced close to the site of tumor inoculum.

**Prophylactic vaccination using Rad.h5T4 does not enhance CR T cell antitumor effect in a Winn assay model system**

Previous studies have indicated that vaccination with Rad.h5T4 before tumor challenge results in a significant delay in tumor growth compared with nonvaccinated controls (26). To confirm this observation and to determine whether prevaccination could be combined with CR-expressing T cells, mice were prevaccinated with 1 × 10⁹ pfu of Rad.h5T4 or Rad.GFP s.c./i.m. at day −7, which was followed by s.c. injection of 2 × 10⁵ B16h5T4 cells.
mixed with $1 \times 10^7$ mock or CR T cells at day 0. The data in Fig. 5A show that mice treated with either CR T cells or Rad.h5T4 could delay tumor growth by ~10 days and significantly prolong the survival rate, which confirmed the effective antitumor function of CR T cells and Rad.h5T4 when used individually ($p < 0.01$) (Fig. 5B, group 2 and group 3). However, when mice were treated with both Rad.h5T4 and CR T cells, there was no additive effect of the two treatments (Fig. 5B, group 4).

Synergistic effect of engineered T cells and vaccination in an active therapy model

Despite the lack of synergy between a prophylactic vaccination schedule and CR T cells in a Winn assay, we sought to determine whether combining therapeutic vaccination with CR T cells could improve overall antitumor activity. In this experiment, $3 \times 10^4$ B16hST4 tumor cells were injected s.c. on day 0, followed by vaccination at day 7, then groups of mice were given $10^7$ 5T4-ε CR T cells per animal per dose on days 8, 9, 11, and 12. On day 13, mice who had received Rad.h5T4 were boosted with unmodified BMDC s.c. Control groups received PBS or CR T cells only or the Rad.h5T4-BMDC vaccination schedule only. Confirming our previous observations, the administration of four doses of 5T4-ε CR T cells alone either systemically (Fig. 6, group 3) or locally (group 2) had at best a marginal effect on established tumor growth (established for 1 wk before therapy). However, vaccination with Rad.h5T4 and BMDC (group 4) significantly delayed tumor growth ($p < 0.05$ vs PBS G1) but critically combining the latter with local injection of 5T4-ε CR T cells (group 5) significantly enhanced survival ($p < 0.001$ vs PBS G1, $p < 0.05$ vs vaccination G4) (Fig. 6). Furthermore, groups of mice receiving systemic 5T4-ε CR T cells together with vaccination also showed a modest statistical improvement in survival rate when compared with the PBS treated group ($p < 0.05$) but not against the vaccination only group 4.

To confirm that both an Ag-specific adenoviral vaccine and Ag-specific 5T4-ε CR T cells were required to generate this improvement in survival in mice challenged with B16h5T4 tumor cells, additional experiments were performed using a control adenovirus (Rad.GFP) and mock T cells. Groups of animals underwent the standard protocol of B16.h5T4 inoculum, followed by vaccination at day 7 with either Rad.h5T4 or Rad.GFP and which was followed by four doses of either 5T4-ε or mock T cells s.c. at days 8, 9, 11, and 12. BMDC were given s.c. at day 13. Mice were sacrificed at day 21 to enable assessment of Ab and proliferation assays vs h5T4. As shown in Fig. 7A, the mean tumor size in groups of mice that had received saline (group 1) or Rad.GFP + mock T cells + BMDC (group 2) or Rad.GFP + 5T4-ε CR T cells + BMDC (group 3) were all similar, although there was some initial difference in growth rate for the group 3 mice. By contrast, the tumor growth was significantly inhibited by the specific vaccination regimen with mock T cells ($p < 0.05$, G4 vs G1, day 15). The regimen of specific therapeutic vaccination combined with 5T4-ε CR T cells showed a further enhanced and significant effect on tumor growth ($p < 0.01$, G5 vs G1; $p < 0.05$, G5 vs G4, day 15).

The Rad.GFP + 5T4-ε CR T cells + BMDC (group 3) and the other control treatments (groups 1 and 2) did not induce a detectable 5T4 Ab response by day 21 (Fig. 7B). Mice treated with Rad.h5T4 (group 4) showed significant h5T4 Ab responses, which were slightly higher in mice treated with Rad.h5T4 + 5T4-ε CR T cells + BMDC (group 5). Interestingly, in the specifically vaccinated animals, splenocytes from mice, which had received 5T4-ε CR T cells (group 5), showed significantly higher levels of h5T4-specific cell proliferation compared with mock T cells (group 4) (G5 vs G4, $p < 0.05$; Fig. 7C), indicating that an enhanced cellular immune response was generated in the combined therapy. There was some evidence of lower level 5T4 proliferative responses in animals treated with mock or 5T4-ε CR T cells and nonspecific vaccination as compared with PBS-treated animals. No specific serological response against the IgG1 component of the chimeric receptor expressed on the T cells could be detected (Fig. 8). To determine whether CR T cells present in the spleens of recipient animals were contributing to this Ag-specific proliferation, RT-PCR was performed for the GFP-expressed marker gene. Although CR T cells could be detected in both groups 3 and 5, the frequency was <0.4%, suggesting that CR T cells in this experiment were not playing a significant role in the proliferation response against 5T4 Ag (Fig. 9).

