Combination Therapy of an Orthotopic Renal Cell Carcinoma Model Using Intratumoral Vector-Mediated Costimulation and Systemic Interleukin-2

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Abstract

Purpose: Interleukin (IL)-2 therapy is currently used for therapy of renal cell carcinoma (RCC). However, it is only effective in approximately 10% to 15% of patients, showing a need for additional therapies. We have previously described a replication-defective fowlpox vector encoding three costimulatory molecules (B7-1, ICAM-1, and LFA-3), designated rF-TRICOM. Here, we show that intratumoral administration of rF-TRICOM in an orthotopic RCC model effectively enhances tumor immunogenicity and reduces tumor burden in mice and the combination of rF-TRICOM and IL-2 is more effective than either therapy alone.

Experimental Design: RCC cells were implanted under the capsule of the kidney, and mice were given rF-TRICOM intratumorally 14 days later. We compared the effect of rF-TRICOM, rF-granulocyte macrophage colony-stimulating factor (GM-CSF), and two doses of IL-2 and combinations of the above on antitumor efficacy and survival. Host CD4+ and CD8+ T-cell responses were also evaluated.

Results: The results show that (a) systemic IL-2 therapy was moderately effective in the reduction of tumor burden in an orthotopic RCC model; (b) a single intratumoral injection of rF-TRICOM and rF-GM-CSF significantly reduced tumor burden; (c) the addition of systemic IL-2 to intratumoral rF-TRICOM/rF-GM-CSF administration resulted in further reduction of tumor burden, decrease in the incidence of metastasis, and extended survival in tumor-bearing mice above that seen with either treatment alone; and (d) CD8+ T cells played a critical role in the antitumor effect seen with rF-TRICOM/rF-GM-CSF + IL-2 therapy. Finally, the addition of systemic recombinant IL-15 or intratumoral vector-delivered IL-15 to intratumoral rF-TRICOM/rF-GM-CSF administration resulted in substantially more tumor-free mice than either therapy alone.

Conclusions: These studies show that intratumoral administration of rF-TRICOM admixed with rF-GM-CSF is effective at reducing tumor burden in mice and the addition of IL-2 further contributes to this effect. These studies thus form the rationale for combination immunotherapy clinical trials in patients with RCC.
greater in patients given high-dose IL-2 (16). However, there was no statistical advantage in overall survival using the high-dose IL-2 regimen versus the low-dose regimen and more toxicity was seen with the high-dose IL-2 regimen (16). Unfortunately, the toxicities seen with this therapy limit the patients who can receive high-dose IL-2 to those who can tolerate the side effects. There is a need for less toxic and more effective therapies for patients with RCC.

Various orthotopic models for renal carcinoma have been established to help identify new immunotherapies. The renal adenocarcinoma cell line Renca of BALB/c mice is one of the most commonly studied models of RCC (13). Recently, new preclinical transplantable orthotopic models have been described that share many ultrastructural features with human RCC (17, 18). In this study, we have used a RCC model that has been developed in BALB/c mice as a result of the carcinogen streptozotocin (18). This model is more reflective of human RCC in that the RCC#15 cell line grows more slowly than many of the murine renal cancer cell lines available (including Renca), thereby simulating tumor growth in humans more accurately. In addition, these tumors grow orthotopically under the capsule of the kidney and spontaneously metastasize to the lymph nodes and lungs, further mimicking the disease seen in humans (18).

Because IL-2 is only partially effective in human RCC therapy, other immunotherapeutic strategies might be used to add to or synergize with the effects of IL-2. RCC cells have been modified to express the costimulatory molecule B7-1 as both allogeneic and syngeneic whole tumor cell vaccines and show increased immunogenicity in vitro (19), whereas the results in a phase I clinical trial could not be differentiated from the effect of IL-2 because of the small number of patients in the study (20, 21). We and others have shown that the introduction of a single T-cell costimulatory molecule into carcinomas can enhance their immunogenicity (22, 23). We have previously described a replication-defective fowlpox virus that encodes three T-cell costimulatory molecules (B7-1, ICAM-1, and LFA-3), designated rF-TRICOM (23). We have shown that introduction of rF-TRICOM into tumor cells can lead to enhanced signaling in T cells and enhanced T-cell killing in vitro (24). We thus hypothesized that intratumoral introduction of rF-TRICOM with IL-2 may enhance antitumor effects.

Few tumor-associated antigens for renal cancer (murine and/or human) have been described (9, 25, 26). Intratumoral administration of rF-TRICOM would directly introduce immunostimulatory molecules into the tumor milieu, thereby potentially exploiting the presence of multiple tumor antigens to induce and potentiate tumor-specific immune responses.

We showed here for the first time that (a) systemic IL-2 therapy (low or high dose) was moderately effective in the reduction of tumor burden in an orthotopic RCC model; (b) a single intratumoral injection of rF-TRICOM significantly reduced tumor burden; (c) the addition of rF-granulocyte macrophage colony-stimulating factor (GM-CSF) augmented the antitumor effect seen with rF-TRICOM; (d) the addition of systemic low-dose IL-2 to intratumoral rF-TRICOM administration resulted in further reduction of tumor burden, decrease in the incidence of metastasis, and extended survival; (e) CD8 cells played an important role in the antitumor response seen in mice treated with rF-TRICOM + IL-2; and (f) antigen-specific T cells generated after rF-TRICOM admixed with rF-GM-CSF + IL-2 produced significantly higher levels of IFN-γ and tumor necrosis factor-α (TNF-α) and lysed additional biologically distinct tumor cell lines much more effectively than T cells from mice treated with rF-TRICOM and rF-GM-CSF alone. Because the safety and biological activity of intratumoral TRICOM administration has now been established in patients (27), the studies reported here thus form the rationale for the clinical evaluation of the combined use of systemic IL-2 and intratumoral rF-TRICOM administration in patients with advanced RCC.

Materials and Methods

Mice. Female BALB/c mice were obtained from the National Cancer Institute-Frederick Cancer Research Animal Facility (Frederick, MD). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6 to 8 weeks of age.

