Influence of CD80, Interleukin-2, and Interleukin-7 Expression in Human Renal Cell Carcinoma on the Expansion, Function, and Survival of Tumor-Specific CTLs

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ABSTRACT

Purpose: **A renal cell carcinoma (RCC) line, RCC-26, has been identified as a suitable candidate for development of an allogeneic tumor cell vaccine based on its expression of a variety of tumor-associated antigens (TAA). To improve immunogenicity, RCC-26 cells were genetically engineered to express CD80 alone or in combination with interleukin (IL)-2 or IL-7. The effect of these modifications on proliferation, function, and survival of autologous and allogeneic tumor-specific CTLs was assessed.**

Experimental Design: **RCC-26 sublines expressing different transgenes were tested for their capacity to reactivate cytokine secretion and cytotoxicity in autologous tumor-infiltrating lymphocytes, to improve proliferation and survival of tumor-associated T cells present in autologous peripheral blood, and to induce tumor-associated responses in naive allogeneic lymphocytes. The expression of several common TAA was quantitated in the RCC-26 sublines using reverse transcription-PCR to identify surrogate markers for immune monitoring in clinical trials.**

Results: **Gene-modified RCC-26 cells showed enhanced immunogenicity. CD80 expression was necessary to induce RCC-associated CTL in blood of healthy allogeneic donors. It also improved proliferation of autologous effector-memory T cells. Further enhancement was achieved with IL-2 through induction of the antiapoptosis protein Bcl-x_L. The candidate vaccine lines overexpressed several common TAA that are suitable markers for immune monitoring.**

Conclusions: **RCC-26 cells coexpressing CD80 and cytokine transgenes display improved immunogenic characteristics, supporting their use as allogeneic tumor cell vaccines for HLA-A2-matched patients with metastatic RCC.**

INTRODUCTION

Renal cell carcinomas (RCC) are classified as immunogenic tumors based on the observation that patients with metastatic RCC show some of the most favorable responses to immunotherapy (1, 2). Both natural killer cells and MHC-restricted CTLs could be specifically recruited to RCC *in vivo* (3, 4). Furthermore, RCC patients showed tumor regression following allogeneic stem cell transplantation with subsequent donor lymphocyte infusions (5, 6). Nevertheless, only a small percentage of patients achieved durable responses following various forms of immunotherapy and the basis of effective antitumor immunity remains unclear. Whether the presence of increased numbers of tumor-infiltrating lymphocytes (TIL) correlates with better prognosis is controversial (7–9), although isolated TIL often killed autologous tumor cells following restimulation *in vitro* (10, 11).

We analyzed the anti-RCC response in a patient with stage I disease ($T_1N_0M_0G_2$) in whom a single brain metastasis appeared 9 years after primary tumor nephrectomy. The TIL of this patient (TIL-26) displayed cytotoxic activity against an autologous tumor cell line (RCC-26) following reactivation *ex vivo*. The TIL-26 line and several clonal derivatives recognized an epitope presented by HLA-A*0201-encoded molecules. This peptide-MHC (pMHC) ligand was expressed by RCC-26 cells but not by autologous cells derived from the normal kidney parenchyma (NKC-26) or by autologous EBV-transformed lymphoblastoid cells (LCL-26; ref. 11). The TIL-26 line contained two dominant CTL clones that expressed highly conserved T-cell receptors (TCR), which enabled them to be traced and quantified based on their characteristic third complementary determining region (CDR3) sequences (12, 13). T cells bearing these two TCRs were prevalent in the RCC-26 tumor *in situ* and were also found to be circulating in the peripheral blood of patient 26 up to 48 months post surgery (12).

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Note: B. Frankenberger, H. Pohla, and E. Noessner made equal contributions to this study.

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We also observed that HLA-A2-restricted TIL derived from unrelated RCC patients were able to recognize RCC-26 cells, showing that these cells displayed several distinct tumor-associated pMHC ligands that were shared by other RCC (3, 11, 12). In addition, RCC-26 cells were recognized by allogeneic natural killer and non-MHC-restricted (natural killer–like) T cells; thus, they could engage effector cells of the innate immune system (4, 14). It would be desirable to specifically tap such reservoirs of effector cells in RCC patients to improve their antitumor immunity. One strategy to mobilize effector cells is to vaccinate patients with genetically modified tumor cells that have sufficient immunogenicity. The characteristics displayed by RCC-26 cells suggest that they may be able to activate important effector cells in an allogeneic vaccination setting.

Although RCC-26 cells showed a striking natural immunogenicity, they did not express positive B7 costimulatory molecules. Several members of the B7 superfamily play key roles in activating T cells (15). When T cells are stimulated through TCR-pMHC interactions, parallel coupling of CD28 receptors to CD80 (B7.1) or CD86 (B7.2) provides positive costimulatory signals that improve the capacity of T cells to produce interleukin-2 (IL-2), to up-regulate their high-affinity IL-2 receptors (CD25), and to undergo clonal expansion. Seminal studies by Chen et al. (16) and Townsend and Allison (17) revealed a central role of B7 expression by tumor cells in inducing tumor-specific CTL responses. Animal models showed that injection of B7-IgG or CD80-expressing tumor cells led to T-cell-mediated rejection of unmodified tumor cells (18–21). CD80 expression by tumor cells also protected effector CTL from activation-induced cell death through induction of the antiapoptosis factor Bcl-x_L (22).

Because RCC-26 cells did not express such positive costimulatory molecules, we genetically engineered them to express CD80 alone or in combination with selected cytokines to enhance their natural immunogenicity. As we show here, these alterations substantially improved their ability to stimulate lymphocyte proliferation, to activate optimal effector cell function, and to induce Bcl-x_L in effector-memory T cells, thereby providing protection against apoptosis. CD80-expressing RCC-26 cells were also able to induce tumor-associated CTL in naive lymphocytes of healthy allogeneic donors. When combined with the feature that RCC-26 cells display ligands recognized by numerous allogeneic TIL in addition to overexpressing several defined tumor-associated antigens (TAA), our findings support clinical testing of gene-modified RCC-26 cells as allogeneic vaccines for HLA-A2-matched RCC patients.

MATERIALS AND METHODS

Gene Modification of RCC-26 Cells. The RCC-26 subline expressing CD80 (B7.1) was generated as described previously (23). To construct bicistronic vectors, human B7.1 and human IL-2 cDNAs were amplified by PCR from WEWAKE II (24) and concanavalin A–stimulated human T cells, respectively. B7.1 cDNA was cloned behind the poliovirus internal ribosome entry site of plasmid pPBS. The IRES-B7.1 fragment was then cloned into plasmid pKEX-2-XR (25), resulting in plasmid pK2B7.1-3. *Bgl*II/*Bam*HI-digested IL-2 amplicon was inserted into plasmid pK2B7.1-3, resulting in plasmid pKEX-IL2-IR-B7. IL-7 cDNA was recovered as an *Eco*RI fragment from plasmid pLhIL7SN (26) and was inserted blunt ended into pK2B7.1-3, resulting in plasmid pKEX-IL7-IR-B7. All cDNA inserts were verified by sequencing. In these constructs, the cytokine (IL-2 or IL-7) and B7.1 cDNAs are connected by the poliovirus internal ribosome entry site and driven by the human cytomegalovirus immediate early promoter, and the Hy^R gene is under the control of the herpes simplex virus thymidine kinase promoter. The plasmids were introduced in RCC-26 cells, cultured as described previously (27), via electroporation (10 μg plasmid cDNA, 2 ms at 420 V and 1,200 μF, Elektroporations-Impulsgenerator, L. Fischer, Germany). Cells were subsequently seeded in dishes and selected with 600 μg/mL hygromycin 24 hours later. Clones were either picked using cloning cylinders or obtained by limiting dilution.