In summary, our data have provided good evidence for an effective antitumor synergy between these therapeutic strategies when applied to an established tumor model.

BMDC are essential in enhancing the antitumor effect of Rad.h5T4 combined with CR T cells.

The previous sets of experiments demonstrated that a delay of growth of the highly aggressive B16h5T4 tumor in vivo could be achieved with a vaccination schedule of Rad.5T4 with BMDC coupled with 5T4-ε T cells. To examine the role of BMDC, groups of mice were inoculated with B16h5T4 as usual and then treated in four defined groups. The first group received BMDC but included Rad.GFP and mock T cells in place of Rad.h5T4 and local 5T4-ε T cells. This group performed the poorest, with all animals surviving no longer than 30 days (Fig. 10A). The second group, which featured Rad.5T4 in place of Rad.GFP, showed an improvement in overall survival as compared with group 1, whereas the best performing group was once again the group that included Rad.5T4 + BMDC + local 5T4-ε T cells (group 3; Fig. 10A); these data confirmed our previous observation as shown in Fig. 6. Somewhat surprisingly, when the BMDC were removed from this vaccination

FIGURE 6. Combination of CR T cells with Rad.h5T4 and BMDC induced a synergistic antitumor effect in an established B16 tumor model. A total of $2 \times 10^3$ B16h5T4 cells was injected s.c. at day 0, then $1 \times 10^7$ pfu of Rad.h5T4 was injected s.c./i.m. at day 7, followed by s.c. or i.v. injection of $1 \times 10^7$ CR T cells at days 8, 9, 11, and 12. At day 13, BMDC were s.c. injected. Control groups were injected with PBS, CR T cells alone, or with Rad.h5T4 and BMDC only. Our results show that CR T cells treatment locally (group 2) or systemically (group 3) a week after tumor challenge did not induce significant antitumor effect, but vaccination with Rad.h5T4 and BMDC (group 4) induced a significant delay of the mice tumor growth, combination vaccination with local-injected CR T cells (group 5) further enhanced the antitumor effect. G1, PBS vs G4, vaccination, $p < 0.01$; G1, PBS vs G5, vaccination + CR Tc(sc), $p < 0.001$; G1, PBS vs G6, vaccination + CR Tc(iv), $p < 0.05$; G4, vaccination vs G5, vaccination + CR Tc(sc), $p < 0.05$.
schedule (group 4), the resulting survival curve was more reminiscent of that of the Rad.5T4 vaccine (group 2), suggesting that the improvement above vaccination alone had been lost in the absence of BMDC.

Proliferation assays performed with splenocytes generated from these animals clearly demonstrated that in the absence of BMDC, there was a reduced h5T4-specific response compared with those animals receiving all three elements (p < 0.05, group 3 vs group 4). Indeed, cells from mice receiving the specific vaccine + BMDC + mock T cells (group 2) induced significantly higher h5T4-specific Ab response compared with other three groups, with a slightly higher level in group 5 mice than the group 4 mice. These results clearly demonstrated that nonmodified BMDC were playing a critical role in the improved survival of animals challenged with B16h5T4 and vaccinated with an adenoviral vector coupled with adoptive local transfer of 5T4-ζ T cells.

**Discussion**

This study demonstrates that mouse T cells can be engineered to express a CR with specificity for the target Ag h5T4. These targeted T cells showed non-MHC-restricted h5T4-specific cytolysis of syngeneic or allogeneic 5T4-expressing target cells in vitro or antitumor activity in vivo. While CR T cells could effectively delay the growth of the highly aggressive B16h5T4 tumor cells when coinjected in a Winn assay, prevaccination of Rad.5T4, which can provide protection vs tumor challenge, surprisingly, did not potentiate the antitumor activity. By contrast, in the active therapy model, the combination of therapeutic vaccination, followed by CR T cells and BMDC resulted in an improved antitumor effect with a significant growth delay observed over any of the components given individually.

