Diverse CD8+ T-Cell Responses to Renal Cell Carcinoma Antigens in Patients Treated with an Autologous Granulocyte-Macrophage Colony-Stimulating Factor Gene-Transduced Renal Tumor Cell Vaccine

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Abstract

A phase I clinical trial with granulocyte-macrophage colony-stimulating factor tumor cell vaccines in patients with metastatic renal cell carcinoma (RCC) showed immune cell infiltration at vaccine sites and delayed-type hypersensitivity (DTH) responses to autologous tumor cells indicative of T-cell immunity. To further characterize RCC T-cell responses and identify relevant RCC-associated antigens, we did a detailed analysis of CD8+ T-cell responses in two vaccinated RCC patients who generated the greatest magnitude of DTH response and also displayed a strong clinical response to vaccination (>90% reduction in metastatic tumor volume). Three separate CD8+ T-cell lines (and subsequent derived clones) derived from patient 24 recognized distinct RCC-associated antigens. One recognized a shared HLA-A*0201-restricted antigen expressed by both renal cancer cells and normal kidney cells. This recognition pattern correlated with a positive DTH test to normal kidney cells despite no evidence of impairment of renal function by the patient's remaining kidney after vaccination. A second line recognized a shared HLA-C7-restricted antigen that was IFN-γ inducible. A third line recognized a unique HLA-A*0101-restricted RCC antigen derived from a mutated *KIAA1440* gene specific to the tumor. In addition, two independent CTL lines and three clones were also generated from patient 26 and they recognized autologous tumor cells restricted through HLA-A*0205, HLA-A/B/C, and HLA-B/C. These results show that paracrine granulocyte-macrophage colony-stimulating factor tumor vaccines may generate a diverse repertoire of tumor-reactive CD8+ T-cell responses and emphasize the importance of polyvalency in the design of cancer immunotherapies. (Cancer Res 2005; 65(3): 1079-88)

Introduction

Technologic advances have led to the identification of numerous tumor-associated antigens, particularly those associated with malignant melanoma (1–3). As a result, a variety of immunization approaches testing the immunogenicity of these identified antigens are now under development for the treatment of melanoma in clinics. However, until significant numbers of nonmelanoma tumor antigens are identified that are the targets of tumor-specific T cells, whole tumor cell–based vaccines are required for successful immune priming against antigenically heterogenous tumor burdens (4). Because they are so highly polyvalent, whole tumor cell vaccines provide an opportunity to reveal the diversity of the tumor-specific T-cell repertoire.

An early comparison study evaluating tumor lines transduced with multiple cytokine genes showed that granulocyte-macrophage colony-stimulating factor (GM-CSF)–transduced tumor cell vaccines induced the most potent, specific, and long-lasting systemic antitumor immunity in poorly immunogenic murine models (5). Subsequently, this vaccine approach has induced antitumor immune responses in patients with renal cell carcinoma (RCC; ref. 6), melanoma (7), prostate cancer (8), and pancreatic cancer (9). The therapeutic activity of GM-CSF is now thought to be due to the paracrine action of the cytokine on dendritic cell differentiation and proliferation at the vaccine site (5–10).

A phase I clinical study in patients with metastatic RCC vaccinated with GM-CSF-transduced autologous tumor cell vaccines showed that this vaccine approach induces local immune responses that are similar to what have been observed in preclinical models (6). Specifically, the vaccination sites were infiltrated with dendritic cells, eosinophils, and neutrophils. In addition, three vaccinated patients receiving a bioactive dose of 4×10^7 vaccine cells mounted delayed-type hypersensitivity (DTH) responses against both autologous tumor and normal kidney cells, but not against autologous peripheral blood lymphocytes (PBL), suggesting that systemic antitumor immunity was established *in vivo* (6). Unlike the vaccine sites, immunohistochemical staining of biopsies of the DTH responses showed intense infiltration of predominantly CD4+ and CD8+ T cells in addition to a prominent eosinophil component.

To further evaluate these CD8+ T-cell responses, CD8+ T cells were isolated and characterized from the PBLs of two patients who showed a postvaccination DTH response that was associated with a partial clinical response. In this report, we show that RCC-specific CD8+ T-cell lines and clones with diverse specificity can be readily

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induced following GM-CSF vaccination. Our results suggest that immunization with the bioactive GM-CSF tumor cell vaccine may induce a diverse T-cell repertoire that recognizes shared, unique, and inducible antigens.

Materials and Methods

Cell Lines and Culture Conditions. RCC cell lines and normal kidney cells (NKC) were derived from surgical samples of patients with RCC enrolled in a phase I clinical trial (6), using methods established in our laboratory (10). Informed consent for this work was obtained from all patients. RCC24 and RCC26 tumor cell lines expressing the costimulatory molecule B7-1 (RCC24B7 and RCC26B7) were developed by retroviral transduction (5, 10). RCC and NKC cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (HyClone, Logan, UT), 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI), 1% nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and antibiotics.

EBV–transformed B-cell lines were generated at the Johns Hopkins EBV Core Facility and maintained in EBV medium consisting of RPMI 1640, 10% fetal bovine serum, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and antibiotics. Melanoma cell line Balmel, EBV-transformed B-cell line Bal-EBV-B, and T-cell clone C-9 recognizing HLA-A*0101-restricted melanoma peptide RSDSQQQARY derived from AIM-2 (antigen isolated from immunoselected melanoma-2) cDNA were previously described (11).

Human Leukocyte Antigen Typing. Human leukocyte antigen (HLA) serotyping of cell lines was done by the Johns Hopkins University Immunogenetics Facility. The HLA class I genotypes for the patients analyzed in this study were HLA-A*0101/0201, B39/62, Cw*07/02 (RCC patient 24); HLA-A*0205/1101, B*3503/5801, Cw*0302/1203 (RCC26); HLA-A*0101, B8, Cw7 (RCC1); HLA-A*0101, A25, B8, B18, Cw7 (RCC4); HLA-A*0101, A2, B8, B17, Cw6, Cw7 (RCC5); HLA-A2, A11, B38, B51, Cw1 (RCC6); HLA-A2, A26, B18, B41, Cw7 (RCC11); HLA-A*0101, A24, B6, B7, Cw7 (RCC18); HLA-A3, B35, B49, Cw7 (RCC27); HLA-A24, A33, B17, B51, Cw1, Cw7 (RCC28); HLA-A2, B27, B39 (RCC-Fr); HLA-A*0101, A25, B7, B57, Cw6, Cw7 (RCC-Lo); HLA-A*0101, A2, B57, B62 (RCC-Sa).

Generation of CTL Lines and Clones. CTL lines were generated from PBLs of patients 24 and 26 at day 92 or 108 post vaccination, respectively. All patients provided informed consent on an Institutional Review Board–approved clinical trial that allowed collection and analysis of PBLs and tumor cells for this purpose. By using anti-CD4–coated AIS CELLector flasks (Applied Immune Sciences, Menlo Park, CA), 1×10^6 $CD4^+$ T cell–depleted PBLs were cocultured with 2×10^5 irradiated (16 Gy) RCC24B7 or RCC26B7 cells per well in 24-well plates in 2 mL of human T-cell medium (RPMI 1640, 10% human serum, 2 mmol/L L-glutamine, 10 mmol/L HEPES, and antibiotics).