Tumor cells. Streptozotocin-induced renal cell tumor line SIRC-1.15 (designated RCC#15) has recently been isolated and characterized (18). The RCC#15 tumor develops spontaneous metastases to lung and mesenteric lymph nodes after implantation into kidneys. These tumor cells were determined not to be of clear cell pathology. No mutations in the von Hippel-Lindau or Ras genes were found. Immunogenicity of the RCC#15 cell line was tested, and the cells were found to be minimally to nonimmunogenic, as vaccination of mice with irradiated RCC#15 cells showed little protection against challenge with viable RCC#15 tumor cells. The RCC#15 cells were analyzed by flow cytometry for MHC class I and II and various costimulatory molecules, and the expression profile was found to be as follows: Kd+ (96% positive cells; mean fluorescence intensity, 308), I-Aα-; B7-1-, B7-2-, ICAM-1- (35%; mean fluorescence intensity, 100), and LFA-3-, CD40- (69%; mean fluorescence intensity, 1,032). Syngeneic tumor cell lines, including Renca (from Dr. R.H. Wiltrout; ref. 11) and colon tumor cell CT26 (ATCC ID CRL-2638; American Type Culture Collection, Manassas, VA), were also used in this study. These cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 50 mmol/L 2-mercaptoethanol (designated 10% FCS/RPMI 1640). These adherent cells were harvested by a scraper and washed in PBS before use.

Recombinant poxvirus vectors. The recombinant fowlpox viruses containing the murine B7-1, ICAM-1, and LFA-3 genes (designated rF-TRICOM), the recombinant fowlpox virus containing the murine GM-CSF gene (designated rF-GM-CSF), and recombinant wild-type fowlpox virus (FP-WT) were used as described elsewhere (23, 28). The recombinant fowlpox virus designated rF-IL-15 was generated by insertion of the murine IL-15 gene under the control of the 40-kb promoter at the FP14 site in the genome. The IL-15 cDNA was amplified from total mouse spleen RNA by reverse transcription-PCR using primers specific for the 5′-dGCTAGGCGTACGAGGTTTTGGGAAUUGAGGA and the 3′-dGTGATTTGTGATGAGGTGTCACGGGTTTTGTTTTATTTTGGAAACCA and the 3′-dCAGCAGCCACCTGGACCTGGAGGTTTTGTTTTATTTTGGAAACCA) ends of the open reading frame. The cDNA was cloned into a poxvirus transfer vector. Sequence analysis confirmed that no mutations were introduced in the cloning process. The recombinant fowlpox virus was generated by methods previously described (23, 28). Therion Biologics Corp. (Cambridge, MA) provided these poxviruses per a National Cancer Institute/Therion Cooperative Research and Development Agreement.

Intratumoral therapy of an orthotopic RCC tumor model using rF-TRICOM/GM and IL-2. RCC#15 tumor cells (1 × 107/50 μl/kidney) were implanted under the capsule of the right kidney of BALB/c mice using 1 mL syringe with 30-gauge needle under anesthesia on day 0. Groups of mice received intratumoral administration (50 μl/tumor/kidney) of rF-TRICOM [1 × 107 plaque-forming units (pfu)] admixed with rF-GM-CSF (1 × 107 pfu) under anesthesia on day 14. The
admixture will be referred to as rF-TRICOM/GM. Control mice received an intratumoral administration of buffer (PBS). The incision was closed with clips immediately after injection. Subsets of mice were injected i.p. with recombinant human IL-2 (Hoffman-La Roche, Inc., Nutley, NJ) at the same time as the intratumoral rF-TRICOM/GM administration. The doses of IL-2 used in these studies were 16,000 IU twice daily for 4 days. This dose (16,000 IU) was used because it is equivalent to the standard of care dose given to humans for the therapy of RCC (29). On day 25 after tumor transplant, the mice were sacrificed and tumor size was measured using calipers. Groups of mice were also observed for survival and tumor volume was calculated as follows: tumor volume (mm$^3$) = 0.5 × length × width$^2$.

Depletion of effector cells from mice. To determine the requirement of effector cells in the antitumor activity induced by rF-TRICOM, mice were treated with anti-CD4 antibody ascitic fluid (GK1.5 hybridoma, 10$^{-4}$ dilution, 100 μL/dose) to deplete CD4$^+$ cells or with anti-CD8 antibody ascitic fluid (Ly2.2 hybridoma, 10$^{-4}$ dilution, 100 μL/dose) to deplete CD8$^+$ cells during the rF-TRICOM therapy. Each antibody was injected i.p. into mice on days 12, 13, and 14 after tumor implantation; mice then received intratumoral rF-TRICOM/GM administration on day 14. The antibody was injected every week for the duration of the experiment. Cell depletion was validated by flow cytometry using FITC-conjugated anti-CD4 monoclonal antibody or CyChrome-conjugated anti-CD8 (BD PharMingen, San Diego, CA); >90% of the relevant cell population was depleted.

Lymphocyte proliferation and cytokine production assays. To evaluate T-cell immune responses to RCC#15 tumors, splenic T cells were tested for cell proliferation and cytokine production in response to irradiated RCC#15 tumor cells. First, spleen cells were dispersed into single-cell suspensions in 10% FCS/RPMI 1640 followed by RBC removal, and lymphocytes were then separated by centrifugation through a Ficoll-Hypaque gradient. The cells at the interface were collected and washed in 10% FCS/RPMI 1640, and CD4$^+$ or CD8$^+$ cells were isolated by negative selection and found to be >90% pure (Miltenyi Biotec, Auburn, CA). The purified T cells (2 × 10$^5$ per well) were cultured with irradiated (with 2,000 rads) naive syngeneic splenocytes as antigen-presenting cells (APC; 5 × 10$^5$ per well) and with irradiated RCC#15 tumor cells (1 × 10$^4$ per well; responder to stimulator ratio, 20:1) in 10% FCS/RPMI 1640 in 96-well flat-bottomed plates for 5 days. T cells and APCs were cultured in medium only as a control. [3H]Thymidine (1 μCi/well) was added to the wells for the last 24 h and harvested using a Tomtec cell harvester (Wallac, Inc., Gaithersburg, MD). The incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1205 Betaplate, Wallac).