Flow Cytometric Analysis of Renal Cell Carcinoma Cells. RCC-26 sublines were tested for surface expression of pan-MHC class I, HLA-A2, CD80, and CD86 molecules by flow cytometry using the following monoclonal antibodies (mAb): directly labeled phycoerythrin mAbs binding to CD80 (clone L307.4, BD PharMingen) and CD86 (clone 2331, BD PharMingen, Heidelberg, Germany). Clone HB-82 specific for HLA-A2 and clone W6/32 specific for pan-MHC class I were unconjugated. Indirect immunofluorescence was done using polyclonal phycoerythrin-conjugated goat anti-mouse $F(ab)_2$ immunoglobulin (Dianova, Hamburg, Germany). The myeloma protein MOPC-21 (Sigma, Deisenhofen, Germany) and mAb IgG1-phycoerythrin (DAKO, Glostrup, Denmark) served as isotype controls. Data acquisition and analysis were done on a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA) using CellQuest Pro software.

Analysis of Cytokine Expression. Expression of cytokines by T cells and RCC-26 cells was tested using the highly sensitive BioPlex Human Cytokine Broad Range Panel (17-Plex, Bio-Rad Laboratories, Inc., Hercules, CA). Data analysis was done using the Bio-Rad Array Operation System (Bio-Rad Laboratories) and applying five-variable logistic regression algorithms. Background values obtained from wells containing RCC-26 sublines without lymphocytes were subtracted in the studies assessing T cells. Expression of vascular endothelial growth factor and transforming growth factor-β1 was tested using the Quantikine ELISA system (R&D Systems, Minneapolis, MN) and analyzed on the Emax (Molecular Devices Corp., Sunnyvale, CA).

ELISPOT Analysis. For ELISPOT analysis, a TIL-26-derived clone (TIL-26-GG) was thawed and plated at 1,000 cells in 50 μL/well in triplicates on nitrocellulose-bottomed microtiter plates (ELIHPSSP10, Millipore, Bedford, MA), precoated overnight at 4°C with 1.5 μg IFN-γ capture antibody in 100 μL/well (clone 1-D1K, Mabtech AB, Nacka, Sweden), and incubated for 2 hours at 37°C in supplemented RPMI 1640 containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, penicillin/streptomycin (100 units/mL), and 10% human AB serum (BioWhittaker, Verviers, Belgium) to block unspecific binding. The different autologous RCC-26 sublines, NKC-26 cells and LCL-26 cells, were then carefully added to the wells

(5,000 cells in 50 μL). For background evaluation, each stimulator cell and the TIL-26 cells were plated alone. For antibody blocking, stimulator cells were preincubated with 3 μg anti-HLA-A/HLA-B/HLA-C (W6/32, DAKO, Hamburg, Germany) for 30 minutes at room temperature before plating. The ELISPOT was then done as described previously (13, 28). Spots were counted using a computer-assisted video image analysis system (KS ELISPOT, Carl Zeiss Jena GmbH, Munich, Germany).

Mixed Lymphocyte Tumor Cell Cultures. *In vitro* primed CTLs were established using autologous peripheral blood mononuclear cells (PBMC) of patient 26 or using allogeneic PBMC of HLA-A*0201 or B*5101-matched healthy control donors and irradiated (100 Gy) tumor cells, plating 0.5×10^6 responding cells and 3×10^4 irradiated tumor cells per well of a 24-well culture plate, in supplemented RPMI 1640 containing 15% heat-inactivated pooled human serum. Four hours after initiation of the cultures, 20 units/mL recombinant IL-2 (Proleukin, Chiron, Emeryville, CA) and 5 units/mL recombinant IL-4 (R&D Systems) were added to all cultures. Responding lymphocytes were restimulated at intervals of 8 of 10 days using irradiated unmodified and gene-modified RCC-26 tumor cells in medium supplemented with exogenous recombinant IL-2 and recombinant IL-4.

In the experiments analyzing the role of IL-2, minor changes were done: 3.0×10^5 responding cells were stimulated with 2×10^4 irradiated tumor cells per well of a 48-well culture plate; during the first round of stimulation, low-dose IL-2 (20 units/mL) was added to all mixed lymphocyte tumor cell culture (MLTC) combinations, whereas starting from the second round of restimulation (day 10) each MLTC combination was split into two fractions and exogenous IL-2 (50 units/mL) was added to only one set of cultures. Limiting dilution was done in 96-well round-bottomed microtiter plates and responder cells were plated at concentrations of 5, 1, or 0.5 cells per well to distinguish T cells with alloreactive versus RCC-associated specificity.

Cell-Mediated Cytotoxicity Assay. Cell-mediated lysis was quantitated in a standard 4-hour chromium-51 release assay. Spontaneous release was determined by incubating target cells alone in complete medium. Total release was determined by directly counting an aliquot of labeled cells. Percentage cytotoxicity was calculated according to the formula: % Lysis = (experimental cpm − spontaneous cpm/total cpm − spontaneous cpm) × 100. Duplicate measurements of three-step titrations of effector cells were used for all experiments.

RNA Preparation and Reverse Transcription-PCR Analysis of AV20 T-Cell Receptor Sequences. A two-step reverse transcription-PCR (RT-PCR) protocol was done for the detection of tumor-associated AV20AJ22 TCR transcripts expressed by two TIL-26-derived clones, designated as TIL-26-GG and TIL-26-LSG cells (12). Quantitative real-time RT-PCR was done by the LightCycler technology (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence. After isolation of total cellular RNA according to the manufacturer's instructions (TriReagent, Biozol, Eching, Germany), an aliquot of 1 μg RNA was reverse transcribed with an oligo$(dT)_{15}$ primer using the avian myeloblastosis virus reverse transcriptase (first-strand synthesis kit for RT-PCR, Roche Diagnostics). A 295-bp amplification product was generated with the LightCycler Fast Start Reaction Mix SYBR Green I using TIL-26-GG CDR3 region-specific primers: Va20.3 (sense 5′-AGTACTTTGAGCCTTGCT-3′) combined with Ja22.3 (antisense 5′-TTGCAGAACCACCCACGAG-3′). The following LightCycler protocol was used for online detection of amplified products: initial denaturation at 95°C for 10 minutes followed by 35 cycles of amplification of 1 second at 95°C, 10 seconds at 56°C, and 25 seconds at 72°C. As an internal control, the housekeeping gene *α-enolase* was generated with the primers α-Eno1 (sense 5′-GTTAGCAA-GAAACTGAACGTCACA-3′) and α-Eno2 (antisense 5′-TGAAGGACTTGTACAGGTCAG-3′). Semiquantitative detection of TCRAV20 transcripts and sequence analysis of the CDR3 was done as described in detail elsewhere (12).