All T-cell clones were generated from the parent T-cell lines by limiting dilution. The 1.24CTL clones 2, 3, 8, 15, 32, and 46 were generated from the 1.24CTL1 line. Clone 5B was derived from the 1.24CTL2 line. The 17/A2 clone was derived from 1.24CTLIFN-γ line that was generated by stimulation of the CD8 T cells with IFN-γ–treated RCC24B7 cells. 1.26CTL1 line and 1.26CTL2 line were generated independently. 1.26CTL clones 3, 24, and 26 were derived from 1.26CTL2 line. Expansion of T-cell clones was carried out in 24-well plates with tumor cells or OKT3 as previously described (12).

Monoclonal Antibodies and Flow Cytometry (Fluorescence-Activated Cell-Sorting) Analysis. Monoclonal antibodies (mAbs) against HLA-A*0101 (0289HA) were purchased from One Lambda (Canoga, CA). Goat anti-mouse immunoglobulin G + immunoglobulin M conjugated with FITC was purchased from Caltag (Burlingame, CA). Flow cytometry analysis was done on a FACScan cytometer using the CellQuest software (Becton Dickinson, San Diego, CA). Hybridomas secreting mAbs including W6/32 (pan anti-HLA-A, B, C), MA2.1 (anti-HLA-A2 and -B17), BB7.2 (anti-HLA-A2), B1.23.2 (anti-HLA-B, C), and MBL (anti-*Schistosoma mansoni* antigen) were purchased from the American Type Culture Collection (Manassas, VA). CR11-351 (anti-HLA-A2 and -A28) was generously provided by Dr. Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY; ref. 13). These mAbs were immunoaffinity purified and used for functional blocking experiments.

Synthetic Peptides and Competitive Peptide-Binding Assay. All peptides with greater than 90% purity were purchased from Macromolecular Resources at Colorado State University (Fort Collins, CO). They were dissolved in DMSO at a concentration of 4 mmol/L and stored at −20°C. Competitive peptide-binding assay was carried out as previously described (14, 15). Briefly, C1R-A1 cells (provided by Dr. Craig Slingluff Jr., University of Virginia, Charlottesville, VA) were incubated at 50,000 cells per well in a 96-well U-bottomed plate with 150 nmol/L of fluorescence-labeled reference peptide CTELK(FLU)LSDY (Biopeptide Co., LLP, San Diego, CA), a known HLA-A*0101-binding peptide derived from influenza $NP_{(44-52)}$ (16), and serial dilutions of peptide substitutes (test peptides) in the presence of 1.5 μg/mL of human β2-microglubulin (Sigma) at 4°C for 24 hours.

Cytolytic and Cytokine Release Assays. T-cell cytotoxicity assay was done using a standard 4-hour chromium (^{51}Cr) release assay (17). Cytokine release assay was done by ELISA kits (R&D Systems, Minneapolis, MN) or tumor necrosis factor (TNF) was detected on WEHI 164 clone 13 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (18).

IFN-γ Enzyme-Linked Immunospot Assay. IFN-γ enzyme-linked immunospot (ELISPOT) assays were done as previously described (19). K562B7A1 (K562 cells transduced with lentiviral human B7.1 and HLA-A1) target cells (1×10^5/well) pulsed with peptide (10 μg/mL) were incubated with Miltenyi bead–purified $CD8^+$ T cells (1×10^5 per well), in total 200 μL T-cell media per well in replicates of six overnight at 37°C. The pool of 32 cytomegalovirus, EBV, and influenza virus (CEF) peptides that stimulate $CD8^+$ T cells to produce IFN-γ were purchased from NIAID, NIH (http://www.aidsreagent.org) and used as positive control peptides (20).

CDNA Library Construction and Screening. Total RNA was extracted from RCC24 cells (passage 10) using TRIZOL reagent (Invitrogen). PolyA RNA was purified from total RNA by the mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ) and converted to cDNA with the cDNA synthesis kit (Stratagene, La Jolla, CA) using an oligodeoxythymidylic acid primer. The cDNA was expressed in pcDNA3.1/His A vector (Invitrogen). Recombinant plasmid was electroporated into *Escherichia coli* DH10B cells (Invitrogen). The library was divided into pools of 100 cDNA clones. Each pool of bacteria was grown in deep-well culture blocks (Edge Biosystems, Gaithersburg, MD), and plasmid was extracted using the QIAprep 96 Miniprep Kit (Qiagen, Valencia, CA).

The cDNA library screening was done in duplicate in 96-well flat-bottom plates using transient transfection of COS-7 cells with LipofectAMINE 2000 (Invitrogen) as previously described (11).

PCR Assay for Sequencing of the Mutation. The *KCAG1* gene mutation was analyzed by subcloning the epitope region (n.t.1130 to 1341) into the PCR Blunt TOPO vector with TOPO kit (Invitrogen). This was done for EBV24, NKC24, RCC24, and other RCC tumor cell lines including RCC1, RCC4, RCC11, RCC18, RCC26, RCC-Fr, RCC-Lo, and RCC-Sa. The sense primer sequence was KCAG1-RT5F, 5′-GTG CAG TGT CTG TGC GAG TT-3′, and the antisense primer sequence was KCAG1-RT5R, 5′-CCG CCA GAA GTA GTC CAG CA-3′. Three clones from each cell line were sent to the Johns Hopkins University DNA Facility for sequencing analysis.

Generation of HLA-A*0101, HLA-A*0201, and Human B7.1 Lentivirus and Gene Transduction. HLA-A*0101 and HLA-A*0201 cDNA were cloned from the total RNA of the RCC24 and EBV24 cells using standard reverse transcription–PCR (RT-PCR) with HLA class I–specific primers (21). HLA-A*0101 or HLA-A*0201 cDNA were expressed in the HIV-1-based lentiviral vector pEF1α-GFPsin, respectively (22, 23). K562B7A1 and K562B7A2 cells were generated by transduction of K562 cells with lentivirus human B7.1. After sorting of B7.1 positive K562 cells by fluorescence-activated cell-sorting analysis, K562B7.1 cells were further transduced with HLA-A1 or HLA-A2 lentivirus. Both lines were confirmed by antibody staining, and presentation of HLA-A1- and HLA-A2-restricted influenza M1 or melanoma AIM-2 antigens (11) to relevant T cells.