To evaluate cytokine production from these cells in response to tumor antigens, the supernatant fluid was collected 72 h after culture and analyzed for IFN-γ and TNF-α using the Cytometric Bead Array kit according to the manufacturer’s instructions. The cytokine released in medium was subtracted from that induced by gp70 peptide, and the gp70-specific responses were depicted as Δ pg/mL.

Use of IL-15 with vaccine for the intratumoral therapy of RCC tumors. RCC#15 tumor cells (1 × 10$^5$/50 μL/kidney) were implanted under the capsule of the right kidney of BALB/c mice using 1 mL syringe with 30-gauge needle under anesthesia on day 0. Groups of mice received intratumoral administration (50 μL/tumor/kidney) of rF-TRICOM (1 × 10$^4$ pfu) admixed with rF-GM-CSF (1 × 10$^4$ pfu) under anesthesia on day 14. Control mice received an intratumoral administration of buffer (PBS). Two modalities of IL-15 were tested: recombinant murine IL-15 and vector-driven IL-15 via rF-IL-15. One subset of mice was injected i.p. with recombinant human IL-15 (PeproTech, Rocky Hill, NJ) at 5 μg/dose, twice daily for 3 days. Another set of mice received intratumoral administration of rF-TRICOM admixed with rF-GM-CSF and 10$^6$ or 10$^5$ pfu rF-IL-15 under anesthesia on day 14. On day 25 after tumor transplant, the mice were sacrificed and tumor size was measured using calipers. Tumor volumes were calculated as follows: tumor volume (mm$^3$) = 0.5 × length × width$^2$.

Statistical analysis. Significance of differences was calculated using ANOVA with repeated measures using StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA). For graphical representation of data, Y-axis error bars indicate the SD of the data for each point on the graph. For antitumor data, the statistics were calculated based on the tumor sizes of each animal as opposed to the mean tumors indicated by the heavy line. Evaluation of survival patterns was done by the Kaplan-Meier method and ranked according to the Mantel-Cox log-rank test using StatView 4.1 software package.

Results

The combination of IL-2 and rF-TRICOM reduces tumor burden in an orthotopic RCC model. Because IL-2 is used as a therapy for RCC (8, 10, 16), we examined the antitumor effect of IL-2 and compared it with intratumoral rF-TRICOM (a recombinant fowlpox virus expressing the costimulatory molecules B7-1, ICAM-1, and LFA-3) administration in an orthotopic model of RCC. In this model, 1 × 10$^6$ RCC#15 tumor cells were implanted under the capsule of the kidney of BALB/c mice, and mice were injected intratumorally on day 14 with PBS (Fig. 1A) or rF-TRICOM admixed with rF-GM-CSF (referred to as rF-TRICOM/GM; Fig. 1B-D). Mice were injected i.p. with 16,000 IU recombinant human IL-2 given twice daily for 4 consecutive days (days 14-17; Fig. 1B and D). As shown in Fig. 1B, IL-2 moderately but significantly reduced tumor burden compared with the PBS control (P = 0.0008 and 0.0047, respectively), but no mice were cured of tumor. IL-2 therapy alone (Fig. 1B), however, was not as effective as rF-TRICOM/GM alone (P = 0.0117; Fig. 1C); moreover, 3 of 15 mice were cured of tumor using rF-TRICOM/GM alone. When IL-2 was added to intratumoral rF-TRICOM/GM administration, there was a significant reduction in tumor burden compared with rF-TRICOM/GM alone (P = 0.0039), and 8 of 15 mice were found to be tumor-free compared with 3 of 15 mice in the rF-TRICOM/GM only group (Fig. 1D). From these data, we conclude that the addition of IL-2 to intratumoral rF-TRICOM/GM administration contributes significantly to the reduction of tumor burden over that of intratumoral rF-TRICOM/GM administration alone.

Effect of the addition of rF-GM-CSF to intratumoral rF-TRICOM administration on orthotopic renal cell tumor growth. To determine the effect of rF-TRICOM administration...
versus that of the vector alone (devoid of TRICOM) on tumor burden, tumor cells were implanted as described in Fig. 1 and mice were injected intratumorally with PBS (data not shown). FP-WT (vector control; Fig. 2A and B), or rF-TRICOM (Fig. 2C and D) 14 days after tumor implantation. Twenty-four days after tumor implantation, mice were sacrificed and tumor size was measured. Treatment of tumors with the FP-WT vector control did not reduce tumor burden in mice compared with the PBS control group ($P = 0.6$). However, treatment with rF-TRICOM significantly reduced tumor burden compared with the PBS control group ($P = 0.02$) or FP-WT treatment ($P = 0.05$; Fig. 2C).

To determine whether GM-CSF could increase antitumor efficacy in the RCC model, $1 \times 10^7$ pfu rF-GM-CSF was admixed with $1 \times 10^8$ pfu of either FP-WT or rF-TRICOM (Fig. 2B and D, respectively). When rF-GM-CSF was given with FP-WT, there was a significant decrease in tumor burden compared with FP-WT alone, although none of the mice were cured of tumor (Fig. 2B, $P = 0.02$, versus Fig. 2A). When rF-GM-CSF was admixed with rF-TRICOM, there was a significant decrease in tumor burden compared with rF-TRICOM admixed with FP-WT (Fig. 2D, $P = 0.04$, versus Fig. 2C); additionally, three of five mice were tumor-free after treatment with rF-TRICOM and rF-GM-CSF, whereas only one of five mice was tumor-free after treatment with rF-TRICOM and FP-WT. From these data, we conclude that intratumoral injection of RCC with rF-TRICOM is more effective than FP-WT at reducing tumor burden and that the addition of rF-GM-CSF further enhances this effect. This observation confirms and extends the earlier findings emphasizing the importance of GM-CSF in eliciting an antitumor response (31). Throughout the rest of this study, when mice received intratumoral injection of rF-TRICOM, it was admixed with $1 \times 10^7$ pfu rF-GM-CSF (rF-TRICOM/GM).