Absolute quantification of Bcl-x_L transcripts was done using a Bcl-x_L-specific PCR primer mix, optimized for use in the LightCycler instrument, according to the manufacturer's instructions (Search LC, Heidelberg, Germany).

Detection of Tumor-Associated Antigens in RCC-26 Sublines. Expression of mRNA encoding TAAs was determined by quantitative real-time RT-PCR. Comparisons of mRNA expression of each single TAA was made with a pool of control healthy normal kidney tissue. Normalization was made using the housekeeping gene *hPBGD* according to the manufacturer's instructions (Roche Diagnostics). PCR amplification was done using the LightCycler Fast Start Reaction Mix SYBR Green I, including a three-segment amplification protocol: initial denaturation at 95°C for 10 minutes followed by 38 cycles of amplification of 1 second at 95°C, 10 seconds at 56°C or 60°C (depending on the primers used), and 25 seconds at 72°C. Final extension was done for 10 minutes at 72°C. Primers used for the PCR amplifications were as follows: carbonic anhydrase IX sense 5′-GTCTCGCTTGGAAGAAATCG-3′ and antisense 5′-CTCCTCCAGCGACAAACAAT-3′, adipophilin sense 5′-GTGAAGACCATCACCTCCGT-3′ and antisense 5′-TTCTCCACACTGCCAGTCAC-3′, preferentially expresses antigen in melanoma sense 5′-ACCTGGAAGCTACCCACCTT-3′ and antisense 5′-AGATGCATCACATCCCCTTC-3′, and oncofetal antigen sense 5′-TTCTGGATTCCCGTCGTAAC-3′ and antisense 5′-GCGCAGAGGAGAATCTGTGT-3′. Survivin mRNA expression for human survivin was analyzed using a primer mix, optimized for use in the LightCycler instrument, according to the manufacturer's instructions (Search LC).

RESULTS

Immunophenotype of RCC-26 Sublines. RCC-26 cells were genetically modified to express CD80 alone or in combination with IL-2 or IL-7. Sublines were derived from the modified cells and selected for the highest CD80 expression and the best transgenic cytokine secretion. CD80 expression was greatest on the CD80 transfectant, and RCC-26/CD80/IL-2 expressed more CD80 than RCC-26/CD80/IL-7 cells (Fig. 1). Analysis of a larger panel of cytokines revealed that the RCC-26 sublines produced a broad array of

factors that could contribute to their immunogenic potential. In addition to their respective expression of IL-2 or IL-7, all RCC-26 sublines secreted particularly high levels of the proinflammatory cytokines IL-6, IL-8, and monocyte-chemoattractant-protein-1, and MCP-1 (Supplementary Table 1). The highest levels were produced by the uncloned parental line; nevertheless, all three gene-modified sublines secreted high amounts of these factors, albeit levels of IL-8 were significantly less. All RCC lines also produced vascular endothelial growth factor and, to a lesser extent, transforming growth factor-β1. As shown in the functional *in vitro* studies described below, the net effect of this complex cytokine mixture was not overtly inhibitory.

TIL-26 Responses to Gene-Modified RCC-26 Cells. The three gene-modified sublines were compared with unmodified RCC-26 cells for their capacity to be recognized by autologous TIL-26 cells in a standard 4-hour chromium-51 release assay (Fig. 2*A*). All four RCC-26-derived target cells were killed at comparable levels, whereas autologous LCL-26 cells and the MHC class I negative target cells, Daudi and K562, were not recognized, demonstrating the well-documented MHC-restricted specificity of the TIL (11). These results revealed that gene modification of RCC-26 cells, particularly CD80 expression, did not directly influence CTL cytotoxic function. It should be noted however that the TIL-26 cells were maintained in culture with exogenous cytokines and underwent regular restimulation with tumor cells so that their cytotoxic potential *in vitro* was held at an optimum.

The RCC-26 sublines coexpressing CD80 and IL-2 or IL-7 were also tested for their capacity to induce IFN-γ secretion by TIL-26 cells as measured in an ELISPOT assay (Fig. 2*B*). Both double-transgenic sublines induced TIL-26 cells to secrete IFN-γ at numbers comparable with unmodified RCC-26 cells. Following irradiation, the cells showed an improved stimulatory capacity. This is an important point with respect to clinical testing in which irradiated vaccine cells would be employed. The TIL-26 responses were specific for tumor cells because cytokine secretion was not seen in response to autologous NKC-26 and LCL-26 cells and the responses were completely blocked in the presence of class I–specific mAb, as expected for MHC-restricted $CD8^+$ T cells.

CD80 Supports Expansion of AV20 T Cells from PBMC-26. The TIL-26 line contained two dominant CTL clones that expressed highly conserved TCR, using AV20α chains, which differed only slightly in their CDR3 sequences (12, 23). Thereby, the two clones, designated as TIL-26-GG and TIL-26-LSG, respectively, could be distinguished and quantified at the molecular level using PCR primers specific for their individual AV20 CDR3 regions. TCR transcripts were detected directly, albeit at very low levels, in PBMC-26 samples obtained