Results

1.24CTL Line and Clones Lyse HLA-A2$^+$ Autologous Tumor Cells, Autologous NKCs, a Panel of Allogeneic RCC Lines, and Nonhematopoietic Cell Lines. Three bulk T-cell lines designated 1.24CTL1, 1.24CTL2, and 1.24CTLIFN-γ were established from lymphocytes isolated from RCC patient 24 post vaccination. To characterize the 1.24CTL1 line, six T-cell clones (2, 3, 8, 15, 32, and 46) were compared with 1.24CTL1 cells from which they derived. As shown in Table 1, all of these T-cell clones have similar target cell recognition for the autologous RCC cells when compared with the parental 1.24CTL1 line. 1.24CTL1 line and clones all recognize the autologous RCC line with or without B7-1 expression as well as an autologous NKC. They also recognize other HLA-A2$^+$ allogeneic RCC lines including RCC5, RCC11, RCC-Fr, and RCC-Sa. Furthermore, All HLA-A2$^-$ RCC lines (RCC1, RCC4, RCC18, RCC28, and RCC-Lo) transduced with HLA-A2 lentiviral vector stimulated TNF-α release by #15 and #32 T-cell clones, whereas all HLA-A2$^-$ RCC lines transduced with green fluorescent protein failed to do so (data not shown). Taken together, these data strongly suggest that the 1.24CTL1 line and its clones recognize an antigen shared by RCC tumors and NKCs. In contrast, these T cells do not recognize EBV-transformed B cells (Table 1) or HLA-A2$^+$ myeloma and leukemia cell lines (data not shown).

To confirm the MHC class I restriction of the 1.24CTL1 line and its clones, ^{51}Cr release and cytokine release assays were done against a panel of target cells pretreated with HLA class I blocking mAbs. The recognition of autologous RCC24, NKC24, and other HLA-A2$^+$ RCC lines by 1.24CTL1 and its clones was almost completely blocked by the anti-HLA-A, B, and C antibody, W6/32, and two anti-HLA-A2-specific mAbs, BB7.2 and CR11-351. In contrast, the anti-HLA-B and C antibody B1.23.2 and an irrelevant mAb MBL did not inhibit T-cell cytolysis or cytokine release (data not shown). These experiments confirm that target cell recognition by the 1.24CTL1 line and its clones are HLA-A2 restricted.

Recognition of HLA-A2-Transfected COS-7 Cells by the 1.24CTL1 Line and Clones. One of our goals is to identify the antigens recognized by these T-cell lines and clones. Therefore, we tested the 1.24CTL1 line and clones for recognition of COS-7 cells to examine the signal-to-noise ratio before cDNA library screening for antigen identification. Consistently, COS-7 cells transfected with HLA-A*0201 cDNA alone stimulated high TNF-α release by the 1.24CTL1 line and its clones. In contrast, mock-transfected COS-7 cells or COS-7 cells transfected with HLA-A*0101 cDNA were not recognized by these T-cell clones (data not shown), suggesting that the antigen recognized by 1.24CTL1 and its clones is also expressed by the COS-7 monkey-derived NKCs. Furthermore, screening of other transfectable cell lines such as 293 (human embryonic kidney cells) transfected with HLA-A*0201 also gave a high background (data not shown). This high background recognition of COS-7 cells and the few other known transfectable cell lines by 1.24CTL1 and clones precludes the identification of this antigen using expression cloning.

1.24CTLIFN-γ-Derived 17/A2 Clone Only Recognizes IFN-γ-Treated RCC Cells. CTL clone 17/A2 was derived from 1.24CTLIFN-γ line that was generated by repeated stimulation with IFN-γ-treated RCC24 cells. The 17/A2 T-cell clone is CD4$^+$CD8$^+$ positive and recognizes both autologous and allogeneic IFN-γ-treated RCC24 cells (Table 2). This clone does not recognize untreated autologous RCC24 or allogeneic RCC cells. This T-cell clone only recognizes IFN-γ-treated RCC cells following prolonged treatment (50 IU/mL for 3-5 days) in both cytokine and ^{51}chromium release assays (Table 2 and data not shown). This recognition is through a TCR-MHC/peptide interaction because the pan HLA-A, B, C antibody W6/32 inhibits 17/A2-mediated recognition (data not shown). Based on an assessment for recognition of a panel of allogeneic RCC lines, it seems that clone 17/A2 recognizes an HLA-C7-restricted antigen (Table 2 and data not shown). This antigen seems to be inducible by IFN-γ treatment because HLA-C locus levels expressed on the RCC tumor cell surface remain similar with and without IFN-γ treatment as measured by staining of the RCC cells with the pan HLA-B, C antibody (data not shown).

Table 1. Target cells recognized by 1.24CTL1 line and clones derived from RCC patient 24

Target cells	Origin	CTL lysis assay (% killing)*				
		1.24CTL1	#2 clone	#3 clone	#8 clone	#32 clone
K562	CML	7	1	0	0	0
EBV11 (A2, A26)	EBV-B cell	5	ND	ND	2	2
EBV24 (A1, A2)	EBV-B cell	0	0	0	2	1
RCC5 (A1, A2)	RCC	46	ND	ND	ND	ND
RCC11 (A2, A26)	RCC	86	ND	ND	ND	ND
RCC18 (A1, A24)	RCC	4	ND	ND	ND	ND
RCC24 (A1, A2)	RCC	52	40	32	44	39
RCC24B7 (A1, A2)	RCC	30	ND	ND	28	26
NKC24 (A1, A2)	NKC	30	60	55	ND	ND
RCC26B7 (A2, A11)	RCC	3	ND	ND	ND	ND
RCC-Fr (A2)	RCC	41	ND	ND	ND	ND
RCC-Sa (A1, A2)	RCC	65	40	28	39	29
Bal-mel (A1, A2)	Melanoma	18	30	ND	ND	ND

Abbreviations: CML, chronic myelogenous leukemia; ND, not done.
*^{51}Cr-release assay [effect or/target (E/T) ratio, 30/1]: the assay was performed with three different E/T ratios and only the values at the highest E/T were shown. Similar data were obtained with the clones, #1 and #4 (data not shown).

Table 2. Recognition of RCC lines by the 17/A2 T-cell clone is IFN-γ dependent and HLA-C locus restricted

Target cells (IFN-γ status)*	HLA class I loci common to RCC24						GM-CSF release (pg/mL)
K562	No HLA class I expression						0
RCC24 (no IFN-γ)	A1	A2	B39	B62	C1	C7	20
RCC24 (IFN-γ)	A1	A2	B39	B62	C1	C7	580
RCC24 (passage 0; IFN-γ)	A1	A2	B39	B62	C1	C7	400
NKC24	A1	A2	B39	B62	C1	C7	10
Allogeneic tumor lines							
RCC26 (IFN-γ)	—	A2	—	—	—	C7	30
RCC1 (no IFN-γ)	A1	—	—	—	—	C7	0
RCC1 (IFN-γ)	A1	—	—	—	—	C7	390
RCC-Lo (no IFN-γ)	A1	—	—	—	C1	C7	0
RCC-Lo (IFN-γ)	A1	—	—	—	C1	C7	550
RCC-Ba (no IFN-γ)	—	A2	B39	—	—	C7	0
RCC-Ba (IFN-γ)	—	A2	B39	—	—	C7	750
RCC-Sa (IFN-γ)	A1	A2	—	B62	—	—	0

*Target cells were pretreated with recombinant IFN-γ (50 IU/mL) for 4 days before assay. Target cells either untreated or pretreated with IFN-γ were incubated with the 17/A2 T-cell clone for 24 hours before performing the GM-CSF assay.