**IL-2 treatment in combination with intratumoral rF-TRICOM administration decreases incidence of metastasis and extends survival in tumor-bearing mice.** To determine whether combination treatment with systemic IL-2 and intratumoral rF-TRICOM/GM administration could increase survival in tumor-bearing mice, RCC cells were implanted orthotopically as described above (day 0) and mice were given intratumoral rF-TRICOM/GM administration on day 14 and/or treated with IL-2 on days 14 to 17. The mice were monitored for survival, and the primary tumor and metastases were observed at death. As shown in Fig. 3, all mice treated with PBS or IL-2 alone had died by 70 days after tumor implantation. Mice treated with rF-TRICOM/GM showed extended survival, with 3 of 10 mice surviving over 180 days after tumor implantation ($P < 0.0001$ versus PBS control). When IL-2 and rF-TRICOM/GM were combined, greater survival was observed, with 6 of 10 mice surviving over 180 days after tumor implantation ($P < 0.0001$ versus PBS control). In this experiment, survival in the rF-TRICOM/GM + IL-2 group was not increased significantly compared with rF-TRICOM/GM alone ($P = 0.2565$). However, the trend shows that extended survival is observed when IL-2 is added to intratumoral rF-TRICOM/GM administration (6 of 10 mice survived past 180 days versus 3 of 10 mice when treated with rF-TRICOM/GM alone).

At the time of death, mice were analyzed for evidence of primary tumor and metastatic deposits. In PBS-treated mice, 60% of mice had lung metastases and 90% had metastasis to the mesenteric (draining) lymph node and 90% had ascites.

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**Fig. 1.** Effect of the addition of IL-2 on intratumoral rF-TRICOM antitumor efficacy. RCC-15 tumor cells were implanted under the capsule of the kidney. Fourteen days later, mice were given rF-TRICOM/GM intratumorally. A, control mice were given PBS under the capsule of the kidney on day 14. B, mice were injected i.p. with IL-2, the dose equivalent to that administered to patients (16,000 IU/dose), twice daily for 4 consecutive days (days 14-17). C, mice were given rF-TRICOM admixed with rF-GM-CSF (rF-TRICOM/GM) intratumorally on day 14. D, mice were given rF-TRICOM/GM intratumorally on day 14. Concurrent with intratumoral rF-TRICOM/GM administration, mice were injected i.p. with IL-2. Twenty-five days after tumor implantation, mice were sacrificed and tumor size was measured. Statistical significance was analyzed by including the tumor volume of each animal and not the mean tumor volume (heavy lines). Data are a compilation of two separate experiments.

**Fig. 2.** Effect of GM-CSF on antitumor efficacy of intratumoral treatment of orthotopic RCC tumors. RCC-15 tumor cells were implanted under the capsule of the kidney of BALB/c mice on day 0. Fourteen days after tumor implantation, mice were given FP-WT or rF-TRICOM/GM intratumorally. A, mice were given FP-WT virus intratumorally. B, mice were given an admixture of FP-WT and rF-GM-CSF intratumorally. C, mice were given an admixture of rF-TRICOM and FP-WT intratumorally. D, mice were given an admixture of rF-TRICOM and rF-GM-CSF intratumorally. Twenty-four days after tumor implantation, mice were sacrificed and tumor size was measured. Statistical significance was analyzed by including the tumor volume of each animal and not the mean tumor volume (heavy lines).
IL-2 treatment alone did not improve this profile, as 78% had lung metastases, 56% had mesenteric lymph node metastases, and 100% had ascites ($P = 0.266$ versus PBS control). Of the mice that did not survive in the groups treated with rF-TRICOM/GM alone, none showed metastasis to the lung, and only one of seven mice showed evidence of ascites and metastasis to the mesenteric lymph node ($P < 0.0001$ versus PBS control and IL-2 alone). Only four mice in the rF-TRICOM/GM + IL-2 therapy group did not survive, and of these mice, none showed evidence of ascites or metastasis to the lung and one of four showed metastasis to the mesenteric lymph node (25%; $P < 0.0001$ versus PBS control and IL-2 alone). Of note, all mice examined at death had primary tumor on the kidney that was $\geq 500$ mm$^3$. Collectively, these data show that whereas IL-2 therapy alone does not increase survival or reduce the incidence of metastasis in tumor-bearing mice, treatment with rF-TRICOM/GM both increases survival and reduces the incidence of metastasis and ascites in mice. Although not statistically significant, these data show an additive therapeutic effect of rF-TRICOM/GM and IL-2, as mice in this group showed extended survival and reduction in the occurrence of metastasis compared with mice treated with rF-TRICOM/GM alone.

**Intratumoral rF-TRICOM administration and IL-2 therapy increase CD4$^+$ and CD8$^+$ T-cell responses.** To determine whether intratumoral rF-TRICOM + IL-2 treatment increased the levels of tumor-specific CD4$^+$ and CD8$^+$ T cells, proliferation and IFN-γ production in response to irradiated RCC#15 tumor were measured for both cell types. RCC tumor cells were implanted as described above, and 14 days after tumor implantation, mice were injected intratumorally with rF-TRICOM/GM and/or treated with IL-2. Fourteen days later (day 28 after tumor implantation), mice were sacrificed and spleen cells were used to assay proliferation and IFN-γ production in both CD4$^+$ and CD8$^+$ populations. CD4$^+$ T cells showed a significant increase in both cell proliferation and IFN-γ production after treatment with IL-2 alone over PBS control ($P = 0.0022$; Fig. 4A and B). Treatment with rF-TRICOM/GM alone showed a marked increase in both proliferation and IFN-γ production over PBS control and IL-2 therapy alone ($P = 0.0008$ versus PBS control). When IL-2 was given with rF-TRICOM/GM, cell proliferation and IFN-γ production slightly increased over that of rF-TRICOM/GM alone, and the increase seen in the cell proliferation assay was statistically significant ($P = 0.0418$). With regard to CD8$^+$ cell proliferation, IL-2 therapy showed an increase over PBS control ($P = 0.0597$) and intratumoral rF-TRICOM/GM administration showed a further increase ($P = 0.0424$; Fig. 4C). In mice treated with rF-TRICOM/GM and IL-2, there was a decrease in cell proliferation compared with rF-TRICOM/GM alone, although this decrease was not significant ($P = 0.0948$). IL-2, rF-TRICOM/GM, and rF-TRICOM/GM + IL-2 therapy resulted in an increase over that of