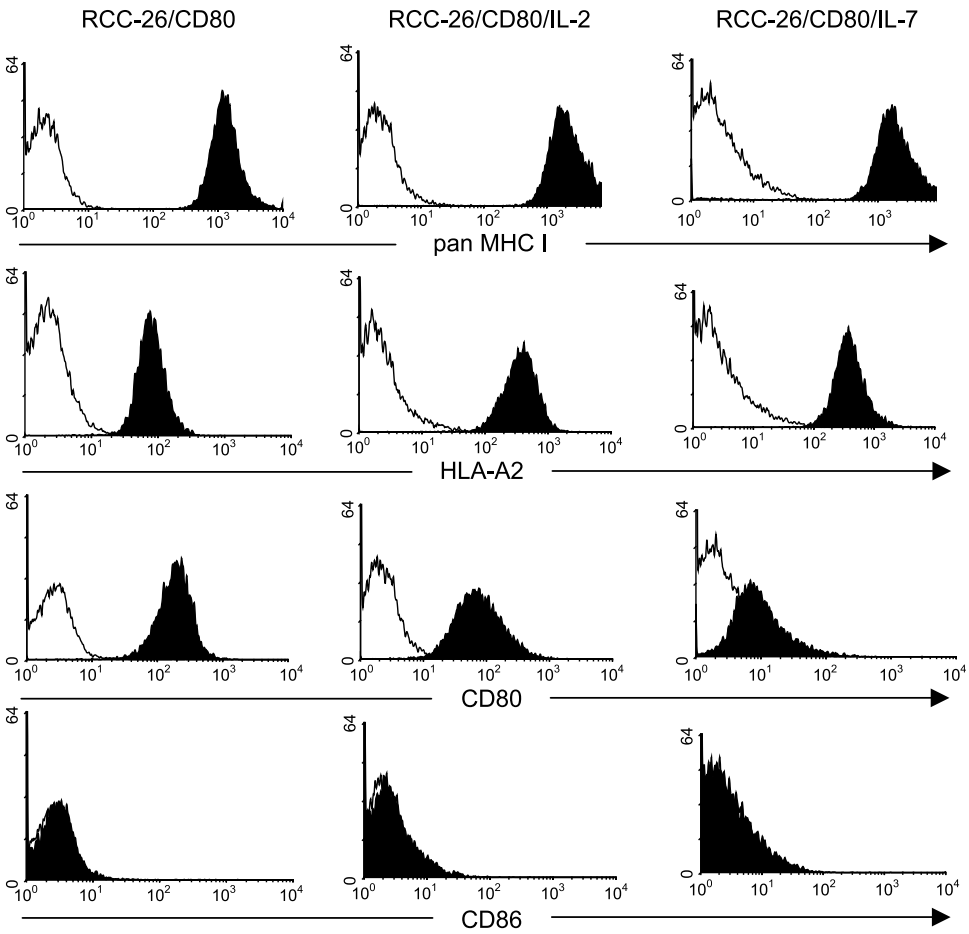

Fig. 1 Flow cytometry of gene-modified RCC-26 cell lines. Cell lines, as indicated, were stained with mAbs to pan-MHC I, HLA-A2, CD80, and CD86 (*black histograms*). Isotype-matched antibodies were used for control staining (*white histograms*). Gates were set on viable propidium iodide–negative cells.

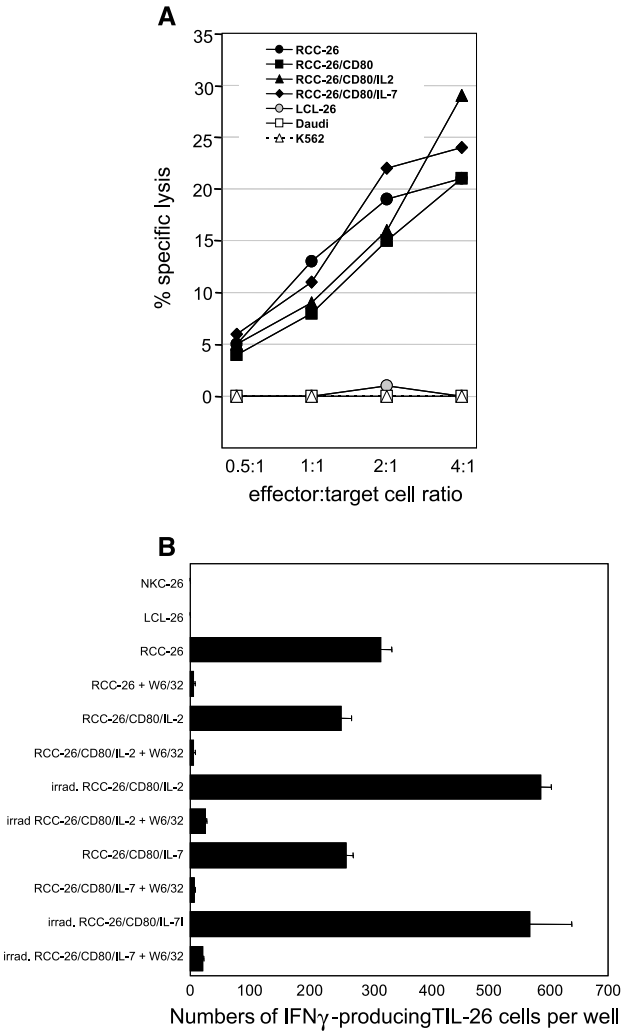

Fig. 2 *A,* cytotoxic activity of autologous uncloned TIL-26 cells. TILs of patient 26 were tested for lysis of four RCC-26-derived target cells, autologous LCL-26 cells, and the MHC class I–negative target cells, Daudi and K562. Assay was done using varying effector-to-target cell ratios. Points, percentages of specific lysis. *B,* IFN-γ-ELISPOT assay of the TIL-26 response to RCC-26 sublines. Triplicates of 1×10^3 TIL-26-GG cells were stimulated against the different autologous RCC-26 sublines, NKC-26 and LCL-26, with 5×10^3 cells per well. For antibody blocking experiments, the RCC-26 sublines were preincubated with 3 μg/well anti-HLA-A/B/C (W6/32). Vaccine cell lines were additionally irradiated at a dose of 120 Gy. No background was observed for TIL-26. Columns, mean of triplicate wells; bars, SD. Representative of three independent experiments.

from patient 26 up to 48 months post surgery (12). Because of the absolute sequence identity of these AV20 TCR transcripts with those expressed by TIL-26 cells, which were also found to be prevalent in the primary tumor *in situ* (12), we presume that the circulating AV20 T cells represent effector-memory T cells. However, due to their low frequency and the lack of an AV20-specific mAb, we have not been able to directly analyze the phenotype of the AV20 T cells circulating among the PBMC-26 to confirm this assumption.

The effect of RCC-26 restimulation on proliferation of AV20 T cells was analyzed by determining increases in AV20 TCR transcripts as a measure of T-cell expansion. Unmodified RCC-26 and CD80-expressing RCC-26 sublines were compared as stimulating cells in autologous MLTC. Because AV20 T-cell proliferation was not detected in the absence of exogenous cytokine, IL-2 (50 units/mL) was added to the MLTC medium. AV20 sequences were assessed by RT-PCR after each round of restimulation using an AV20-Cα primer pair. Because the amplicons were not cloned before sequencing, substantial expansion of AV20 T cells was required before the TCR transcripts could be detected in PCR (12). Normally, AV20-specific PCR amplicons could be visualized on agarose gels and characteristic TCR sequences could be detected after three rounds of restimulation in the MLTC. Table 1 summarizes the data acquired after the fourth round of restimulation. The GG or LSG clonotypic TCR transcripts could be detected following stimulation with either unmodified RCC-26 cells or the RCC-26/CD80 subline; thus, CD28-CD80 costimulation was not essential to induce proliferation of these T cells, a finding that is consistent with an effector-memory phenotype. Proliferation of AV20 T cells was strictly dependent on restimulation with tumor cells because AV20 amplicons were not detected in the MLTC combinations using autologous LCL-26 or NKC-26 cells as stimulating cells.

Although these experiments provided qualitative information about AV20 T-cell expansion, it could not be determined whether there were quantitative differences in the stimulatory capacities of the two lines. Therefore, the emerging AV20 TCR transcripts were quantified by real-time RT-PCR after each round of restimulation (Fig. 3*A*). Here, we found that RCC-26/CD80 cells indeed induced superior expansion of AV20 T cells; significantly more AV20 transcripts were detected compared with unmodified tumor cells in two independent experiments (see Fig. 3*A*). Therefore, costimulation by CD80 was not essential for effector-memory T-cell expansion; nevertheless, it strongly enhanced autologous AV20 T-cell proliferation *ex vivo*.