It appears that a single vaccination of Rad.h5T4 can generate the predominant effect upon which the combination of CR T cells and BMDC appeared to improve efficacy. While the exact mechanisms underlying this phenomenon are not known, it is tempting to speculate that the Th1 environment known to be generated as a result of a single Rad.h5T4 vaccination in tumor-bearing mice (26) may be facilitating the activity of the adoptively transferred T cells and BMDC. The Ag specificity of the vaccination and CR T cells both are important since Rad.GFP combined with CR T cells or Rad.h5T4 combined with mock-transduced T cells failed to show
any improvement in tumor delay, suggesting that it is not only the potential presence of a facilitating environment that is critical for the observed effect. Indeed, other studies using the adoptive transfer of Ag-specific T cells (melanoma-specific pmel-1 transgenic T cells) demonstrated that in vivo T cell activation achieved through vaccination using a fowlpox virus vaccine and IL-2 support was essential to facilitate T cell trafficking through all tissues, proliferation, and for tumor-specific destruction (42). Furthermore, the combination of Ag-specific DC with adoptively transferred Ag-specific T cells can provide for a more robust antitumor response in vivo possibly through enhanced proliferation and tumor infiltration by adoptively transferred T cells (43).

In our studies, optimal splenocyte proliferative responses against hST4 Ag were only observed when adenovirus vaccine, CR T cells, and BMDC were combined. Indeed, when BMDC were removed, a significant reduction in ST4 proliferative response was observed, indicating that BMDC are a critical component of the combination therapy, albeit that the tumor models are limited in their scope for assessing the optimal kinetics of such treatments. It is possible that any early tumor cytotoxicity mediated by the ST4-ξ T cells could result in the release of large amounts of tumor debris, providing the Ag source for the induction of antitumor immunity (44). However, tumor debris alone is insufficient to induce a potent antitumor response based on the results of vaccination studies with irradiated autologous tumor cells or tumor lysates (45) and supported by our own observation (Fig. 3C) that the tumor growth curves in CR T cell-treated mice do not differ from the control mice once tumors are apparent. Importantly, ST4 expression was observed in the tumors from all groups, indicating that loss of Ag expression was not a factor facilitating tumor growth in these experiments (data not shown).

It is interesting to speculate that the BMDC in this model could be acting in the optimal presentation of the local source of Ag provided by the action of the CR T cells, thereby sustaining or boosting antitumor responses. Particularly when Ags are provided from tumor cells, DC would have the potential to present a broad range of tumor-associated Ags and on both MHC class I and II products. It has been reported that in mice B16 models, BMDC charged with tumor necrotic or apoptotic cells induced long-lived combined CD4 and CD8 immunity against tumor cells (46).

**FIGURE 8.** Sera from mice in the experiment (Fig. 7) were tested for the hST4-specific Ab response (hST4-Fc as the coating protein), and human IgG (hIgG) was used as the control capture protein. Data show here that only group 5 treated with Rad.hST4+ CR T cells induced high levels of hST4-specific Ab response (square line), but no response against hIgG (cross line), which suggests that the Ab response is hST4 specific, but not against hIgG1 Fc in the CR construct. The readings of the positive control of hST4-Fc and hIgG were 0.27 ± 0.005 and 0.36 ± 0.018 (average of 4 wells ± SD).

**FIGURE 9.** A, Mouse T cells were transduced with retrovirus encoding a chimeric receptor coexpressing the marker eGFP by an internal ribosome entry site element. After transduction and culture for 3 days, these cells were serially diluted with C57BL/6 splenocytes to achieve populations of splenocytes spiked with eGFP-expressing cells with the relative proportion of eGFP+ cells determined by flow cytometry (range of 5−0.0% eGFP-positive cells). RNA was generated from these cell populations (RNeasy; Qiagen), and 2.5 mg of RNA was subjected to reverse transcription using oligo(dT) primer and one-tenth of the product used to prime a PCR with primers specific for GFP and mouse β-actin. After 25 cycles, one-fifth of the product was electrophoresed through a 2% agarose gel and visualized under UV illumination. The quality of RNA was low in these cell populations, as determined by amplification of the β-actin control; however, a specific dose-dependent product was observed for GFP with a signal detectable down to a ratio of 0.4% GFP+ cells. B, RNA was generated from splenocytes used in the proliferation assays shown in Fig. 7 and 2.5 mg of RNA used to prime RT-PCR for GFP and β-actin as above. A strong β-actin signal in all groups demonstrated high levels of RNA, whereas a weak GFP signal was detected in groups G3 and G5 (both CR*) groups while no signal was detected in mock cells or relevant controls. A comparison of the intensity of GFP+ product compared with β-actin signal suggests that the frequency of CR+ T cells in groups G3 and G5 was <0.4% as determined by comparison with the eGFP titration series in A.
Unfortunately, we were unable to test the long-term antitumor effect because of the aggressive nature of the tumor model in our current study; future investigation using a less aggressive model may help to address this issue.