**Table 1. Occurrence of metastasis at death**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Metastases observed at death</th>
<th>Total incidence</th>
<th>$P$ (vs PBS)</th>
<th>$P$ (vs IL-2)</th>
<th>$P$ (vs rF-TRICOM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Mesenteric lymph node</td>
<td>Ascites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>60% (6/10)</td>
<td>90% (9/10)</td>
<td>90% (9/10)</td>
<td>80% (24/30)</td>
<td>0.266</td>
</tr>
<tr>
<td>IL-2</td>
<td>78% (7/9)</td>
<td>56% (5/9)</td>
<td>100% (9/9)</td>
<td>78% (21/27)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>rF-TRICOM</td>
<td>0% (0/7)</td>
<td>14% (1/7)</td>
<td>14% (1/7)</td>
<td>10% (2/21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Combination</td>
<td>0% (0/4)</td>
<td>25% (1/4)</td>
<td>0% (0/4)</td>
<td>8% (1/12)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: Mice given RCC tumor cells were treated with PBS, IL-2, rF-TRICOM/GM, or the combination of rF-TRICOM/GM + IL-2 14 d after tumor implantation (see Fig. 3). Mice were observed for evidence of metastasis at death. Number in parentheses represents number of mice with metastases/number of total mice.
the PBS control in the amount of IFN-γ production from CD8+ T cells to similar levels (Fig. 4D). Taken together, these data show that, whereas treatment with rF-TRICOM/GM alone markedly increased the ability of CD4+ and CD8+ T cells to proliferate and produce IFN-γ in response to tumor, the addition of IL-2 to rF-TRICOM/GM did not further augment this effect.

Role of CD4+ and CD8+ T cells in the antitumor response induced by rF-TRICOM/GM and IL-2. To determine the requirement of CD4+ or CD8+ T cells in the antitumor response after rF-TRICOM/GM and IL-2 treatment, BALB/c mice were implanted with RCC tumor cells as described above and treated with rF-TRICOM/GM and IL-2 on day 14 after implantation. On days 12, 13, and 14 after tumor implantation and once weekly thereafter for the duration of the experiment, mice were injected i.p. with either anti-CD4 or anti-CD8 antibodies to deplete their respective cell populations. Mice were sacrificed 24 days after tumor implantation and observed for the presence of tumors. As shown in Fig. 5B, 9 of 10 mice treated with PBS after rF-TRICOM/GM and IL-2 treatment were found to be tumor-free. Similarly, when treated with anti-CD4 antibody, 10 of 10 mice were tumor-free after rF-TRICOM/GM and IL-2 treatment, suggesting that CD4 cells may not be required for antitumor efficacy (Fig. 5C). However, when mice were treated with anti-CD8 antibody, only 2 of 10 mice were tumor-free and tumor burden was significantly increased over PBS treatment (Fig. 5D, P = 0.001, versus Fig. 5B). These data suggest that CD8+, but not CD4+, T cells play a role in the antitumor response seen during rF-TRICOM/GM and IL-2 treatment.

rF-TRICOM/GM + IL-2 therapy enhances cross-presentation. gp70 has been identified as an antigen expressed on certain murine tumor cells that can be recognized by tumor-specific CTL (32). To determine whether treatment with rF-TRICOM/GM and IL-2 could amplify a gp70-specific CD8+ T-cell response, spleens were harvested 180 days after tumor implantation from mice cured of tumor after rF-TRICOM/GM + IL-2 treatment (from the experiment shown in Fig. 3). It should be pointed out that mice treated with IL-2 alone were not available because none survived this therapy at day 180 (see Fig. 3). Splenic lymphocytes were stimulated for 6 days with the gp70 peptide (30), and cytokine production and CTL activity were assayed. CD8+ T cells from mice treated with rF-TRICOM/GM alone showed no increase in IFN-γ, TNF-α, or IL-10 production above that of PBS-treated mice. However, CD8+ T cells from mice treated with both rF-TRICOM/GM and IL-2 showed an increase in antigen-specific IFN-γ, TNF-α, and IL-10 production (Fig. 6A). No detectable levels of IL-6, IL-12, or MCP-1 were found in any samples.

To determine whether lymphocytes from mice treated with rF-TRICOM/GM and IL-2 could kill the RCC tumor cells more effectively than lymphocytes from mice treated with rF-TRICOM/GM alone, a CTL killing assay was done on RCC#15 tumor cells. Tumor cells were labeled with chromium-51 and incubated with lymphocytes previously stimulated...
IL-2 produced much higher levels of IFN-γ which contributed to a dramatic effect seen when RCC#15 cells were incubated in addition to IL-2 treatment. CTLs from mice treated with rF-TRICOM/GM and IL-2 kill gp70-positive tumor cells much more effectively than CTLs from mice treated with rF-TRICOM/GM alone.