1.24CTL2-Derived 5B T-Cell Clone Specifically Recognizes Only Autologous Tumor. The 5B T-cell clone was generated from a third independent T-cell bulk culture, 1.24CTL2. This CTL clone recognizes autologous RCC24 cells but not NKC24 (Fig. 1*A*). This T-cell clone does not recognize the HLA-A*0101-matched allogeneic RCC lines RCC1 and RCC4, or the HLA-A*0201-matched allogeneic RCC lines RCC-Fr and RCC11 (Fig. 1*B*). In addition, HLA-A*0101$^+$ RCC lines RCC18 and RCC-Sa, and pancreatic cancer line Panc2.2 are not recognized by this T-cell clone (data not shown). This T-cell clone seems to be HLA-A*0101 restricted because lysis of RCC24 was blocked by the pan HLA-A, B, C antibody W6/32 but not by the HLA-A2 blocking antibodies CR11-351 and MA2.1 or the pan HLA-B, C blocking antibody B1.23.2 (Fig. 1*C*). These data suggested that the antigen recognized by this 5B clone is a unique tumor antigen only expressed by autologous RCC24 cells.

To confirm that this unique tumor antigen expressed by RCC24 is not an *in vitro* tissue culture–induced artifact, we tested T-cell clone 5B for its ability to lyse uncultured RCC24 tumor cells. Frozen and previously uncultured RCC24 cells were thawed and cultured for 3 days. Subsequent analysis showed that the 5B T-cell clone lyses both the original and the long-term cultured RCC24 line. However, as expected, NKC24 line was not lysed (Fig. 1*D*). These experiments show that the unique tumor antigen recognized by the 5B T-cell clone was most likely generated *in vivo*.

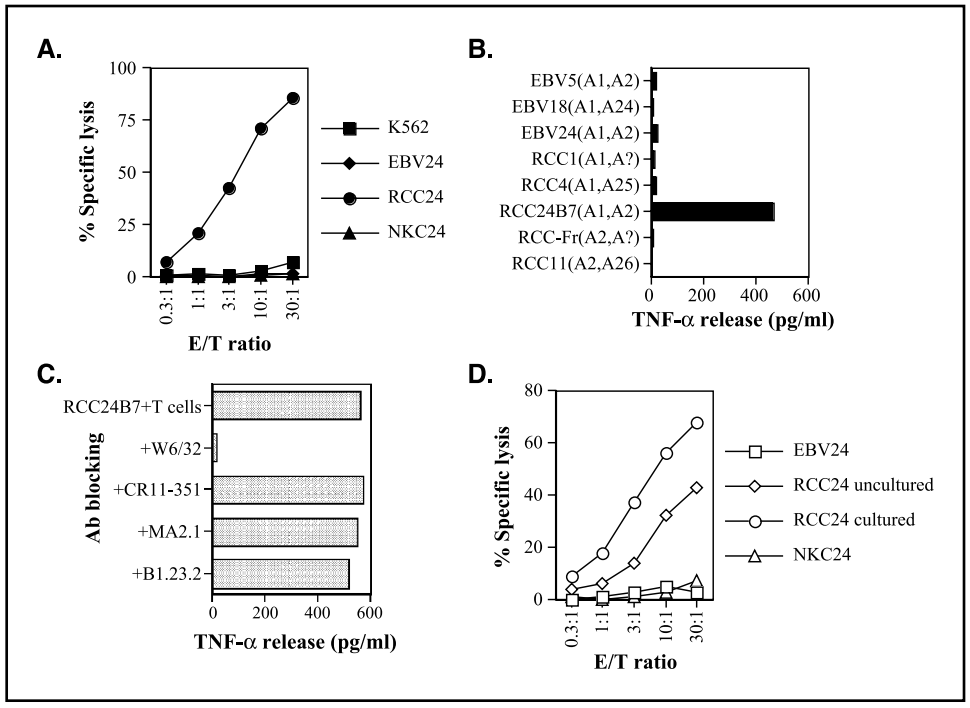

Figure 1. The 5B T-cell clone is HLA-A*0101 restricted and only recognizes autologous RCC24 tumor cells. *A,* a standard 4-hour ^{51}Cr release assay was done to test specific killing of target cells by 5B T cells at different effector to target (E/T) ratios. *B,* measurements of 5B T-cell responses were determined against a panel of allogeneic RCC cell lines with known HLA class I expression in a TNF-α release assay. *C,* antibody blocking studies were done to determine HLA restriction. RCC24B7 cells were preincubated with the pan anti-HLA-A, B, C antibody (W6/32), the anti-HLA-A2 antibodies (CR11-351 and MA2.1), or the anti-HLA-B, C antibody (B1.23.2) at a concentration of 50 μg/mL for 45 to 60 minutes at 37°C before addition of T cells. T-cell activity was determined in a TNF-α release assay. Similar results were obtained in at least three independent assays. *D,* a standard 4-hour ^{51}Cr release assay was done to confirm that clone 5B T cells can recognize and lyse previously uncultured RCC24 tumor cells. *Ab,* antibody.

Isolation of a cDNA Clone Encoding the Antigen Recognized by the 5B T-Cell Clone. Of approximately 60,000 cDNA screened, 1 positive cDNA pool of 100 cDNA was recognized by the 5B clone (absorbance values were 0.866 and 1.111 for the same cDNA pool assayed in duplicate versus <0.095 background). To isolate the single cDNA clone from the positive pool, 300 single cDNA clones were screened and 4 positive, single cDNA clones were identified. The 5B T-cell clone only recognized the single cDNA when cotransfected with the *HLA-A*0101* gene but not with the *HLA-A*0201* and *HLA-A24* genes (data not shown). DNA sequence analysis revealed that all 4 positive cDNA clones were 1736 bp long and 100% identical (Fig. 2*A*). This positive cDNA clone was designated *KCAG1* (kidney cancer antigen 1). A BLAST search revealed that *KCAG1* gene sequence had a 98% homology to nucleotides 1 to 538 of the partial *KIAA1440* cDNA sequence (24). *KIAA1440* cDNA (accession no. AB037861) was first cloned by Nagase and colleagues from human adult and fetal brain cDNA libraries (24). It is 4434 bp long and its predicted protein length is 1377 amino acids long. The nucleotides 329 through 406 and 717 through 846 of the *KCAG1* had 100% homology with nucleotides 1 through 78 and 79 through 208 of the *KIAA1440* gene, respectively. However, a nucleotide mutation (A→G) in *KCAG1* was found at position 1321 that corresponds to nucleotide 465 in the *KIAA1440* gene (Fig. 2*B*). A human genome data base search revealed that other nucleotide sequences (1 through 329, 406 through 717, 846 through 1065 and 1394 through 1736) of the mutated *KCAG1* completely matched the genomic DNA sequences on human chromosome 7 p22.3 (data not shown), suggesting that *KCAG1* is an incomplete splicing variant of the *KIAA1440*.