***Ex vivo* AV20 T-Cell Expansion Is Dependent on Interleukin-2.** The double-transfectant sublines were also examined for their capacity to promote expansion of AV20 T cells from PBMC-26 in MLTC experiments. As shown in Table 1, specific AV20 amplicons were detected when RCC-26/CD80/IL-2 cells, but not RCC-26/CD80/IL-7 cells, were used as stimulating cells. This distinction was noted in repeated

Table 1 *Ex vivo* expansion of AV20-positive T cells in autologous MLTC

Responder	Stimulator	CDR3 sequence*
PBMC-26	LCL-26	ND†
	NKC-26	ND†
	RCC-26	GG
	RCC-26/VC	LSG
	RCC-26/CD80	GG/LSG‡
	RCC-26/CD80/IL-2	GG
	RCC-26/CD80/IL-7	ND†

*CDR3 sequences represent the variable regions of the TCR of T-cell clones, TIL-26-GG and TIL-26-LSG. Listed are the first amino acids that are characteristic for the TIL-26-GG or TIL-26-LSG CDR3 regions.

†Characteristic TIL-26 TCR sequences were not detected (ND).

‡Different clones emerged in separate experiments.

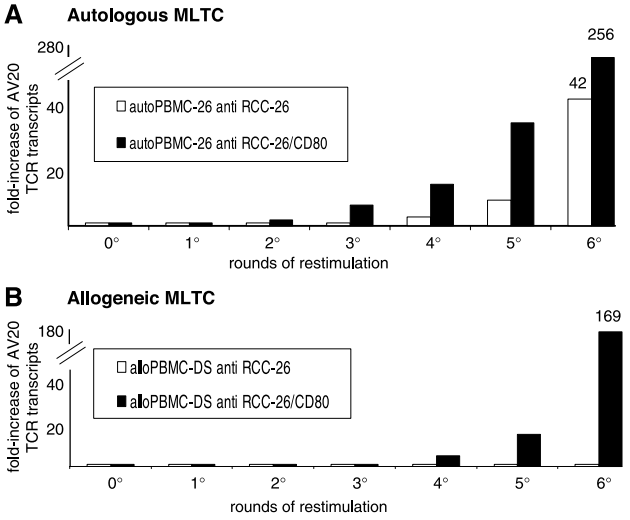

Fig. 3 Quantification of AV20 TCR transcripts by real-time RT-PCR. In an autologous MLTC experiment, PBMCs of patient 26 were stimulated with parental unmodified RCC-26 cells and RCC-26/CD80 cells. After each round of restimulation, the emergence of tumor-associated AV20AJ22 TCR transcripts was assessed quantitatively by real-time RT-PCR using primers specific for the TCR-CDR3 region of TIL-26-GG. Costimulation by CD80 was not essential for autologous effector-memory T-cell expansion; nevertheless, it strongly enhanced AV20 T-cell proliferation *ex vivo* (*A*). Representative of two independent experiments. Mean fold increase and range after each round of simulation was as follows: mean 1-fold (*0-3°*); mean 3.5-fold, range 3-4-fold (*4°*); mean 6-fold, range 3-9-fold (*5°*); mean 66-fold, range 42-90-fold (*6°*) for the RCC-26 stimulated MLTC; mean 1-fold (*0-2°*); mean 7-fold (*3°*); mean 16-fold, range 14-18-fold (*4°*); mean 27.5-fold, range 21-34-fold (*5°*); mean 330-fold, range 256-404-fold (*6°*) for the RCC-26/CD80 stimulated MLTC. *B,* quantification of AV20 TCR transcripts of an allogeneic MLTC using unmodified RCC-26 cells or RCC-26/CD80 cells for stimulation of PBMC derived from one healthy HLA-A*0201 control donor. Expansion of AV20 T cells required expression of CD80 by tumor cells because TCR transcripts were not detected in MLTC using unmodified RCC-26 cells. Representative of two independent experiments using two different HLA-A*0201 donors. Mean fold increase and range after each round of stimulation using the RCC-26/CD80 cells was as follows: mean 1-fold (*0-3°*); mean 19.5-fold, range 9-30-fold (*4°*); mean 37.5-fold, range 19-56-fold (*5°*); mean 329.5-fold, range 169-490-fold (*6°*).

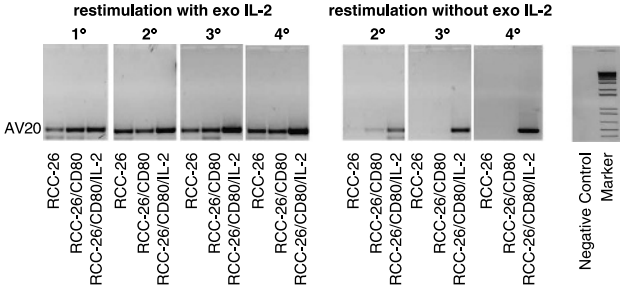

Fig. 4 *Ex vivo* AV20 T-cell expansion is dependent on IL-2. PBMC-26 were stimulated with unmodified and gene-modified RCC-26 cells for four rounds. During the first round of stimulation, low-dose IL-2 was present in all MLTC. Each MLTC was then split into two fractions and exogenous IL-2 was added to only one set of cultures. PCR amplicons represent the specific AV20 TCR transcripts. PCR in the negative control was done with water instead of cDNA.

experiments, although both sublines could be recognized equally well as target cells by TIL-26 cells (see Fig. 2*A*). They could also induce comparable cytokine secretion in the TIL-26 cells (Fig. 2*B*; Supplementary Table 2). Thus, the requirements for inducing *ex vivo* expansion of the AV20 T cells differed from those required for triggering their cytotoxic function and cytokine secretion.

The role of endogenous IL-2 expression by RCC-26/CD80/IL-2 cells could not be elucidated in these experiments because the MLTCs were supplemented with exogenous IL-2. Therefore, an experimental adaptation was made in the MLTC strategy to allow us to directly assess the role of endogenous IL-2 production. Low-dose exogenous IL-2 was added to the initial MLTC, but after 7 days each combination was split into two fractions and exogenous IL-2 was provided to only one set of cultures. Increases in AV20 transcripts were then analyzed following further rounds of restimulation in the MLTC. The results of one of three representative experiments using this strategy are presented in Fig. 4. AV20 amplicons were detected through all rounds of restimulation in the cultures supplemented with exogenous IL-2, but AV20 band intensities were consistently greater when RCC-26/CD80/IL-2 cells were used as stimulating cells. In contrast, a continual decrease in AV20 amplicon intensity occurred with RCC-26 and RCC-26/CD80 stimulation in the absence of exogenous IL-2, and by the third round of restimulation, an AV20 band was only detected when RCC-26/CD80/IL-2 cells were used for stimulation. Thus, the RCC-26/CD80/IL-2 subline provided sufficient endogenous IL-2 to maintain AV20 T-cell expansion. The requirement for an initial startup with exogenous IL-2 may be due to the fact that the tumor cells