The combination of IL-15 and rF-TRICOM reduces tumor burden in an orthotopic RCC model. Although IL-2 has been approved for use in patients with metastatic RCC, it has been suggested that IL-2 is not optimal for inhibiting tumor growth because, in the presence of IL-2, TIL may recognize tumor as self and undergo activation-induced cell death or the immune response might be inhibited by IL-2-dependent regulatory T cells (Tregs; ref. 33). In contrast, IL-15 has been reported to activate T cells and natural killer cells, inhibit activation-induced cell death, and not support Tregs. It has been suggested that IL-15 could become a better choice than IL-2 for the treatment of RCC (33–35). To determine whether IL-15 could increase antitumor efficacy in the RCC model, we examined the antitumor effect of IL-15 alone and in combination with intratumoral rF-TRICOM administration. Two modalities of IL-15 were tested: recombinant murine IL-15 (systemic) and vector-driven IL-15 via rF-IL-15 (intratumoral). BALB/c mice were implanted with RCC tumor cells as described above.

with the gp70 peptide (as in Fig. 6A) for 5 h, and radioactivity in the supernatant was measured and percentage lysis was calculated as described in Materials and Methods. The most dramatic effect was seen when RCC#15 cells were incubated with lymphocytes from mice treated with rF-TRICOM/GM and IL-2 and 80% to 90% lysis was seen (P = 0.0001 versus PBS control; Fig. 6B). By contrast, lysis by lymphocytes taken from mice treated with PBS or rF-TRICOM/GM alone was much lower, ranging from 0% to 15%, although the increase over control was significant at an E:T ratio of 80:1 (P = 0.0347). Immune responses were also analyzed against two additional tumor cell lines: Renca, a RCC line, and CT26, a biologically distinct murine colon carcinoma cell line. As shown in Fig. 6C, significantly more lysis of Renca tumor cells was seen with cells from rF-TRICOM/GM–treated mice than PBS-treated mice (P = 0.0353), and lymphocytes from mice treated with rF-TRICOM/GM and IL-2 displayed ~50% lysis of Renca cells (P = 0.0001, compared with both control and rF-TRICOM/GM alone). When CT26 cells were used as targets, there was a significant increase in lysis by cells from mice treated with rF-TRICOM/GM (up to 50% compared with 20% seen with control; P = 0.0008) and lymphocytes from rF-TRICOM/GM– and IL-2–treated mice lysed from 80% to 100% of the tumor cell targets (P = 0.0001, compared with both control and rF-TRICOM/GM alone; Fig. 6D). Taken together, these data show cross-presentation of antigen from destroyed tumor cells in that CD8+ T cells from mice cured of tumor by rF-TRICOM/GM and IL-2 produce much higher levels of IFN-γ, TNF-α, and IL-10 in response to a tumor-associated peptide (gp70) than cells from mice that received rF-TRICOM/GM treatment alone. In addition, CTLs from mice treated with rF-TRICOM/GM and IL-2 kill gp70-positive tumor cells much more effectively than CTLs from mice treated with rF-TRICOM/GM alone.

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Fig. 7. Effect of the addition of IL-15 on intratumoral rF-TRICOM antitumor efficacy. RCC#15 tumor cells were implanted under the capsule of the kidney. A, control mice were injected intratumorally with PBS on day 14. B, mice were given rF-TRICOM admixed with rF-GM-CSF (rF-TRICOM/GM) intratumorally on day 14. C, mice were injected i.p. with recombinant murine IL-15 (rIL-15; 5 μg/dose) twice daily for 3 consecutive days (days 14-16). Mice were given rF-TRICOM admixed with rF-GM-CSF (rF-TRICOM/GM) intratumorally on day 14. D, mice were given rF-TRICOM/GM intratumorally on day 14. Concurrent with intratumoral rF-TRICOM/GM administration, mice were injected i.p. with recombinant IL-15. E, mice were given rF-IL-15 intratumorally on day 14. F, mice were given rF-TRICOM/GM intratumorally on day 14. Concurrent with intratumoral rF-TRICOM/GM administration, mice were injected intratumorally with rF-IL-15. Twenty-five days after tumor implantation, mice were sacrificed and tumor size was measured. Statistical significance was analyzed by including the tumor volume of each animal. Asterisks, statistical significance based on tumor incidence at day 25.

When mice were treated intratumorally with rF-TRICOM/GM alone, there was a significant decrease in tumor burden compared with no treatment (Fig. 7B, P = 0.002, versus Fig. 7A); additionally, two of five mice were tumor-free. These results are similar to those depicted in Figs. 1C, 2C, and 5B. When recombinant murine IL-15 was administered systemically (i.p.), there was a reduction of tumor burden compared with mice receiving no treatment (Fig. 7C), although there were no tumor-free mice. The combination of systemic recombinant IL-15 with intratumoral vaccine resulted in a further reduction of tumor burden compared with no treatment (Fig. 7D, P = 0.001, versus Fig. 7A). When mice were treated with intratumoral rF-IL-15 alone, there was no decrease in tumor burden to mice receiving no treatment (Fig. 7E). However, the greatest antitumor activity was noted with the combination of rF-IL-15 with intratumoral vaccine, which resulted in marked reduction of tumor burden compared with no treatment (Fig. 7F, P < 0.001, versus Fig. 7A). There were no differences noted between mice treated with 10^7 or 10^8 pfu rF-IL-15; therefore, the data are presented together (Fig. 7F). More importantly, this combination treatment resulted in 80% of the mice being tumor-free (Fig. 7F, tumor incidence at day 25, P = 0.046, versus Fig. 7B). From these data, we conclude that intratumoral injection of RCC with rF-TRICOM/GM is effective at reducing tumor burden and that the addition of rIL-15 further enhances this effect.

Discussion

IL-2 is currently used for RCC therapy. However, along with the potential benefits of this therapy are significant toxicities that also limit patient eligibility to those who can withstand these toxicities. Several clinical trials have shown the safety and provided evidence of clinical activity of recombinant TRICOM vectors injected s.c. or s.c./intratumoral; these include rF-CEA-TRICOM (36), rF-CEA-MUC1-TRICOM (37), and rF-PSA-TRICOM (38). In addition, intratumoral rV-TRICOM administration in patients with melanoma showed safety and evidence of clinical activity (27). These studies have also provided evidence of the potential clinical benefit of adding GM-CSF to TRICOM therapy (36). An ongoing clinical trial is also showing the safety and feasibility of intraprostatic administration of rF-PSA-TRICOM in patients with locally advanced prostate cancer. Here, we sought to determine whether the combination of rF-TRICOM/GM and IL-2 could reduce tumor burden in mice more effectively than either therapy alone. The data presented here show that the combination of rF-TRICOM/GM and IL-2 not only is more effective at reducing primary tumor burden and distal metastases in mice than either therapy alone but also induces greater T-cell responses to tumor and increases cross-presentation of tumor antigen.