Epitope Mapping to Identify the HLA-A*0101 Binding Peptide. The *KCAG1* cDNA contains at least four ATG sites at

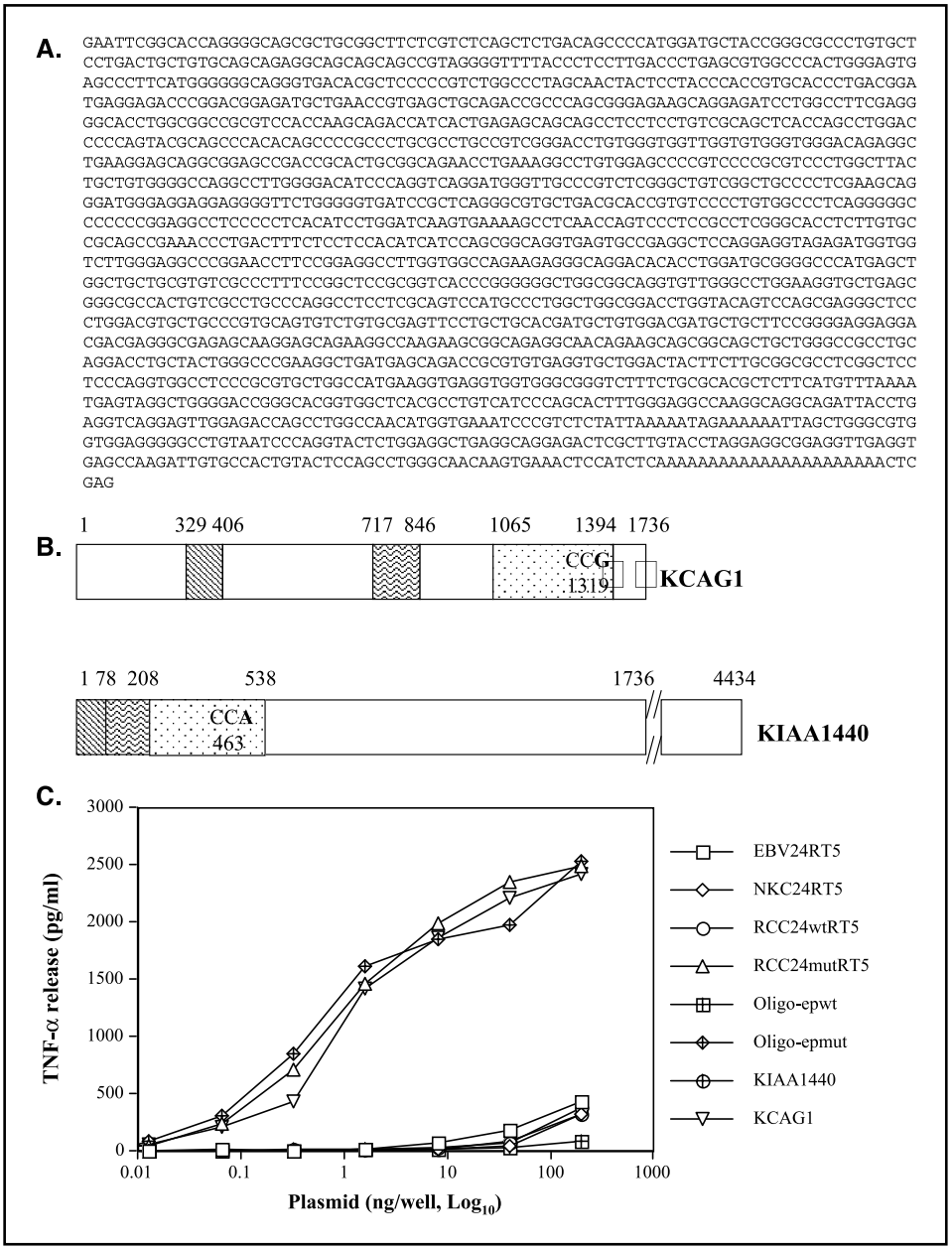

Figure 2. *KCAG1* antigen recognized by 5B T cell is derived from an incomplete splicing variant of *KIAA1440* with A→G mutation. *A,* the nucleotide sequence of *KCAG1* that was isolated from RCC24 cDNA library screening is shown. Four positive single cDNA clones were isolated and their DNA sequences were all identical. *B,* comparison of *KCAG1* with *KIAA1440* cDNA (24). Two DNA sequences at nucleotides 329 to 406 and nucleotides 717 to 846 in *KCAG1* had 100% homology to that at nucleotides 1 to 78 and 79 to 208 in the *KIAA1440* gene. A single nucleotide mutation (A→G) at position 465 in the *KIAA1440* gene was found in the *KCAG1* spliced variant. The rest of the DNA sequence in the *KCAG1* gene matches the genomic sequence on chromosome 7 p22.3 at 100% homology. *C,* a point mutation in *KCAG1* is responsible for generating the antigenic epitope recognized by T-cell clone 5B. Sequencing analysis of the region containing the point mutation in the *KCAG1* gene was done in different cell lines. Total RNA was extracted from EBV24, NKC24, and RCC24 cells and RT-PCR was used to amplify the region with the point mutation (A to G at nucleotide 1321) in *KCAG1*. PCR fragments were then sequenced. RT-PCR fragments derived from EBV24 (*EBV24RT5*), NKC24 (*NKC24RT5*), and RCC24 (*RCC24wtRT5* and *RCC24mutRT5*) cells were subcloned into pcDNA3. The oligonucleotide with a point mutation (*Oligo-epmut*) and the *KIAA1440* cDNA were also expressed in pcDNA3. The 5-fold diluted plasmids ranging from 200 to 0.0128 ng/well were cotransfected with HLA-A*0101 into COS-7 cells and incubated with 5B T cells for 18 to 24 hours before determining recognition in a TNF-α release assay.

nucleotide positions 260, 954, 1079, and 1308 that may initiate transcription and translation and result in epitope generation. Using unique restriction enzyme sites in *KCAG1*, the *KCAG1* gene was cut into two fragments: *KCAG1*-F (forward) and *KCAG1*-R (rear). The two fragments were then cotransfected with the HLA-A*0101 plasmid into COS-7 cells and transfectants were tested for stimulation of TNF-α release by the 5B T-cell clone. Only the *KCAG1*-R stimulated the 5B T-cell clone to release TNF-α.