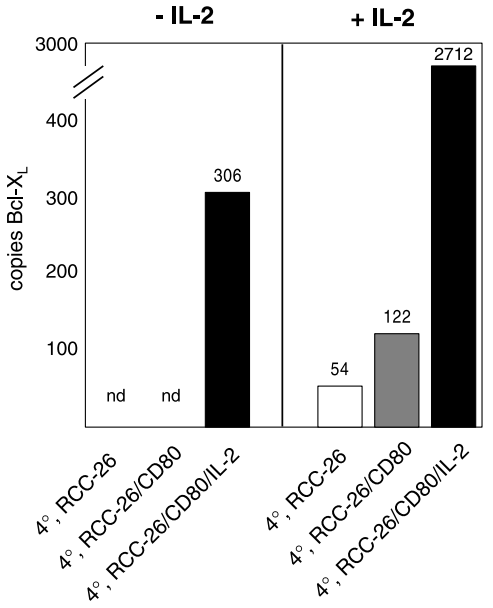

Fig. 5 Induction of Bcl-x_L by CD80 costimulation and IL-2. Expression of the antiapoptotic Bcl-x_L message in activated lymphocytes was assessed by real-time RT-PCR after four rounds of restimulation in cells derived from the autologous MLTC shown in Fig. 4. Transcripts were not detected (*nd*).

may not secrete adequate amounts of IL-2 to initiate proliferation of quiescent T cells, but after initial reactivation of the T cells the levels of IL-2 production were sufficient to maintain AV20 T-cell expansion. The results of these studies revealed the essential role of IL-2 in the expansion of AV20 T cells.

Because long-term proliferation and survival of activated T cells is associated with expression of the antiapoptosis factor Bcl-x_L (13, 29), we quantified the Bcl-x_L transcripts in the lymphocytes obtained from the MLTC experiment shown in Fig. 4 after the fourth restimulation (Fig. 5). In MLTC lacking exogenous IL-2, Bcl-x_L was only detected when RCC-26/CD80/IL-2 cells were used for stimulation, indicating that IL-2R signaling in the responding cells was essential to retain Bcl-x_L expression. Substantially more transcripts were detected in cultures supplemented with exogenous IL-2; however, only low-copy numbers were present in the MLTC using RCC-26 and RCC-26/CD80 stimulating cells, whereas the MLTC with RCC-26/CD80/IL-2 cells had a substantially higher Bcl-x_L copy number. These results revealed that IL-2 affected not only the quantity but also the quality of the expanding AV20 T cells.

Induction of Tumor-Associated CTL in Allogeneic Mixed Lymphocyte Tumor Cell Cultures. To test the capacity of RCC-26 cells to induce RCC-associated CTL in allogeneic donors, we established MLTC using unmodified RCC-26 cells and the different RCC-26/CD80-expressing sublines as stimulating cells for PBMC derived from two unrelated healthy control donors, who shared either HLA-A*0201 or HLA-B*5101 with RCC-26 cells. When the bulk MLTC were tested for CTL activity after four to six rounds of restimulation, high levels of alloreactivity directed against both RCC-26 and LCL-26 cells were found (data not shown). Because the activity of allospecific CTL will mask detection of tumor-associated CTL, we next established limiting dilution cultures from bulk MLTC and screened for CTL recognizing both RCC-26 and LCL-26 cells (i.e., alloreactive T cells) versus CTL recognizing only RCC-26 cells (i.e., tumor-associated T cells). The majority of T cells were allospecific but some cultures derived using 1 or 0.5 cells per well showed strong killing of unmodified RCC-26 cells but not LCL-26 cells (Table 2). Such RCC-associated CTLs were only detected following priming with CD80-expressing RCC-26 cells, as would be expected for naive T cells that require TCR as well as CD28 costimulatory signals. No overt differences were noted between the RCC-26 cell lines coexpressing IL-2 or IL-7 with respect to their *de novo* priming capacity to stimulate naive T cells to develop into CTL with alloreactive or RCC-associated specificity.

Interestingly, when we analyzed the TCR of the various tumor-associated clones of donor DS, we found that clone DS-0.5E8 expressed a AV20 TCR sequence that was strikingly similar to the AV20 sequences characteristic of the TIL-26 cells. Therefore, we could use our AV20-specific primers to quantify the emergence of one type of RCC-associated T cell by measuring AV20 transcripts over time. Figure 3*B* shows the amplification of AV20 transcripts in an independent allogeneic MLTC established by stimulating PBMC-DS with unmodified RCC-26 cells or RCC-26/CD80 cells. Expansion of AV20 T cells required expression of CD80 by the tumor cells because TCR transcripts were not detected in the MLTC using RCC-26 cells, although high numbers of alloresponsive CTL were induced (data not shown). Expansion of AV20 T cells in a second HLA-A*0201$^+$ donor was also found to be CD80 dependent (see Fig. 3). These experiments revealed that unmodified RCC-26

Table 2 Priming of RCC-associated allogeneic CTL with gene-modified RCC-26 tumor cells

Clones	Cytokine*	% Lysis LCL-26	% Lysis RCC-26	Classification
DS-5E3†	IL-2	24	4	Allogeneic‡
DS-0.5G6	IL-2	28	10	Allogeneic
DS-0.5F10	IL-2	7	34	RCC-assoc.§
DS-0.5E8‖	IL-2	0	39	RCC-assoc.
JB-1E9	IL-2	11	26	Allogeneic
JB-05F4	IL-2	13	11	Allogeneic
JB-0.5F10	IL-2	15	21	Allogeneic
JB-1E8	IL-2	1	31	RCC-assoc.
JB-0.5E6	IL-2	1	47	RCC-assoc.
DS-5C5	IL-7	18	11	Allogeneic
DS-5D3	IL-7	21	11	Allogeneic
DS-5D6	IL-7	53	36	Allogeneic
DS-1D9	IL-7	16	4	Allogeneic
DS-1C4	IL-7	7	25	RCC-assoc.
DS-0.5D6	IL-7	1	26	RCC-assoc.
DS-0.5C4	IL-7	0	23	RCC-assoc.
DS-0.5D3	IL-7	1	58	RCC-assoc.
JB-5C9	IL-7	47	34	Allogeneic
JB-5C4	IL-7	54	31	Allogeneic
JB-5D9	IL-7	65	47	Allogeneic
JB-1D10	IL-7	3	40	RCC-assoc.

*Cytokine: expressed by the RCC-26/CD80 gene-modified line.
†The first number represents number of plated cells per well (5, 1, or 0.5 cells per well).
‡Kill both LCL-26 and RCC-26.
§Kill only RCC-26.
‖Expresses AV20 TCR.

Table 3 Expression of TAA candidates in RCC-26 tumor cells

TAA	RCC-26/ CD80/ IL-2	RCC-26/ CD80/ IL-7	References
Carbonic anhydrase IX (G250)	23	46	(39, 40)
Preferentially expressed antigen in melanoma (PRAME)	20	36	(41)
Oncofetal antigen immature laminin receptor (OTA)	18	26	(42)
Survivin	4,705	6,746	(43)
Adipophilin	41	67	(44)

NOTE. *n*-fold overexpression: quantitative comparisons of mRNA expression of each TAA were made in RCC-26 sublines and compared with control healthy normal kidney tissue using real-time RT-PCR. Candidate molecules were selected from the literature that were described to be overexpressed in RCC or in a variety of tumors.

cells had an inherent immunogenic potential to induce allospecific CTL responses *de novo*, whereas induction of tumor-associated CTL from naive T-cell repertoires required that RCC-26 cells also expressed CD80.