We chose to use a streptozotocin-induced renal cancer cell line of BALB/c mice (RCC#15) in our studies (18). Although many of the renal cancer cell lines available exhibit rapid growth in mice, the RCC#15 cell line grows more slowly, simulating tumor growth in humans more accurately. In addition, these tumors grow orthotopically under the capsule of the kidney and spontaneously metastasize to the lymph nodes and lungs (Table 1), further mimicking the disease seen in humans (18). It is important to note that, although the orthotopic model used here more accurately simulates tumor growth in humans than other renal cancer cell lines, pathology shows that these cells are not clear cell renal carcinoma. Additionally, it is important to state that no mutations were found in exons 1, 2, or 3 of the von Hippel-Lindau tumor suppressor gene or in codons 12, 13, and 61 of the Ras oncogene, the sites of most frequent Ras mutations.

In a preclinical study to determine which of the 10 cytokines expressed by irradiated tumor cells elicited the most potent antitumor immunity, a vaccine consisting of irradiated tumor cells expressing GM-CSF was the only therapy that protected against tumor challenge (39). In a phase I clinical trial in patients with metastatic RCC, vaccination with autologous tumor cells expressing GM-CSF showed infiltration of dendritic cells, macrophages, neutrophils, and T cells at the vaccination site (40). These studies and others provided the rationale to administer rF-TRICOM and rF-GM-CSF intratumorally. This way, GM-CSF and
the costimulatory molecules present in rF-TRICOM would be presented directly into the tumor milieu, exploiting the presence of multiple tumor antigens and acting de facto, as a whole tumor cell immunogen in situ. We and others have previously shown that rF-GM-CSF enhances T-cell responses and antitumor activity when given as an admixture with CEA/TRICOM vectors (28, 41, 42), and when mice were vaccinated intratumorally, the omission of rF-GM-CSF dramatically reduced the antitumor effects induced by the vaccine regimen (43). This study confirmed these previous findings, as mice given rF-GM-CSF showed significantly reduced tumor burden compared with mice that did not receive rF-GM-CSF (Fig. 2).

Because of the toxicity of high-dose IL-2 in humans, a clinical trial was done comparing toxicity and antitumor efficacy of high-dose IL-2 and low-dose IL-2 (one tenth that given with high-dose IL-2). Whereas toxicity was reduced with low-dose IL-2, the response rate, durability, and survival in complete responders were lower than that seen with high-dose IL-2 (16). However, there was no difference in overall survival between the high-dose and low-dose groups. In this study, we compared the efficacy of IL-2 or vaccine alone to the combination of the two modalities. As shown in Fig. 1, IL-2 given with rF-TRICOM/GM resulted in a significantly lower tumor burden and more tumor-free mice than either therapy alone, showing that intratumoral rF-TRICOM/GM administration and IL-2 therapy have a synergistic effect on reducing tumor burden.

Extended survival was observed in mice receiving rF-TRICOM/GM, and although the addition of IL-2 to rF-TRICOM/GM resulted in a larger number of mice surviving past 180 days (30% with rF-TRICOM/GM versus 60% with rF-TRICOM/GM + IL-2; Fig. 3), this difference was not significant. Similarly, the incidence of metastasis at death was significantly less in mice treated with rF-TRICOM/GM ± IL-2 compared with PBS or IL-2 alone, but the combination of rF-TRICOM/GM and IL-2 did not significantly reduce the incidence of metastases compared with rF-TRICOM/GM alone (Table 1). Although the differences between rF-TRICOM/GM with or without IL-2 were not statistically significant, the trends favored the addition of IL-2 to rF-TRICOM/GM in both survival and presence of metastases. Additionally, combination therapy was shown to be superior to rF-TRICOM/GM alone when immune responses to the endogenous antigen gp70 were tested (Fig. 6).

When examining the T-cell response to the various therapies used, it was interesting to observe that, although reduction in tumor burden and mouse survival was increased when IL-2 was added to rF-TRICOM/GM, the addition of IL-2 did not result in an increase in either the proliferation or IFN-γ production by CD4+ and CD8+ T cells (Fig. 4). It has previously been shown that the avidity of T cells plays an important role in antitumor and antiviral responses (44, 45). We have found that vaccination with rV-TRICOM and rF-GM-CSF greatly enhances the avidity of T cells produced in both foreign and self-antigen systems (46). In addition, we have found that the addition of a recombinant fowlpox virus expressing IL-2 (rF-IL2) to rV-CEA/TRICOM + rF-GM-CSF enhances the avidity of carcinoembryonic antigen and gp70-specific T cells compared with vaccination without rF-IL2.3 Therefore, although the quantity of the T-cell response did not increase when IL-2 was added, it is possible that the quality (avidity) was greatly increased, resulting in a greater and longer-lived antitumor response. Future studies will include a systematic examination of the effect of rF-TRICOM/GM + recombinant human IL-2 on T-cell avidity.