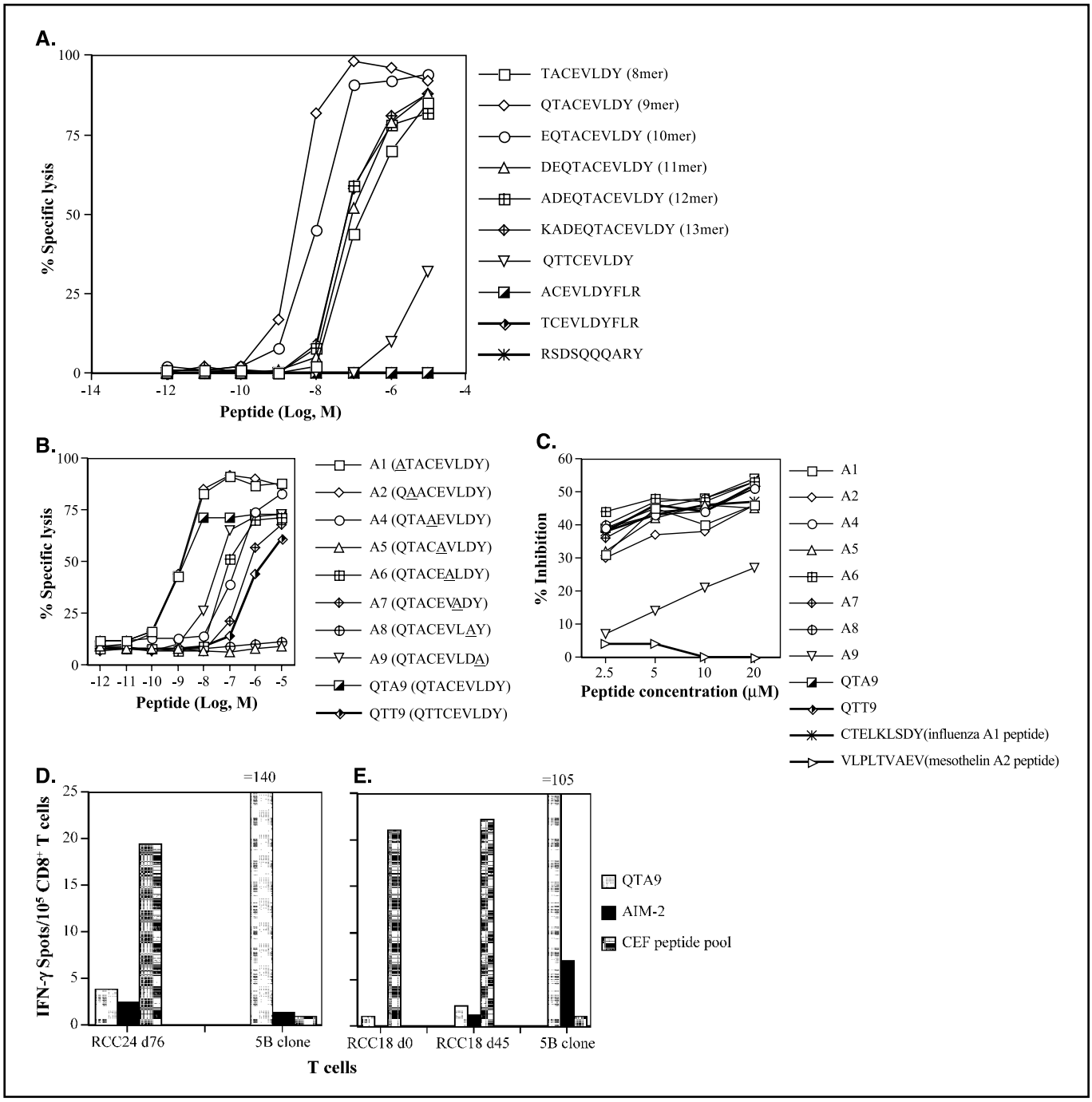

Figure 3. Characterization of the antigenic epitope recognized by 5B T-cell clone. *A,* a nonamer QTACEVLDY is the antigenic peptide recognized by 5B CTL clone. Different lengths of the antigenic peptide (QTACEVLDY) were titrated and pulsed onto ^{51}Cr-labeled EBV24 target cells for 1 hour before addition of the 5B T-cell clone at an E/T ratio of 60:1. QTTCEVLDY, corresponding wild-type peptide. ACEVLDYFLR and TCEVLDYFLR, mutant and wild-type sequence of *KIAA1440*, respectively. RSDSQQQARY, a HLA-A*0101-restricted melanoma peptide (11). This experiment was repeated twice with similar results. *B,* T-cell recognition of alanine-substituted peptide variants. The 10-fold diluted peptides were pulsed onto ^{51}Cr-labeled EBV24 target cells and incubated for 4 hours with the 5B T cells at an E/T ratio of 50:1. Similar results were obtained in two independent cytotoxicity and TNF-α release assays (data not shown). *C,* binding affinity of peptide variants to HLA-A*0101 was determined using a competition binding assay. This experiment was repeated twice with the similar results. *D,* vaccination may slightly increase antigen-specific CD8$^+$ T cells response *in vivo*. An IFN-γ ELISPOT assay was done to test antigen (QTA9)-specific CD8$^+$ T-cell response from PBL post vaccination (day 76) of the patient 24. 5B CTL clone, isolated from the same patient, was used as a positive control. AIM-2 (RSDSQQQARY) is a HLA-A*0101-restricted melanoma peptide (11). CEF is a pool of 32 peptides from cytomegalovirus, EBV, and influenza virus (20). *E,* an ELISPOT assay was used to test antigen-specific CD8$^+$ T-cell response from PBL before (day 0) and post vaccination (day 45) of an irrelevant HLA-A1$^+$ RCC patient 18. This experiment was repeated twice with similar results.

Three additional truncated *KCAG1*-R cDNA were constructed and subsequently tested for recognition by the 5B clone in the TNF-α release assay. Based on this analysis, it was possible to narrow down 5B T-cell clone recognition of the *KCAG1* gene to 207 nucleotides (termed *KCAG1*-R-P) that contain the antigenic epitope (data not shown). To identify the antigenic epitope within the 99-bp region of the *KCAG1* gene, 19 peptides overlapping by one amino acid starting from methionine were synthesized and tested in two independent CTL assays. Surprisingly, all 19 peptides titrated from 10^{-5} to 10^{-12} mol/L concentrations and pulsed onto autologous EBV-B cells as antigen-presenting cells failed to sensitize the 5B clone in both a TNF-α release and ^{51}Cr-release assay (data not shown). This experiment suggested an angiotensin-independent mechanism for the generation of the antigenic epitope derived from *KCAG1*.