Expression of Tumor-Associated Antigens in RCC-26. To identify TAAs that could be used for immune monitoring, we analyzed the RCC-26 double-transfectant sublines for their expression of mRNA encoding various TAA. These candidates were compared in the two sublines foreseen for clinical study using quantitative real-time RT-PCR. Substantial differences in expression were noted for all candidate TAAs when the two potential vaccine sublines were compared with normal kidney tissue controls (Table 3), demonstrating their potential relevance for differential T-cell recognition. Due to the substantial overexpression of these TAA in both vaccine variants, pMHC ligands derived from these TAAs may be able to induce T-cell responses *in vivo*. Thereby, these TAA are potential surrogate markers for immune monitoring of vaccine trials based on the use of whole tumor cells.

DISCUSSION

Extensive effort has been invested in developing autologous gene-modified tumor cell vaccines for various types of malignancy; however, strong limitations in feasibility and cost are associated with this approach (30). Therefore, generic strategies are needed, such as the use of allogeneic tumor cell vaccines, which can be applied in multiple patients. The natural immunogenicity of RCC-26 cells favors their use for vaccine development. We found that RCC-26 cells induced CTL in the PBMC of healthy allogeneic donors, whereby induction of allospecific CTL did not require CD80 expression by the tumor cells but CD80 expression was required to induce CTL with tumor-associated specificity. The capacity of RCC-26/CD80 cells to activate surprisingly high numbers of RCC-associated CTL indicates that stimulation of alloreactivity did not strongly impede development of antitumor immunity.

To better dissect the effect of gene modification on the immunogenicity of RCC-26 cells, we analyzed autologous T-cell responses to avoid influences of the rich cytokine milieu associated with ongoing alloresponses. We followed the development of AV20 T cells as a surrogate marker to track the development of T-cell responses. We found that expansion of autologous tumor-specific T cells bearing AV20-specific TCR only occurred in MLTC that included RCC-26 cells. Therefore, pMHC-TCR signaling was required to initiate proliferation of these T cells. Because AV20 T cells expanded in the presence of exogenous IL-2 following stimulation with unmodified RCC-26 cells, CD28 signaling was not essential to stimulate their proliferation. Nevertheless, CD80 stimulation supported T-cell expansion because AV20 transcripts were detected at earlier times and greater numbers of transcripts were present when RCC-26 cells expressed CD80.

Additional RCC-26 sublines were studied which expressed various cytokines that influence lymphocyte responses, including IFN-γ, IFN-α, IL-2, and IL-7 (refs. 31, 32; data not shown). After initial screening studies, we elected to create RCC-26 vaccine variants expressing CD80 in combination with IL-2 or IL-7. IL-2 was of interest because of its well-established clinical effect in immunotherapy of RCC (1, 2) and IL-7 was chosen because of its important role in supporting the development of antigen-independent CD4 T-cell responses, its contribution to cross-presentation, and its effect on establishing long-term T-cell memory (33).

We observed that both double-transgenic RCC-26 sublines were able to activate cytokine release and induce cytotoxicity in TIL-26 effector cells. However, they differed in their ability to stimulate expansion of autologous effector-memory AV20 T cells circulating in PBMC of patient 26. Optimal AV20 T-cell proliferation occurred using RCC-26/CD80/IL-2 cells and only this subline supported expansion in the absence of exogenous IL-2. Clearly, pMHC signaling alone was not sufficient to drive T-cell proliferation and lack of IL-2 was not compensated by CD80 stimulation alone. Similar studies of Wells et al. (34) showed that T cells receiving a TCR signal in the absence of CD28 costimulation entered a state of anergy that could be overcome by IL-2 but not by CD28 stimulation. The failure of RCC-26/CD80/IL-7 cells to support AV20 T-cell expansion revealed that IL-7 could not substitute for IL-2. It remains unclear why this is the case. If AV20 T cells expressing IL-7R do exist *in vivo*, most likely they represent central memory T cells that may not be circulating in the peripheral blood of patient 26 (35). In contrast, the RCC-26/CD80/IL-7 cells were equally potent to the RCC-26/CD80/IL-2 cells in *de novo* priming of allospecific and RCC-associated CTL in naive PBMC. This revealed that the differences with respect to levels of CD80 and cytokine expression did not affect substantially their overall stimulation capacity. Stimulation of allospecific PBMC by both sublines also led to up-regulation of CD40L, revealing the capacity of both vaccine candidates to induce full T-cell activation.[7] Therefore, both lines are suitable vaccine candidates for use in an allogeneic setting.

Both CD28 and IL-2R signaling were shown previously to support long-term survival of activated CTL by enhancing expression of the antiapoptotic factor Bcl-x_L (36–38). IL-2 expression by RCC-26 cells played by far the strongest role in increasing expression of Bcl-x_L in the lymphocytes responding in MLTC (Fig. 5). This contention was supported by the

[7] B. Frankenberger, unpublished observations.

observation that Bcl-x_L was only detected when RCC-26/CD80/IL-2 cells were used for stimulation in cultures lacking exogenous IL-2. If RCC patients have preexisting effector-memory CTL whose corresponding ligands are expressed by RCC-26/CD80/IL-2 cells, they can receive simultaneous signals that may allow their reactivation, expansion, and expression of Bcl-x_L to protect them from apoptosis *in vivo*.

The use of whole tumor cells as allogeneic vaccines presents several challenges for immune monitoring. The detection of allospecific CTL can be used as one measure of the ability of tumor vaccination to induce immune responses in patients with advanced disease. Nevertheless, a better understanding of antitumor immunity would be gained if responses to specific TAA could be analyzed. We identified several candidate molecules to be expressed by our RCC-26 vaccine cells, including carbonic anhydrase IX (G250), adipophilin, preferentially expressed antigen in melanoma, oncofetal antigen, or survivin. Some of these TAAs have already been shown to induce tumor-associated T cells in RCC patients (39–42) or in patients with other solid tumors (43, 44). Because HLA-A2-restricted epitopes are known for several of these TAAs, synthetic peptides can be used to monitor the emergence of specific T cells in vaccinated patients using ELISPOT assays, cytokine capture assays, or tetramer staining. Finally, AV20-expressing T cells with RCC specificity may also emerge in some patients following vaccination with gene-modified RCC-26 cells as seen following *in vitro* priming of PBMC of allogeneic donors. Therefore, molecular tracking of TCR sequences provides yet another tool to follow immune responses developing in patients vaccinated with the RCC-26-based vaccines.

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REFERENCES

1. Motzer RJ. Renal cell carcinoma: a priority malignancy for development and study of novel therapies. J Clin Oncol 2003;21:1193–4.

2. Kim HL, Belldegrun AS, Figlin RA. Immune gene therapy for kidney cancer: the search for a magic trigger. Mol Ther 2003;7:153–4.