We have previously shown that CD4+ and CD8+ T cells play a role in the antitumor effect of CEA/TRICOM + rF-GM-CSF and IL-2 in a s.c. self-antigen tumor model (47). To determine which cell population was responsible for the antitumor effect seen with rF-TRICOM/GM + IL-2 in an orthotopic RCC model, we depleted the CD4+ or CD8+ cell populations during intratumoral rF-TRICOM/GM administration and observed the effect on tumor growth. Whereas the depletion of CD4+ T cells had no effect on the reduction of tumor burden, the depletion of CD8+ T cells significantly reduced the number of tumor-free mice, and average tumor burden was higher than when these cells were not depleted (Fig. 5). Although the mechanism for the antitumor efficacy of IL-2 is not fully elucidated, this effect is thought to be mediated through activated T cells and natural killer cells (8), which is consistent with our results. However, the antibody that we used to deplete CD4 cells would also deplete CD4+CD25+ cells or Tregs. It is possible that, by depleting these Tregs, the immune response was augmented against the tumor. It has been shown that depleting Tregs while giving a vaccine consisting of a B7.1 fusion protein suppressed tumor growth to a greater extent than that seen with either modality alone (48). Additionally, i.v. administration of the Treg-depleting drug Ontak (Ligand Pharmaceuticals, San Diego, CA) before vaccination with RCC RNA-transfected dendritic cells enhanced antitumor immunity (49). Previous work in our laboratory, depleting Tregs along with giving vaccine, has shown that, although the combination of depleting Tregs and giving vaccine increased immune responses compared with vaccine alone, this combination did not augment antitumor efficacy (50). Therefore, although it is a possibility that the depletion of Tregs contributed to the antitumor efficacy seen in Fig. 5C, it is unlikely that this accounts for the whole effect and still suggests a role for CD8+ cells.

Previously, there was not a well-established tumor-associated antigen for murine RCC (25). Therefore, we hypothesized that direct introduction of immunostimulatory molecules into the tumor milieu by intratumoral administration of rF-TRICOM/GM would exploit multiple tumor antigens that would then induce and potentiate tumor-specific immune responses. By PCR analysis, we found that the RCC#15 tumor cell line used in these studies expressed gp70 (data not shown). As shown in Fig. 6, CD8+ T cells from mice treated with rF-TRICOM/GM + IL-2 produced significantly higher levels of IFN-γ, TNF-α, and IL-10 in response to stimulation with gp70 peptide than T cells from mice treated with rF-TRICOM/GM alone. Furthermore, these CD8+ T cells could mediate lysis not only of the original RCC line but also from another RCC line, Renca, and an unrelated tumor cell line, CT26. These data show that intratumoral rF-TRICOM administration can induce immune responses to an endogenous tumor antigen and suggest that intratumoral rF-TRICOM administration can be used to induce antitumor T-cell responses in cancers where the tumor antigens are largely unknown. We have previously shown that vaccinating intratumorally can induce immune responses to antigens only in the original RCC line.
not given in the vaccine (antigen cascade; ref. 50). The data in Fig. 6 support the idea that, in the clinic, TRICOM vectors could be given intratumorally in the primary tumor (as an adjuvant to surgery, or administered laparoscopically), and immune responses would be generated against other tumor antigens. This phenomenon of antigen cascade could aid in the elimination of distant metastases as well as the primary tumor.

In the past year, two new drugs have been approved for use in metastatic RCC. Both sunitinib (Sutent, Pfizer, Inc., Cambridge, MA) and sorafenib (Nexavar, Bayer Pharmaceuticals, West Haven, CT) are tyrosine kinase inhibitors targeting platelet-derived growth factor receptor and vascular endothelial growth factor receptor, among other kinases (51). Sunitinib was approved for use in metastatic RCC in January 2006 after a phase III trial showed a significant improvement in progression-free survival and objective response rate over that seen with IFN-α (52). A phase III trial showed an increase in progression-free survival and median overall survival in advanced RCC patients treated with sorafenib compared with placebo (53, 54), and consequently, the drug was granted fast-track Food and Drug Administration approval in December 2005. Although the approval of these two drugs is a great advance in therapy for RCC, tyrosine kinase inhibitors have several disadvantages, including the ability of a tumor cell to redirect signals through other pathways or mutate the tyrosine kinase in question, becoming resistant to the tyrosine kinase inhibitor (51). It has been shown that, when tyrosine kinase inhibitors are combined with chemotherapy or radiation, synergistic antitumor effects are seen (55–58). In light of this, and because clinical trials with TRICOM vectors in RCC may now be conducted in patients who have failed sorafenib or sunitinib, future studies examining the combination of these tyrosine kinase inhibitors with vaccine and IL-2 therapy may need to be conducted.

The antitumor effect of IL-2 most likely results from its ability to expand T-cell populations and increase the activity of these populations. However, it has recently been suggested that IL-2 is not optimal for inhibiting tumor growth because, in the presence of IL-2, CTL may recognize tumor as self and undergo activation-induced cell death or the immune response might be inhibited by IL-2–dependent Tregs (33). In contrast, IL-15 has been reported to activate T cells and natural killer cells, inhibit activation-induced cell death, and not support Tregs. IL-15 could become a better choice than IL-2 for the treatment of RCC (33–35). As shown in Fig. 7, tumor-bearing mice treated systemically with recombinant IL-15 had a significant reduction in tumor burden (Fig. 7C). These data are similar to that of Kobayashi et al. (59) who described IL-15 preventing experimental MC38 pulmonary metastases in IL-15 transgenic mice that had large quantities of IL-15 in their serum. The addition of systemic IL-15 to intratumoral vaccine resulted in more mice being tumor-free by day 25 (0 of 5 versus 3 of 5; Fig. 7C and D). Local IL-15 delivered to the tumor by intratumoral rIF-IL-15 did not result in a significant reduction in tumor burden. However, rIF-IL-15, given concurrently with vaccine, resulted in the greatest antitumor activity, with substantially more tumor-free mice than vaccine alone or rIF-IL-15 alone, showing that intratumoral rIF-TRICOM/GM administration and IL-15 therapy have a synergistic effect on reducing tumor burden. The use of IL-15 in combination with vaccine will be further explored and compared with IL-12 in terms of toxicity and T-cell maintenance.

Taken together, our data show that rIF-TRICOM/GM effectively reduces RCC tumor burden in mice when given alone and show a synergistic effect when given with standard of care IL-2. These data suggest that rIF-TRICOM/GM could be given to renal cell cancer patients alone or in addition to IL-2 to induce antitumor immune responses and reduce tumor burden with minimal toxicity.

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