There is obviously a need to further optimize the therapeutic potential of both components of the CR T cells and vaccine in the combination treatments. There are several aspects of CR design, manipulation, and transduction methodology, which may improve the action of CR T cells. Thus, murine CR T cells with a low proportion (~10%) of the CD4+ subset perform less effectively against established tumor in vivo than mixtures of CR T cells given with a 1:1 CD4:CD8 ratio (47). The inference is that ensuring a high prevalence of CD4+ T cells in the CR population may improve overall antitumor responses. Recent developments in chimeric receptor technology have focused on the introduction of multiple signaling domains into the receptor, including the CD28 receptor (48, 49), and T cells armed with these improved receptors show improved function against established tumors (50). The development of such receptors against 5T4 is a route of future research to investigate in these models. Within this study, the highest dose of T cells available was used. The points above indicate some of the likely routes of improvement to CR technology, and armed with these developments, an important area for future work will be defining the optimal dose of CR T cells. There are a number of reasons why this is important, but in terms of future clinical translation, a lower number of highly active T cells would be more attractive as compared with the issues the surrounding need to generate vast numbers of T cells under quality-controlled conditions.

With respect to vaccination, the number and timing of immunizations with respect to delivery of engineered T cells needs to be further explored, including the use of other immunostimulatory molecules (51). Whether Ag-pulsed BMDC could improve upon the survival of animals with established tumors as observed in other models (43) or whether the BMDC are simply boosting the local immune response and could be effectively replaced by a vaccine boost or whether DC function is critical to the overall efficacy of the combination therapy will need to be tested. The necessity for improved localization of CR T cells given systemically is exemplified by other studies that have shown that T cells armed with CD3ζ chimeric receptors could induce significant delays in tumor growth when given systemically within 6 h of tumor injection (12) but failed to impact upon the growth of tumors established over a longer time frame (50). One interesting question is whether optimal vaccination may condition the tumor to receive the CR T cell more optimally. This might be the result of the expression of tumor-specific chemokine receptors, which might also be exploited as a means of retargeting the migration pattern of T cells (52). Conditioning regimes consisting of low-dose irradiation or cyclophosphamide improved the homing of CR T cells to bone marrow in an established prostate cancer metastasis model (14). Importantly, significant clinical responses against melanoma have been documented when Ag-specific T cells are given after a nonmyeloablative preconditioning regime (5). These observations suggest that Ag-specific T cell function is facilitated when used as a combination therapy.

There are several factors that could account for the synergistic effect of the combination of CR T cells, adenoviral vaccination, and BMDC in established tumor therapy. We hypothesize that Ag-specific vaccination plays a critical role in generating an optimal environment for the sustained antitumor activity of the CR effector and the development of additional adaptive immunity. In the absence of any vaccination, the tumor generates a Th2 type response, but the Rad.h5T4 vaccination shifts the balance to a Th1 response (26). This is advantageous to the overall function of the CR T cells, which, on reaching the tumor, are cytotoxic, release TAA (5T4), and locally delivered BMDC facilitated by the Th1 environment can then generate further an antitumor response. It has been shown that local delivery of the Ag-pulsed BMDC can be effective in providing antitumor immunity as a result of subsequent accumulation in the draining LN (53). In this study, “Ag” pulsing occurs in vivo as a result of both the adenoviral vaccination and the CR T cells actions locally. BMDC in this situation may be acting to support CR T cells either by cell-cell contact mechanisms or through the production of cytokines. Determining the regional distribution and the in vivo survival of adoptively transferred cells will be an important step toward further dissecting the mechanisms invoked through the combination of vaccination, CR T cells, and BMDC.
We have designed a series of experiments that have tested the potential for chimeric receptor T cells to deliver therapy to ST4-specific targets in vitro and in vivo. In doing this, we have used the Winn assay as the starting point in vivo as this represents the potential of 100% of effectors targeting the tumor. Subsequently, we have shown that giving the chimeric T cells locally, 1–3 days but not at later times, after tumor inoculation has some impact upon tumor growth. The novel component to this study is that with specific vaccination at 1 wk after tumor inoculation, chimeric T cells given between days 8 and 12 systemically or locally, followed by BMDC on day 13, can still produce significant effects upon tumor growth. However, vaccination of animals before tumor challenge completely abolishes any protective effect of coadministered chimeric T cells in a Winn assay. These experiments demonstrate that the interaction between passive and active immunity for providing potential combination therapeutic effect is complex and not simply additive and provides the context for further studies. Ultimately, optimizing and validating this approach to tumor immunotherapy will require a truly autologous model showing efficacy with systemic delivery of chimeric T cells. This study provides the impetus to invest in this approach.

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Disclosures

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References