3. Schendel DJ, Oberneder R, Falk CS, et al. Cellular and molecular analyses of MHC-restricted and non-MHC-restricted effector cells recognizing renal cell carcinomas: problems and perspectives for immunotherapy [review]. J Mol Med 1997;75:400–13.

4. Schleypen JS, Von Geldern M, Weiss EH, et al. Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. Int J Cancer 106:905–12.

5. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N Engl J Med 2000;343:750–8.

6. Roigas J, Massenkeil G. Nonmyeloablative allogeneic stem cell transplantation in metastatic renal cell carcinoma: a new therapeutic option or just a clinical experiment? World J Urol. In press.

7. Figlin RA, Pierce WC, Kaboo R, et al. Treatment of metastatic renal cell carcinoma with nephrectomy, interleukin-2 and cytokine-primed or CD8(+) selected tumor infiltrating lymphocytes from primary tumor. J Urol 1997;158:740–5.

8. Nakano O, Sato M, Naito Y, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res 2001;61:5132–6.

9. Bromwich EJ, McArdle PA, Canna K, et al. The relationship between T-lymphocyte infiltration, stage, tumour grade and survival in patients undergoing curative surgery for renal cell cancer. Br J Cancer 2003;89:1906–8.

10. Belldegrun A, Muul LM, Rosenberg SA. Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. Cancer Res 1988;48:206–14.

11. Schendel DJ, Gansbacher B, Oberneder R, et al. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines. J Immunol 1993;151:4209–20.

12. Jantzer P, Schendel DJ. Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence *in vivo*. Cancer Res 1998;58:3078–86.

13. Becker C, Pohla H, Frankenberger B, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. Nat Med 2001;7:1159–62.

14. Falk CS, Noessner E, Weiss EH, Schendel DJ. Retaliation against tumor cells showing aberrant HLA expression using lymphokine activated killer-derived T cells. Cancer Res 2002;62:480–7.

15. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol 2002;2:116–26.

16. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 1992;71:1093–102.

17. Townsend SE, Allison JP. Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science 1993;259:368–70.

18. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990;248:1349–56.

19. Sturmhoefel K, Lee K, Gray GS, et al. Potent activity of soluble B7-IgG fusion proteins in therapy of established tumors and as vaccine adjuvant. Cancer Res 1999;59:4964–72.

20. Lee MJ, Choi SH, Shim YH, Cho SH, Kim CW. Tumor removal enhances immunity induced by B7.1. Pathobiology 2001;69:281–8.

21. Cayeux S, Beck C, Dorken B, Blankenstein T. Coexpression of interleukin-4 and B7.1 in murine tumor cells leads to improved tumor rejection and vaccine effect compared to single gene transfectants and a classical adjuvant. Hum Gene Ther 1996;7:525–9.

22. Daniel PT, Schulze-Osthoff K, Belka C, Guner D. Guardians of cell death: the Bcl-2 family proteins. Essays Biochem 2003;39:73–88.

23. Schendel DJ, Frankenberger B, Jantzer P, et al. Expression of B7.1 (CD80) in a renal cell carcinoma line allows expansion of tumor-associated cytotoxic T lymphocytes in the presence of an alloresponse. Gene Ther 2000;7:2007–14.

24. Uckert W, Willimsky G, Pedersen T, Blankenstein T, Pedersen L. RNA levels of human retrovirus receptors Pit1 and Pit2 do not correlate with infectibility by three retroviral vector pseudotypes. Hum Gene Ther 1998;9:2619–27.

25. Rittner K, Stöppler H, Pawlita M, Sczakiel G. Versatile eucaryotic vectors for strong and constitutive transient and stable expression. Methods Mol Cell Biol 1991;2:176–81.

26. Westermann J, Aicher A, Qin Z, et al. Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. Gene Ther 1998;5:264–71.

27. Schendel DJ, Gansbacher B. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes: modulation of recognition through retroviral transduction of tumor cells with interleukin 2 complementary DNA and exogenous α interferon treatment. Cancer Res 1993;53:4020–5.

28. Pohla H, Frankenberger B, Stadlbauer B, et al. Allogeneic

vaccination for renal cell carcinoma: development and monitoring. Bone Marrow Transplant 2000;25:83–7.

29. Gett AV, Sallusto F, Lanzavecchia A, Geginat J. T cell fitness determined by signal strength. Nat Immunol 2003;4:355–60.

30. Parmiani G, Rodolfo M, Melani C. Immunological gene therapy with *ex vivo* gene-modified tumor cells: a critique and a reappraisal. Hum Gene Ther 2000 Jun 10;11:1269–75.

31. Noessner E, Falk CS, Schendel DJ. Interferon α and γ differentially alter the susceptibility of tumor cells to lysis by MHC-restricted and non-MHC-restricted cytotoxic effector cells. In: Hiddemann W, Büchner T, Ritter J, Unterhalt M, Haferlach T, editors. New York: Springer-Verlag; 2003. p. 497–503.

32. Schendel DJ, Falk CS, Nossner E, et al. Gene transfer of human interferon γ complementary DNA into a renal cell carcinoma line enhances MHC-restricted cytotoxic T lymphocyte recognition but suppresses non-MHC-restricted effector cell activity. Gene Ther 2000 Jun;7:950–9.

33. Cayeux S, Richter G, Noffz G, Dorken B, Blankenstein T. Influence of gene-modified (IL-7, IL-4, and B7) tumor cell vaccines on tumor antigen presentation. J Immunol 1997;158:2834–41.

34. Wells AD, Walsh MC, Bluestone JA, Turka LA. Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. J Clin Invest 2001;108:895–903.

35. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol 2003;12(4):1191–8.

36. Kerstan A, Hunig T. Cutting edge: distinct TCR- and CD28-derived signals regulate CD95L, Bcl-X_L, and the survival of primary T cells. J Immunol 2004;172:1341–5.

37. Akbar AN, Borthwick NJ, Wickremasinghe RG, et al. Interleukin-2 receptor common γ-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-x_L) but not pro-apoptotic (bax, bcl-x_S) gene expression. Eur J Immunol 1996;26:294–9.

38. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. Immunity 1995;3:87–98.

39. Vissers JL, De Vries IJ, Schreurs MW, et al. The renal cell carcinoma-associated antigen G250 encodes a human leukocyte antigen (HLA)-A2.1-restricted epitope recognized by cytotoxic T lymphocytes. Cancer Res 1999 Nov 1;59:5554–9.

40. Su Z, Dannull J, Reiser A, et al. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res 2003;63:2127–33.

41. Kessler JH, Beekman NJ, Bres-Vloemans SA, et al. Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. J Exp Med 2001;193:73–88.

42. Höltl L, Zelle-Reiser C, Gander H, et al. Immunotherapy of metastatic renal cell carcinoma with tumor lysate-pulsed autologous dendritic cells. Clin Cancer Res 2002;8:3369–76.

43. Schmidt SM, Schag K, Muller MR, et al. Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells. Blood 2003;102:571–6.

44. Schmidt SM, Schag K, Muller MR, et al. Induction of adipophilin-specific cytotoxic T lymphocytes using a novel HLA-A2-binding peptide that mediates tumor cell lysis. Cancer Res. 2004;64:1164–70.