A more detailed analysis of the *KCAG1* gene sequence was therefore done to address whether the point mutation (A→G) in *KCAG1* is critical in the formation of the epitope. RT-PCR analysis was done on RCC24, autologous EBV B cells, and autologous NKCs to amplify the region between nucleotides 1130 and 1341, which encompasses the point mutation in *KCAG1*. DNA sequencing results showed that both autologous EBV-B and NKCs had a wild-type sequence of *KIAA1440* in both alleles (w/w). However, the tumor cell RCC24 had a heterozygous phenotype: one allele with a point mutation and the other allele with a normal sequence (wt/mut, CCA/CCG). Further analysis showed that this point mutation is autologous tumor cell specific because we were unable to find this mutation in other RCC lines including RCC1, RCC4, RCC11, RCC18, RCC26, RCC-Fr, RCC-Lo, and RCC-Sa (data not shown). Furthermore, COS-7 cells transfected with the RT-PCR fragments from EBV24, NKC24, and RCC24 that contained only the wild-type sequence could not activate the 5B T-cell clone to release TNF-α. However, COS-7 cells transfected with the RT-PCR fragment containing the point mutation in RCC24 induced 1000-fold greater TNF-α release by the 5B T-cell clone than the wild-type fragment (Fig. 2*C*). These data support our hypothesis that this point mutation in RCC24 tumor cells leads to the generation of a new antigenic epitope recognized by 5B T cells.

Identification of the Antigenic Peptide. Using an HLA-A*0101 peptide prediction algorithm (http://bimas.dcrt.nih.gov/molbio/hla_bind/), we searched for peptides bearing an HLA-A*0101-binding motif and encompassing the mutation site. Two peptides, QTACEVLDY (termed QTA9) and ACEVLDYFLR (termed ACE10), were listed with the highest binding scores (6.25 for 9 mer versus 45.00 for 10 mer). These two mutant peptides were compared with the corresponding wild-type peptides, QTTCEVLDY (termed QTT9) and TCEVLDYFLR (termed TCE10), for recognition by the 5B T-cell clone in a TNF-α release assay. Only the nonapeptide QTA9 with the alanine mutant residue at position 3 was recognized. However, half-maximal TNF-α release was obtained at a peptide concentration of 1×10^{-6} mol/L, which is low in comparison with antiviral CD8$^+$ T cells (which typically recognize their cognate peptide at concentrations between 1×10^{-9} and 1×10^{-12} mol/L; Fig. 3*A*; ref. 25). This could be due either to low peptide-MHC affinity or to low TCR affinity for the peptide-MHC ligand. To confirm that the QTA9 peptide length is the optimal peptide length, different lengths of QTA9 peptide ranging from 8 to 13 mer were synthesized and tested in both a cytokine release (data not shown) and CTL lysis assay (Fig. 3*A*). These studies confirmed that the 9 mer is the optimal activator of the 5B T cells. In addition, the half-maximal lysis still required 5×10^{-8} mol/L peptide. As reported by others, our data confirmed that peptide concentration required for half-maximal CTL killing is generally lower than that for cytokine release (25). Regardless, the QTA9 peptide is the optimal antigenic peptide recognized by the 5B T cells.

Optimization of the Antigenic Peptide and Peptide Binding to HLA-A*0101. Several studies have shown that altered peptide ligands can stabilize either the MHC-peptide or MHC-peptide-TCR complex and induce more potent T-cell responses against the original antigen both *in vitro* and *in vivo* (26, 27). We therefore attempted to characterize the ability of alanine-substituted variant peptides of the antigenic peptide QTA9 to stabilize the HLA-A*0101 MHC-peptide complex and enhance T cell recognition. As shown in Fig. 3*B*, alanine substitutions at positions 1 and 2 had no effect on CTL recognition when compared with the natural ligand QTA9. In contrast, peptides with substituted residues at positions 4, 6, 7, and 9 partially antagonized T-cell recognition and alanine substitutions at positions 5 and 8 completely abolished T-cell recognition. As shown earlier, the QTA9 peptide with a mutation at position 3 induced 1000-fold greater target cell sensitization for recognition by the T cells than did the wild-type QTT9 peptide.

Next we did a peptide competition assay to compare the HLA-A*0101 binding of the natural antigenic peptide and its alanine-substituted variants. Our results showed that all alanine-substituted peptides except for the one at position 9 (A9) bind to HLA-A*0101 as efficiently as the naturally expressed QTA9 antigenic peptide. Interestingly, no difference in peptide binding to HLA-A*0101 between the wild-type and mutant peptide was found although the mutant peptide is much more efficiently recognized by the 5B T-cell clone than the wild-type peptide (Fig. 3*C*). We therefore conclude that the peptide residues at positions 3, 5, and 8 are TCR contact sites, whereas position 9 is a MHC anchor residue.

ELISPOT Analysis of CD8$^+$ T-Cell Responses to the Antigenic Peptide. An IFN-γ ELISPOT assay was done to determine whether CD8$^+$ T-cell responses to the antigenic peptide QTA9 can be detected and quantitated. Due to the lack of prevaccination samples for this patient, only postvaccination PBL at day 76 was tested. The results show detection of QTA9-specific T cells, albeit only at a 2-fold increase of CD8$^+$ T cells when compared with the melanoma AIM-2 peptide. CD8 T-cell responses to the positive control viral peptide pool CEF (20) was also detected (Fig. 3*D*). Pre- and postvaccination lymphocytes from a second HLA-A1$^+$ patient (patient 18), whose tumor does not express this antigenic epitope, was also tested for recognition of QTA9 epitope. As expected, there was no difference in detected CD8 T cells specific for the antigenic peptide between pre- and postvaccinated PBL from patient 18 (Fig. 3*E*). It therefore remains to be determined whether GM-CSF-tumor cell vaccine can induce QTA9 antigen-specific antitumor CD8 T-cell responses *in vivo*.

1.26CTL Lines and Clones Recognize Autologous RCC Restricted by Multiple MHC Class I Molecules. To test whether it is possible to generate CTL from another RCC patient who responded to the vaccine, PBL from patient 26 (108 days post vaccination) were stimulated with RCC26B7 cells *in vitro*. The 1.26CTL1 line was generated and it recognized autologous RCC26B7 cells and RCC26E6/E7 (RCC26 transduced with the retroviral vector containing the human papillomavirus E6 and E7 oncogenes) but not RCC24 cells in both ^{51}Cr-release (data not shown) and cytokine release assays (Fig. 4*A*). Furthermore, this recognition was completely inhibited by anti-MHC class I (W6/32)

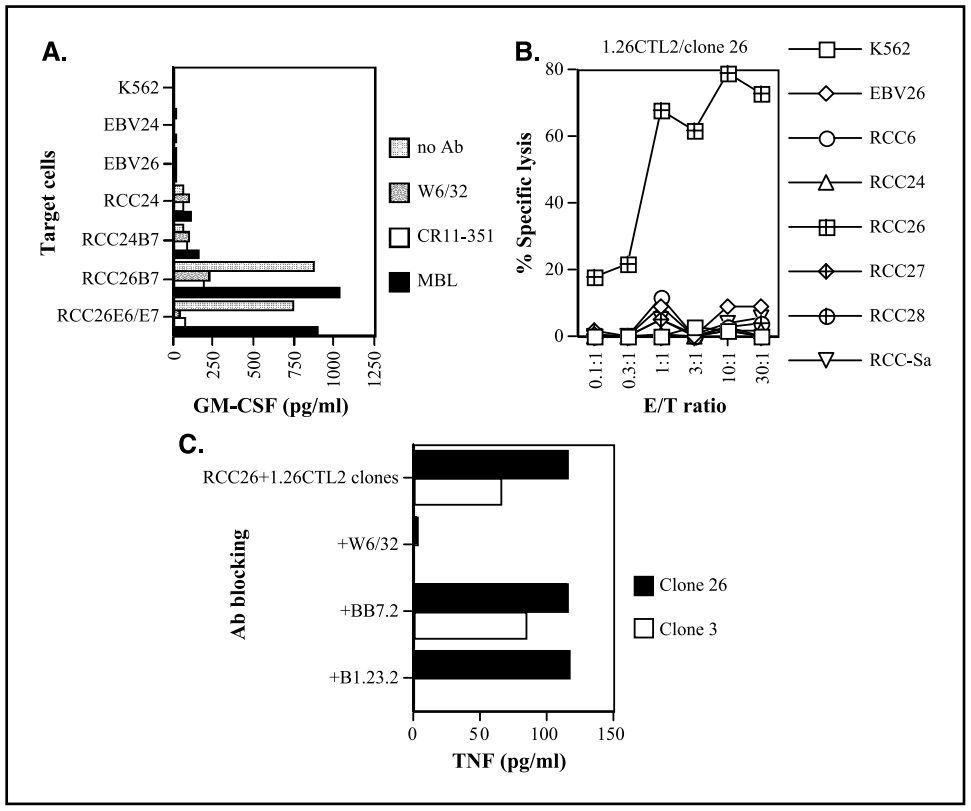

Figure 4. 1.26CTL lines and clones recognize autologous tumor cells in a multiple MHC class I–restricted manner. *A,* 1.26CTL1 line produced GM-CSF in response to target cells through HLA-A2 restricted fashion. *B,* 1.26CTL2 line–derived clone 26 killed RCC26 tumor cells in a ^{51}Cr-release assay. Similar data were obtained with clone 3 (data not shown). *C,* an Ab blocking assay was used to test MHC class I restricting elements.

and anti-HLA-A2 (CR11-35) antibodies, suggesting that 1.26CTL1 line recognized autologous RCC tumor antigen presented by HLA-A2. A second independent line 1.26CTL2 and three clones (3, 24, and 26) derived from this line were also generated. A ^{51}Cr-release assay showed that the 1.26CTL2 line–derived clones 3, 24 (data not shown), and 26 killed autologous RCC26 but not other HLA-A2-matched RCC lines (Fig. 4*B*). A mAb blocking experiment showed that recognition of RCC26 by these three CTL clones was inhibited by the anti-HLA-A, B, C antibody, W6/32, whereas the anti-HLA-B, C antibody, B1.23.2 only blocked recognition of clone 3 but not clone 26 (Fig. 4*C*). These results show that a diverse repertoire of tumor-reactive CTL can be generated in a second vaccinated patient.

Discussion

The data presented in this report suggest three observations concerning the T-cell responses induced by an autologous GM-CSF secreting tumor vaccine in two patients who had an associated clinical response (6). First, the GM-CSF tumor cell vaccine may induce a diverse CD8$^+$ T-cell repertoire that seems to recognize both shared and unique RCC antigens presented by a diversity of MHC class I molecules. In addition, the antigen-recognition pattern of a subset of the CD8$^+$ T-cell responses measured *in vitro* correlate with the postvaccination DTH responses observed *in vivo*. Second, the unique tumor antigen recognized by a subset of CD8$^+$ T cells (5B CTL clone) is generated by a point mutation in the previously reported *KIAA1440*. Third, currently the target cells used in cDNA library screening techniques to identify tumor antigens give high background in T-cell assays using T cells that recognize shared renal tumor antigens and are therefore not suitable for identifying shared renal antigens.

In the present study, we were able to readily generate CD8$^+$ T-cell lines and clones against the autologous tumor cell line derived from patients 24 and 26 who had a remarkable clinical response to an autologous GM-CSF tumor vaccine. These CD8$^+$ T-cell responses target a broad spectrum of tumor antigens presented by several HLA class I molecules. This diversity in antigen types recognized by immunized lymphocytes from these two patients with renal cancer is similar to what has been observed in patients with malignant melanoma (1–3). A broad CD4$^+$ T-cell repertoire toward RCC has also been shown in these two patients.[3] These CD4$^+$ T-cell lines and clones also seem to recognize both shared and unique antigens through multiple MHC class II elements. The ability to induce T-cell responses to multiple antigens may play an important role in avoiding the development of tumor antigen loss variants and in maintaining effective antitumor immunity *in vivo*.

We cannot be absolutely certain that an autologous GM-CSF tumor cell vaccine truly induces renal cancer–specific CD8 T-cell responses that we detected *in vitro* because we were unable to directly analyze QTA9-specific CD8 T cells in the prevaccination PBL from the RCC patient 24 due to lack of the samples (Fig. 4*D* and *E*). It is possible that this patient and other RCC patients may have preexisting T cells specific for renal cancer antigens in view of recent publications demonstrating the presence of melanoma- or prostate epitope–specific CTL responses in a high number of untreated patients with cancer (28, 29). Therefore, additional studies are required to determine whether GM-CSF–engineered tumor cell vaccines are capable of generating T-cell responses toward multiple tumor antigens in other vaccinated patients.

[3] Mautner J., Pardoll D.M., and Jafee E.M., unpublished data.

In this report we also show that one population of T cells, 1.24CTL1 and its clones, recognize both the autologous tumor cells and NKCs. This *in vitro* observation correlates with this patient's *in vivo* postvaccination DTH response to both uncultured and cryopreserved autologous tumor cells and NKCs (6). Recognition of both NKC and RCC cells by 1.24CTL1 line and clones raise a question of whether antitumor immunity and autoimmunity may codevelop in patients that are successfully treated with antigen-targeted vaccines. Clinically, we observed that a strong antitumor immunity was induced in this patient after vaccination that was associated with a partial clinical response but without evidence of renal toxicity (6). In contrast to our findings, preclinical and clinical vaccination studies targeting melanoma self-antigens such as gp100 (30, 31) and TRP-1 (32) showed that the deliberate induction of self-reactivity can lead to tumor destruction that is associated with autoimmune vitiligo (30–33). Interestingly, vaccination with another self-antigen, TRP-2, results in melanoma eradication but not vitiligo, suggesting that it is possible to induce protective antitumor immunity against self-antigens expressed on tumor cells without inducing an associated autoimmune response (33). Further analyses are required to determine whether the antigen recognized by the 1.24CTL1 line and its clones only induce antitumor immunity without associated autoimmune responses.

A major goal of these studies was to isolate genes encoding tumor rejection antigens that can ultimately be used to develop renal tumor antigen–based vaccines. Although a large number of melanoma-associated antigens have been identified, very few renal tumor antigens are known. So far, eight RCC expressed antigens have been reported (34–41). However, like *KCAG1*, most of these other known antigens are rarely expressed in RCC and, therefore, unlikely candidates for developing widely applicable antigen-based vaccines for the treatment of patients with RCC.

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